Enzyme Biocatalysis

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Editor

Enzyme Biocatalysis

Principles and Applications

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Foreword

This book was written with the purpose of providing a sound basis for the design of enzymatic reactions based on kinetic principles, but also to give an updated vision of the potentials and limitations of biocatalysis, especially with respect to recent applications in processes of organic synthesis. The first five chapters are structured in the form of a textbook, going from the basic principles of enzyme structure and function to reactor design for homogeneous systems with soluble enzymes and heterogeneous systems with immobilized enzymes. The last chapter of the book is divided into six sections that represent illustrative case studies of biocatalytic processes of industrial relevance or potential, written by experts in the respective fields.

We sincerely hope that this book will represent an element in the toolbox of graduate students in applied biology and chemical and biochemical engineering and also of undergraduate students with formal training in organic chemistry, biochemistry, thermodynamics and chemical reaction kinetics. Beyond that, the book pretends also to illustrate the potential of biocatalytic processes with case studies in the field of organic synthesis, which we hope will be of interest for the academia and professionals involved in R&D&I. If some of our young readers are encouraged to engage or persevere in their work in biocatalysis this will certainly be our more precious reward.

Too much has been written about writing. Nobel laureate Gabriel García Márquez wrote one of its most inspired books by writing about writing (*Living to Tell the Tale*). There he wrote "life is not what one lived, but what one remembers and how one remembers it in order to recount it". This hardly applies to a scientific book, but certainly highlights what is applicable to any book: its symbiosis with life. Writing about biocatalysis has given me that privileged feeling, even more so because enzymes are truly the catalysts of life. Biocatalysis is hardly separable from my life and writing this book has been certainly more an ecstasy than an agony.

A book is an object of love so who better than friends to build it. Eleven distinguished professors and researchers have contributed to this endeavor with their knowledge, their commitment and their encouragement. Beyond our common language, I share with all of them a view and a life-lasting friendship. That is what lies behind this book and made its construction an exciting and rewarding experience.

Chapters 3 to 5 were written with the invaluable collaboration of Claudia Altamirano and Lorena Wilson, two of my former students, now my colleagues, and my bosses I am afraid. Chapter 4 also included the experience of José Manuel Guisán, Roberto Fernández-Lafuente and César Mateo, all of them very good friends who were kind enough to join this project and enrich the book with their world known expertise in heterogeneous biocatalysis. Section 6.1 is the result of a cooperation sustained by a CYTED project that brought together Sonia Barberis, also a former graduate student, now a successful professor and permanent collaborator and, beyond that, a dear friend, Fanny Guzmán, a reputed scientist in the field of peptide synthesis who is my partner, support and inspiration, and Josep López, a well-known scientist and engineer but, above all, a friend at heart and a warm host. Section 6.3 was the result of a joint project with Gregorio Alvaro, a dedicated researcher who has been a permanent collaborator with our group and also a very special friend and kind host. Section 6.4 is the result of a collaboration, in a very challenging field of applied biocatalysis, of Dr. Guisan's group with which we have a long-lasting academic connection and strong personal ties. Section 6.5 represents a very challenging project in which Josep López and Gregorio Alvaro have joined Pere Clapés, a prominent researcher in organic synthesis and a friend through the years, to build up an updated review on a very provocative field of enzyme biocatalysis. Finally, section 6.6 is a collaboration of a dear friend and outstanding teacher, Juan Lema, and his research group that widens the scope of biocatalysis to the field of environmental engineering adding a particular flavor to this final chapter.

A substantial part of this book was written in Spain while doing a sabbatical in the Universitat Autònoma de Barcelona, where I was warmly hosted by the Chemical Engineering Department, as I also was during short stays at the Institute of Catalysis and Petroleum Chemistry in Madrid and at the Department of Chemical Engineering in the Universidad de Santiago de Compostela.

My recognition to the persons in my institution, the Pontificia Universidad Católica de Valparaíso, that supported and encouraged this project, particularly to the rector Prof. Alfonso Muga, and professors Atilio Bustos and Graciela Muñoz. Last but not least, my deepest appreciation to the persons at Springer: Marie Johnson, Meran Owen, Tanja van Gaans and Padmaja Sudhakher, who were always delicate, diligent and encouraging.

Dear reader, the judgment about the product is yours, but beyond the product there is a process whose beauty I hope to have been able to transmit. I count on your indulgence with language that, despite the effort of our editor, may still reveal our condition of non-native English speakers.

Andrés Illanes
Valparaíso, May 15, 2008

Chapter 1
Introduction

Andrés Illanes

1.1 Catalysis and Biocatalysis

Many chemical reactions can occur spontaneously; others require to be catalyzed to proceed at a significant rate. Catalysts are molecules that reduce the magnitude of the energy barrier required to be overcame for a substance to be converted chemically into another. Thermodynamically, the magnitude of this energy barrier can be conveniently expressed in terms of the free-energy change. As depicted in Fig. 1.1, catalysts reduce the magnitude of this barrier by virtue of its interaction with the substrate to form an activated transition complex that delivers the product and frees the catalyst. The catalyst is not consumed or altered during the reaction so, in principle, it can be used indefinitely to convert the substrate into product; in practice, however, this is limited by the stability of the catalyst, that is, its capacity to retain its active structure through time at the conditions of reaction.

Biochemical reactions, this is, the chemical reactions that comprise the metabolism of all living cells, need to be catalyzed to proceed at the pace required to sustain life. Such life catalysts are the enzymes. Each one of the biochemical reactions of the cell metabolism requires to be catalyzed by one specific enzyme. Enzymes are protein molecules that have evolved to perform efficiently under the mild conditions required to preserve the functionality and integrity of the biological systems. Enzymes can be considered then as catalysts that have been optimized through evolution to perform their physiological task upon which all forms of life depend. No wonder why enzymes are capable of performing a wide range of chemical reactions, many of which extremely complex to perform by chemical synthesis. It is not presumptuous to state that any chemical reaction already described might have an enzyme able to catalyze it. In fact, the possible primary structures of an enzyme protein composed of n amino acid residues is 20^n so that for a rather small protein molecule containing 100 amino acid residues, there are 20^{100} or 10^{130} possible

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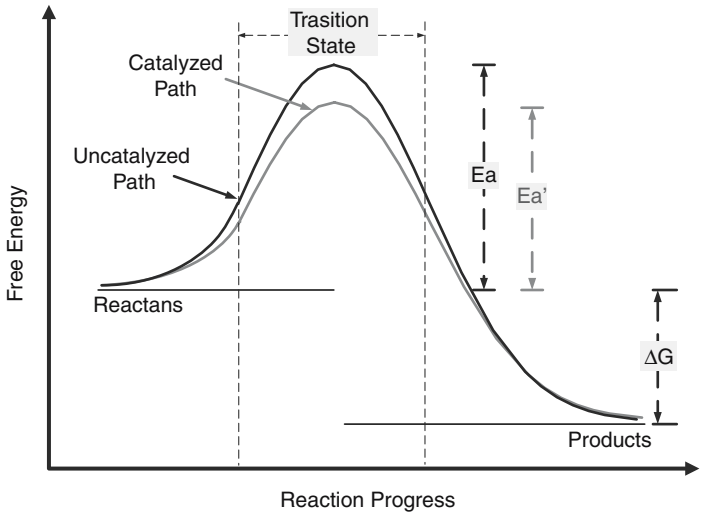


Fig. 1.1 Mechanism of catalysis. Ea and Ea′ are the energies of activation of the uncatalyzed and catalyzed reaction. ΔG is the free energy change of the reaction

amino acid sequences, which is a fabulous number, higher even than the number of molecules in the whole universe. To get the right enzyme for a certain chemical reaction is then a matter of search and this is certainly challenging and exciting if one realizes that a very small fraction of all living forms have been already isolated. It is even more promising when one considers the possibility of obtaining DNA pools from the environment without requiring to know the organism from which it comes and then expressed it into a suitable host organism (Nield et al. 2002), and the opportunities of genetic remodeling of structural genes by site-directed mutagenesis (Abián et al. 2004).

Enzymes have been naturally tailored to perform under physiological conditions. However, biocatalysis refers to the use of enzymes as process catalysts under artificial conditions (in vitro), so that a major challenge in biocatalysis is to transform these physiological catalysts into process catalysts able to perform under the usually tough reaction conditions of an industrial process. Enzyme catalysts (biocatalysts), as any catalyst, act by reducing the energy barrier of the biochemical reactions, without being altered as a consequence of the reaction they promote. However, enzymes display quite distinct properties when compared with chemical catalysts; most of these properties are a consequence of their complex molecular structure and will be analyzed in section 1.2. Potentials and drawbacks of enzymes as process catalysts are summarized in Table 1.1.

Enzymes are highly desirable catalysts when the specificity of the reaction is a major issue (as it occurs in pharmaceutical products and fine chemicals), when the catalysts must be active under mild conditions (because of substrate and/or product instability or to avoid unwanted side-reactions, as it occurs in several reactions of organic synthesis), when environmental restrictions are stringent (which is now a

Table 1.1 Advantages and Drawbacks of Enzymes as Catalysts

Advantages	Drawbacks
High specificity	High molecular complexity
High activity under moderate conditions	High production costs
High turnover number	Intrinsic fragility
Highly biodegradable	
Generally considered as natural products	

rather general situation that gives biocatalysis a distinct advantage over alternative technologies) or when the label of natural product is an issue (as in the case of food and cosmetic applications) (Benkovic and Ballesteros 1997; Wegman et al. 2001). However, enzymes are complex molecular structures that are intrinsically labile and costly to produce, which are definite disadvantages with respect to chemical catalysts (Bommarius and Broering 2005).

While the advantages of biocatalysis are there to stay, most of its present restrictions can be and are being solved through research and development in different areas. In fact, enzyme stabilization under process conditions is a major issue in biocatalysis and several strategies have been developed (Illanes 1999) that include chemical modification (Roig and Kennedy 1992; Özturk et al. 2002; Mislovičová et al. 2006), immobilization to solid matrices (Abián et al. 2001; Mateo et al. 2005; Kim et al. 2006; Wilson et al. 2006), crystallization (Häring and Schreier 1999; Roy and Abraham 2006), aggregation (Cao et al. 2003; Mateo et al. 2004; Schoevaart et al. 2004; Illanes et al. 2006) and the modern techniques of protein engineering (Chen 2001; Declerck et al. 2003; Sylvestre et al. 2006; Leisola and Turunen 2007), namely site-directed mutagenesis (Bhosale et al. 1996; Ogino et al. 2001; Boller et al. 2002; van den Burg and Eijsink 2002; Adamczak and Hari Krishna 2004; Bardy et al. 2005; Morley and Kazlauskas 2005), directed evolution by tandem mutagenesis (Arnold 2001; Brakmann and Johnsson 2002; Alexeeva et al. 2003; Boersma et al. 2007) and gene-shuffling based on polymerase assisted (Stemmer 1994; Zhao et al. 1998; Shibuya et al. 2000; Kaur and Sharma 2006) and, more recently, ligase assisted recombination (Chodorge et al. 2005). Screening for intrinsically stable enzymes is also a prominent area of research in biocatalysis. Extremophiles, that is, organisms able to survive and thrive in extreme environmental conditions are a promising source for highly stable enzymes and research on those organisms is very active at present (Adams and Kelly 1998; Davis 1998; Demirjian et al. 2001; van den Burg 2003; Bommarius and Riebel 2004; Gomes and Steiner 2004). Genes from such extremophiles have been cloned into suitable hosts to develop biological systems more amenable for production (Halldórsdóttir et al. 1998; Haki and Rakshit 2003; Zeikus et al. 2004).

Enzymes are by no means ideal process catalysts, but their extremely high specificity and activity under moderate conditions are prominent characteristics that are being increasingly appreciated by different production sectors, among which the pharmaceutical and fine-chemical industry (Schmid et al. 2001; Thomas et al. 2002; Zhao et al. 2002; Bruggink et al. 2003) have added to the more traditional sectors of food (Hultin 1983) and detergents (Maurer 2004).

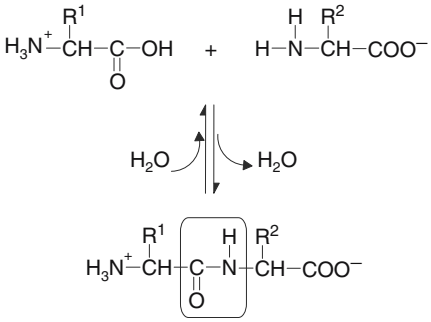


Fig. 1.2 Scheme of peptide bond formation between two adjacent α-amino acids

1.2 Enzymes as Catalysts. Structure–Functionality Relationships

Most of the characteristics of enzymes as catalysts derive from their molecular structure. Enzymes are proteins composed by a number of amino acid residues that range from 100 to several hundreds. These amino acids are covalently bound through the peptide bond (Fig. 1.2) that is formed between the carbon atom of the carboxyl group of one amino acid and the nitrogen atom of the α-amino group of the following. According to the nature of the R group, amino acids can be non-polar (hydrophobic) or polar (charged or uncharged) and their distribution along the protein molecule determines its behavior (Lehninger 1970).

Every protein is conditioned by its amino acid sequence, called *primary structure*, which is genetically determined by the deoxyribonucleotide sequence in the structural gene that codes for it. The DNA sequence is first transcribed into a mRNA molecule which upon reaching the ribosome is translated into an amino acid sequence and finally the synthesized polypeptide chain is transformed into a three-dimensional structure, called *native structure*, which is the one endowed with biological functionality. This transformation may include several post-translational reactions, some of which can be quite relevant for its functionality, like proteolytic cleavage, as it occurs, for instance, with *Escherichia coli* penicillin acylase (Schumacher et al. 1986) and glycosylation, as it occurs for several eukaryotic enzymes (Longo et al. 1995). The three-dimensional structure of a protein is then genetically determined, but environmentally conditioned, since the molecule will interact with the surrounding medium. This is particularly relevant for biocatalysis, where the enzyme acts in a medium quite different from the one in which it was synthesized than can alter its native functional structure. Secondary three-dimensional structure is the result of interactions of amino acid residues proximate in the primary structure, mainly by hydrogen bonding of the amide groups; for the case of globular proteins, like enzymes, these interactions dictate a predominantly ribbon-like coiled configuration termed α-*helix*. Tertiary three-dimensional structure is the result of interactions of amino acid residues located apart in the primary structure that produce a compact and twisted configuration in which the surface is rich in polar amino acid

residues, while the inner part is abundant in hydrophobic amino acid residues. This tertiary structure is essential for the biological functionality of the protein. Some proteins have a quaternary three-dimensional structure, which is common in regulatory proteins, that is the result of the interaction of different polypeptide chains constituting subunits that can display identical or different functions within a protein complex (Dixon and Webb 1979; Creighton 1993).

The main types of interactions responsible for the three-dimensional structure of proteins are (Haschemeyer and Haschemeyer 1973):

- Hydrogen bonds, resulting from the interaction of a proton linked to an electronegative atom with another electronegative atom. A hydrogen bond has approximately one-tenth of the energy stored in a covalent bond. It is the main determinant of the helical secondary structure of globular proteins and it plays a significant role in tertiary structure as well.
- Apolar interactions, as a result of the mutual repulsion of the hydrophobic amino acid residues by a polar solvent, like water. It is a rather weak interaction that does not represent a proper chemical bond (approximation between atoms exceed the van der Waals radius); however, its contribution to the stabilization of the three-dimensional structure of a protein is quite significant.
- Disulphide bridges, produced by oxidation of cysteine residues. They are especially relevant in the stabilization of the three-dimensional structure of low molecular weight extracellular proteins.
- Ionic bonds between charged amino acid residues. They contribute to the stabilization of the three-dimensional structure of a protein, although to a lesser extent, because the ionic strength of the surrounding medium is usually high so that interaction is produced preferentially between amino acid residues and ions in the medium.
- Other weak type interactions, like van der Waals forces, whose contribution to three-dimensional structure is not considered significant.

Proteins can be conjugated, this is, associated with other molecules (*prosthetic groups*). In the case of enzymes which are conjugated proteins (*holoenzymes*), catalysis always occur in the protein portion of the enzyme (*apoenzyme*). Prosthetic groups may be organic macromolecules, like carbohydrates (in the case of glycoproteins), lipids (in the case of lipoproteins) and nucleic acids (in the case of nucleoproteins), or simple inorganic entities, like metal ions. Prosthetic groups are tightly bound (usually covalently) to the apoenzyme and do not dissociate during catalysis. A significant number of enzymes from eukaryotes are glycoproteins, in which case the carbohydrate moiety is covalently linked to the apoenzyme, mainly through serine or threonine residues, and even though the carbohydrate does not participate in catalysis it confers relevant properties to the enzyme.

Catalysis takes place in a small portion of the enzyme called the *active site*, which is usually formed by very few amino acid residues, while the rest of the protein acts as a scaffold. Papain, for instance, has a molecular weight of 23,000 Da with 211 amino acid residues of which only cysteine (Cys 25) and histidine (His 159)

are directly involved in catalysis (Allen and Lowe 1973). Substrate is bound to the enzyme at the active site and doing so, changes in the distribution of electrons in its chemical bonds are produced that cause the reactions that lead to the formation of products. The products are then released from the enzyme which is ready for the next catalytic cycle. According to the early *lock and key* model proposed by Emil Fischer in 1894, the active site has a unique geometric shape that is complementary to the geometric shape of the substrate molecule that fits into it. Even though recent reports provide evidence in favor of this theory (Sonkaria et al. 2004), this rigid model hardly explains many experimental evidences of enzyme biocatalysis. Later on, the *induced-fit* theory was proposed (Koshland 1958) according to which the substrate induces a change in the enzyme conformation after binding, that may orient the catalytic groups in a way prone for the subsequent reaction; this theory has been extensively used to explain enzyme catalysis (Youseff et al. 2003). Based on the transition-state theory, enzyme catalysis has been explained according to the hypothesis of enzyme transition state complementariness, which considers the preferential binding of the transition state rather than the substrate or product (Benković and Hammes-Schiffer 2003).

Many, but not all, enzymes require small molecules to perform as catalysts. These molecules are termed *coenzymes* or *cofactors*. The term *coenzyme* is used to refer to small molecular weight organic molecules that associate reversibly to the enzyme and are not part of its structure; coenzymes bound to enzymes actually take part in the reaction and, therefore, are sometime called *cosubstrates*, since they are stoichiometric in nature (Kula 2002). Coenzymes often function as intermediate carriers of electrons (i.e. NAD^+ or FAD^+ in dehydrogenases), specific atoms (i.e. coenzyme Q in H atom transfer) or functional groups (i.e. coenzyme A in acyl group transfer; pyridoxal phosphate in amino group transfer; biotin in CO_2 transfer) that are transferred in the reaction. The term *cofactor* is commonly used to refer to metal ions that also bind reversibly to enzymes but in general are not chemically altered during the reaction; cofactors usually bind strongly to the enzyme structure so that they are not dissociated from the holoenzyme during the reaction (i.e. Ca^{++} in α-amylase; Co^{++} or Mg^{++} in glucose isomerase; Fe^{+++} in nitrile hydratase). According to these requirements, enzymes can be classified in three groups as depicted in Fig. 1.3:

(i) those that do not require of an additional molecule to perform biocatalysis,
(ii) those that require cofactors that remain unaltered and tightly bound to the enzyme performing in a catalytic fashion, and
(iii) those requiring coenzymes that are chemically modified and dissociated during catalysis, performing in a stoichiometric fashion.

The requirement of cofactors or coenzymes to perform biocatalysis has profound technological implications, as will be analyzed in section 1.4.

Enzyme activity, this is, the capacity of an enzyme to catalyze a chemical reaction, is strictly dependent on its molecular structure. Enzyme activity relies upon the existence of a proper structure of the active site, which is composed by a reduced number of amino acid residues close in the three-dimensional structure of

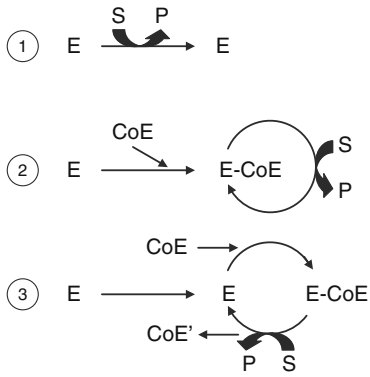


Fig. 1.3 Enzymes according to their cofactor or coenzyme requirements. 1: no requirement; 2: cofactor requiring; 3: coenzyme requiring

the protein but usually far apart in the primary structure. Therefore, any agent that promotes protein unfolding will move apart the residues constituting the active site and will then reduce or destroy its biological activity. Adverse conditions of temperature, pH or solvent and the presence of chaotropic substances, heavy metals and chelating agents can produce this loss of function by distorting the proper active site configuration. Even though a very small portion of the enzyme molecule participates in catalysis, the remaining of the molecule is by no means irrelevant to its performance. Crucial properties, like enzyme stability, are very much dependent on the enzyme three-dimensional structure. Enzyme stability appears to be determined by undefined irreversible processes governed by local unfolding in certain labile regions denoted as *weak spots*. These regions prone to unfolding are the determinants of enzyme stability and are usually located in or close to the surface of the protein molecule, which explains why the surface structure of the enzyme is so important for its catalytic stability (Eijsink et al. 2004). These regions have been the target of site-specific mutations for increasing stability. Though extensively studied, rational engineering of the enzyme molecule for increased stability has been a very complex task. In most cases, these weak spots are not easy to identify so it is not clear to what region of the protein molecule should one be focused on and, even though properly selected, it is not clear what is the right type of mutation to introduce (Gaseidnes et al. 2003). Despite the impressive advances in the field and the existence of some experimentally based rules (Shaw and Bott 1996), rational improvement of the stability is still far from being well established. In fact, the less rational approaches of directed evolution using error-prone PCR and gene shuffling have been more successful in obtaining more stable mutant enzymes (Kaur and Sharma 2006). Both strategies can combine using a set of rationally designed mutants that can then be subjected to gene shuffling (O'Fágáin 2003).

A perfectly structured native enzyme expressing its biological activity can lose it by unfolding of its tertiary structure to a random polypeptide chain in which the amino acids located in the active site are no longer aligned closely enough to perform its catalytic function. This phenomenon is termed *denaturation* and it may be reversible if the denaturing influence is removed since no chemical changes

have occurred in the protein molecule. The enzyme molecule can also be subjected to chemical changes that produce irreversible loss of activity. This phenomenon is termed *inactivation* and usually occurs following unfolding, since an unfolded protein is more prone to proteolysis, loss of an essential cofactor and aggregation (O'Fágáin 1997). These phenomena define what is called *thermodynamic* or *conformational stability*, this is the resistance of the folded protein to denaturation, and *kinetic* or *long-term stability*, this is the resistance to irreversible inactivation (Eisenthal et al. 2006). The overall process of enzyme inactivation can then be represented by:

$$N \overset{K}{\longleftrightarrow} U \overset{k}{\longrightarrow} I$$

where N represents the native active conformation, U the unfolded conformation and I the irreversibly inactivated enzyme (Klibanov 1983; Bommarius and Broering 2005). The first step can be defined by the equilibrium constant of unfolding (K), while the second is defined in terms of the rate constant for irreversible inactivation (k).

Stability is not related to activity and in many cases they have opposite trends. It has been suggested that there is a trade-off between stability and activity based on the fact that stability is clearly related to molecular stiffening while conformational flexibility is beneficial for catalysis. This can be clearly appreciated when studying enzyme thermal inactivation: enzyme activity increases with temperature but enzyme stability decreases. These opposite trends make temperature a critical variable in any enzymatic process and make it prone to optimization. This aspect will be thoroughly analyzed in Chapters 3 and 5.

Enzyme specificity is another relevant property of enzymes strictly related to its structure. Enzymes are usually very specific with respect to its substrate. This is because the substrate is endowed with the chemical bonds that can be attacked by the functional groups in the active site of the enzyme which posses the functional groups that anchor the substrate properly in the active site for the reaction to take place. Under certain conditions conformational changes may alter substrate specificity. This has been elegantly proven by site-directed mutagenesis, in which specific amino acid residues at or near the active site have been replaced producing an alteration of substrate specificity (Colby et al. 1998; diSioudi et al. 1999; Parales et al. 2000), and also by chemical modification (Kirk Wright and Viola 2001).

1.3 The Concept and Determination of Enzyme Activity

As already mentioned, enzymes act as catalysts by virtue of reducing the magnitude of the barrier that represents the energy of activation required for the formation of a transient active complex that leads to product formation (see Fig. 1.1). This thermodynamic definition of enzyme activity, although rigorous, is of little practical significance, since it is by no means an easy task to determine free energy changes for molecular structures as unstable as the enzyme–substrate complex. The direct

consequence of such reduction of energy input for the reaction to proceed is the increase in reaction rate, which can be considered as a kinetic definition of enzyme activity. Rates of chemical reactions are usually simple to determine so this definition is endowed with practicality. Biochemical reactions usually proceed at very low rates in the absence of catalysts so that the magnitude of the reaction rate is a direct and straightforward procedure for assessing the activity of an enzyme. Therefore, for the reaction of conversion of a substrate (S) into a product (P) under the catalytic action of an enzyme (E):

$$S \xrightarrow{E} P$$

$$v = -\frac{ds}{dt} = \frac{dp}{dt} \tag{1.1}$$

If the course of the reaction is followed, a curve like the one depicted in Fig 1.4 will be obtained.

This means that the reaction rate (slope of the p vs t curve) will decrease as the reaction proceeds. Then, the use of Eq. 1.1 is ambiguous if used for the determination of enzyme activity. To solve this ambiguity, the reasons underlying this behavior must be analyzed. The reduction in reaction rate can be the consequence of desaturation of the enzyme because of substrate transformation into product (at substrate depletion reaction rate drops to zero), enzyme inactivation as a consequence of the exposure of the enzyme to the conditions of reaction, enzyme inhibition caused by the products of the reaction, and equilibrium displacement as a consequence of the law of mass action. Some or all of these phenomena are present in any enzymatic reaction so that the catalytic capacity of the enzyme will vary throughout the course of the reaction. It is customary to identify the enzyme activity with the initial rate of reaction (initial slope of the "p" versus "t" curve) where all the above mentioned

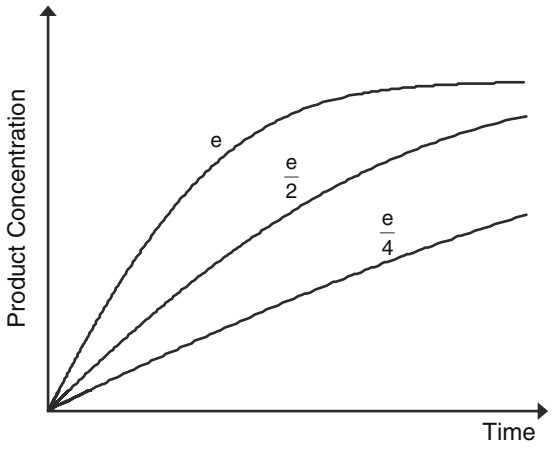


Fig. 1.4 Time course of an enzyme catalyzed reaction: product concentration versus time of reaction at different enzyme concentrations (e)

phenomena are insignificant. According to this:

$$a = v_{t\to 0} = -\left(\frac{ds}{dt}\right)_{t\to 0} = \left(\frac{dp}{dt}\right)_{t\to 0} \tag{1.2}$$

This is not only of practical convenience but fundamentally sound, since the *enzyme activity* so defined represents its *maximum catalytic potential* under a given set of experimental conditions. To what extent is this catalytic potential going to be expressed in a given situation is a different matter and will have to be assessed by modulating it according to the phenomena that cause its reduction. All such phenomena are amenable to quantification as will be presented in Chapter 3, so that the determination of this maximum catalytic potential is fundamental for any study regarding enzyme kinetics. Enzymes should be quantified in terms of its catalytic potential rather than its mass, since enzyme preparations are rather impure mixtures in which the enzyme protein can be a small fraction of the total mass of the preparation; but, even in the unusual case of a completely pure enzyme, the determination of activity is unavoidable since what matters for evaluating the enzyme performance is its catalytic potential and not its mass. Within the context of enzyme kinetics, reaction rates are always considered then as initial rates. It has to be pointed out, however, that there are situations in which the determination of initial reaction rates is a poor predictor of enzyme performance, as it occurs in the determination of degrading enzymes acting on heterogeneous polymeric substrates. This is the case of cellulase (actually an enzyme complex of different activities) (Montenecourt and Eveleigh 1977; Illanes et al. 1988; Fowler and Brown 1992), where the more amorphous portions of the cellulose moiety are more easily degraded than the crystalline regions so that a high initial reaction rate over the amorphous portion may give an overestimate of the catalytic potential of the enzyme over the cellulose substrate as a whole. As shown in Fig. 1.4, the initial slope o the curve (initial rate of reaction) is proportional to the enzyme concentration (it is so in most cases). Therefore, the enzyme sample should be properly diluted to attain a linear product concentration versus time relationship within a reasonable assay time.

The experimental determination of enzyme activity is based on the measurement of initial reaction rates. Substrate depletion or product build-up can be used for the evaluation of enzyme activity according to Eq. 1.2. If the stoichiometry of the reaction is defined and well known, one or the other can be used and the choice will depend on the easiness and readiness for their analytical determination. If this is indifferent, one should prefer to measure according to product build-up since in this case one will be determining significant differences between small magnitudes, while in the case of substrate depletion one will be measuring small differences between large magnitudes, which implies more error. If neither of both is readily measurable, enzyme activity can be determined by coupling reactions. In this case the product is transformed (chemically or enzymatically) to a final analyte amenable for analytical determination, as shown:

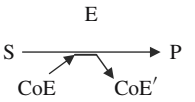
$$S \xrightarrow{E} P \xrightarrow{A} X \xrightarrow{B} Y \xrightarrow{C} Z$$

In this case enzyme activity can be determined as:

$$a = v_{t\to 0} = -\left(\frac{ds}{dt}\right)_{t\to 0} = \left(\frac{dp}{dt}\right)_{t\to 0} = \left(\frac{dz}{dt}\right)_{t\to 0} \tag{1.3}$$

provided that the rate limiting step is the reaction catalyzed by the enzyme, which implies that reagents A, B and C should be added in excess to ensure that all P produced is quantitatively transformed into Z.

For those enzymes requiring (stoichiometric) coenzymes:



activity can be determined as:

$$a = v_{t\to 0} = -\left(\frac{dcoe}{dt}\right)_{t\to 0} = \left(\frac{dcoe'}{dt}\right)_{t\to 0} \tag{1.4}$$

This is actually a very convenient method for determining activity of such class of enzymes, since organic coenzymes (i.e. FAD or NADH) are usually very easy to determine analytically. An example of a coupled system considering coenzyme determination is the assay for lactase (β-galactosidase; EC 3.2.1.23). The enzyme catalyzes the hydrolysis of lactose according to:

$$\text{Lactose} + H_2O \rightarrow \text{Glucose} + \text{Galactose}$$

Glucose produced can be coupled to a classical enzymatic glucose kit, that is: hexoquinase (Hx) plus glucose 6 phosphate dehydrogenase (G6PD), in which:

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{Hx}} \text{Glucose 6Pi} + \text{ADP}$$

$$\text{Glucose 6Pi} + \text{NADP}^+ \xrightarrow{\text{G6PD}} \text{6PiGluconate} + \text{NADPH}$$

where the initial rate of NADPH (easily measured in a spectrophotometer; see ahead) can be then stoichiometrically correlated to the initial rate of lactose hydrolysis, provided that the auxiliary enzymes, Hx and G6PD, and co-substrates are added in excess.

Enzyme activity can be determined by a continuous or discontinuous assay. If the analytical device is provided with a recorder that register the course of reaction, the initial rate could be easily determined from the initial slope of the product (or substrate, or coupled analyte, or coenzyme) concentration versus time curve. It is not always possible or simple to set up a continuous assay; in that case, the course of reaction should be monitored discontinuously by sampling and assaying at predetermined time intervals and samples should be subjected to inactivation to stop the reaction. This is a drawback, since the enzyme should be rapidly, completely and irreversibly inactivated by subjecting it to harsh conditions that can interfere with the

analytical procedure. Data points should describe a linear "p" versus "t" relationship within the time interval for assay to ensure that the initial rate is being measured; if not, enzyme sample should be diluted accordingly. Assay time should be short enough to make the effect of the products on the reaction rate negligible and to produce a negligibly reduction in substrate concentration. A major issue in enzyme activity determination is the definition of a control experiment for discriminating the non-enzymatic build-up of product during the assay. There are essentially three options: to remove the enzyme from the reaction mixture by replacing the enzyme sample by water or buffer, to remove the substrate replacing it by water or buffer, or to use an enzyme placebo. The first one discriminates substrate contamination with product or any non-enzymatic transformation of substrate into product, but does not discriminate enzyme contamination with substrate or product; the second one acts exactly the opposite; the third one can in principle discriminate both enzyme and substrate contamination with product, but the pitfall in this case is the risk of not having inactivated the enzyme completely. The control of choice depends on the situation. For instance, when one is producing an extracellular enzyme by fermentation, enzyme sample is likely to be contaminated with substrate and or product (that can be constituents of the culture medium or products of metabolism) and may be significant, since the sample probably has a low enzyme protein concentration so that it is not diluted prior to assay; in this case, replacing substrate by water or buffer discriminates such contamination. If, on the other hand, one is assaying a preparation from a stock enzyme concentrate, dilution of the sample prior to assay makes unnecessary to blank out enzyme contamination; replacing the enzyme by water or buffer can discriminate substrate contamination that is in this case more relevant. The use of an enzyme placebo as control is advisable when the enzyme is labile enough to be completely inactivated at conditions not affecting the assay. An alternative is to use a double control replacing enzyme in one case and substrate in the other by water or buffer. Once the type of control experiment has been decided, control and enzyme sample are subjected to the same analytical procedure, and enzyme activity is calculated by subtracting the control reading from that of the sample, as illustrated in Fig. 1.5.

Analytical procedures available for enzyme activity determinations are many and usually several alternatives exist. A proper selection should be based on sensibility, reproducibility, flexibility, simplicity and availability. Spectrophotometry can be considered as a method that fulfils most, if not all, such criteria. It is based on the absorption of light of a certain wavelength as described by the Beer–Lambert law:

$$A_\lambda = \varepsilon \cdot 1 \cdot c \tag{1.5}$$

where:

$$A_\lambda = \log \frac{I}{I_0} \tag{1.6}$$

The value of ε can be experimentally obtained through a calibration curve of absorbance versus concentration of analyte, so that the reading of A_λ will allow the determination of its concentration. Optical path width is usually 1 cm. The method is based on the differential absorption of product (or coupling analyte or modified

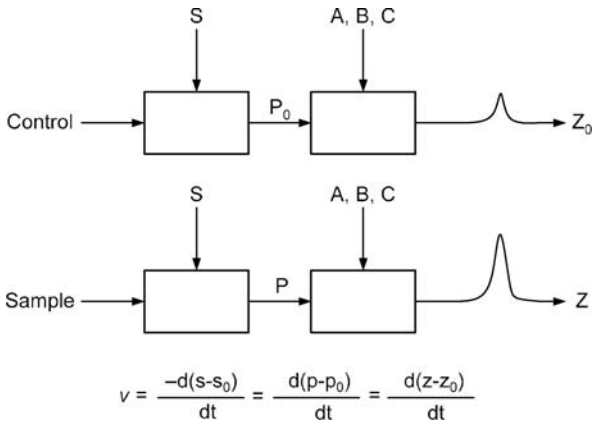


Fig. 1.5 Scheme for the analytical procedure to determine enzyme activity. S: substrate; P: product; P_0: product in control; A, B, C: coupling reagents; Z: analyte; Z_0: analyte in control; s, p, z are the corresponding molar concentrations

coenzyme) and substrate (or coenzyme) at a certain wavelength. For instance, the reduced coenzyme NADH (or NADPH) has a strong peak of absorbance at 340 nm while the absorbance of the oxidized coenzyme NAD^+ (or $NADP^+$) is negligible at that wavelength; therefore, the activity of any enzyme producing or consuming NADH (or NADPH) can be determined by measuring the increase or decline of absorbance at 340 nm in a spectrophotometer. The assay is sensitive, reproducible and simple and equipment is available in any research laboratory. If both substrate and product absorb significantly at a certain wavelength, coupling the detector to an appropriate high performance liquid chromatography (HPLC) column can solve this interference by separating those peaks by differential retardation of the analytes in the column. HPLC systems are increasingly common in research laboratories, so this is a very convenient and flexible way for assaying enzyme activities.

Several other analytical procedures are available for enzyme activity determination. Fluorescence, this is the ability of certain molecules to absorb light at a certain wavelength and emit it at another, is a property than can be used for enzymatic analysis. NADH, but also FAD (flavin adenine dinucleotide) and FMN (flavin mononucleotide) have this property that can be used for those enzyme requiring that molecules as coenzymes (Eschenbrenner et al. 1995). This method shares some of the good properties of spectrophotometry and can also be integrated into an HPLC system, but it is less flexible and the equipment not so common in a standard research laboratory.

Enzymes that produce or consume gases can be assayed by differential manometry by measuring small pressure differences, due to the consumption of the gaseous substrate or the evolution of a gaseous product that can be converted into substrate or product concentrations by using the gas law. Carboxylases and decarboxylases are groups of enzymes that can be conveniently assayed by differential manometry in a respirometer. For instance, the activity of glutamate decarboxylase

(EC 4.1.1.15), that catalyzes the decarboxylation of glutamic acid to γ-aminobutyric acid and CO_2, has been assayed in a differential respirometer by measuring the increase in pressure caused by the formation of gaseous CO_2 (O'Learys and Brummund 1974).

Enzymes catalyzing reactions involving optically active compounds can be assayed by polarimetry. A compound is considered to be optically active if polarized light is rotated when passing through it. The magnitude of optical rotation is determined by the molecular structure and concentration of the optically active substance which has its own specific rotation, as defined in Biot's law:

$$\alpha = \alpha_0 \cdot l \cdot c \tag{1.7}$$

Polarimetry is a simple and accurate method for determining optically active compounds. A polarimeter is a low cost instrument readily available in many research laboratories. The detector can be integrated into an HPLC system if separation of substrates and products of reaction is required. Invertase (β-D-fructofuranoside fructohydrolase; EC 3.2.1.26), a commodity enzyme widely used in the food industry, can be conveniently assayed by polarimetry (Chen et al. 2000), since the specific optical rotation of the substrate (sucrose) differs from that of the products (fructose plus glucose).

Some depolymerizing enzymes can be conveniently assayed by viscometry. The hydrolytic action over a polymeric substrate can produce a significant reduction in kinematic viscosity that can be correlated to the enzyme activity. Polygalacturonase activity in pectinase preparations (Gusakov et al. 2002) and endo β1–4 glucanase activity in cellulose preparations (Canevascini and Gattlen 1981; Illanes and Schaffeld 1983) have been determined by measuring the reduction in viscosity of the corresponding polymer solutions.

A comprehensive review on methods for assaying enzyme activity has been recently published (Bisswanger 2004).

Enzyme activity is expressed in units of activity. The Enzyme Commission of the International Union of Biochemistry recommends to express it in international units (IU), defining 1 IU as the amount of an enzyme that catalyzes the transformation of 1 μmol of substrate per minute under standard conditions of temperature, optimal pH, and optimal substrate concentration (International Union of Biochemistry). Later on, in 1972, the Commission on Biochemical Nomenclature recommended that, in order to adhere to SI units, reaction rates should be expressed in moles per second and the *katal* was proposed as the new unit of enzyme activity, defining it as the catalytic activity that will raise the rate of reaction by 1 mol/second in a specified assay system (Anonymous 1979). This latter definition, although recommended, has some practical drawbacks. The magnitude of the katal is so big that usual enzyme activities expressed in katals are extremely small numbers that are hard to appreciate; the definition, on the other hand, is rather vague with respect to the conditions in which the assay should be performed. In practice, even though in some journals the use of the katal is mandatory, there is reluctance to use it and the former IU is still more widely used.

Going back to the definition of IU there are some points worthwhile to comment. The magnitude of the IU is appropriate to measure most enzyme preparations, whose activities usually range from a few to a few thousands IU per unit mass or unit volume of preparation. Since enzyme activity is to be considered as the maximum catalytic potential of the enzyme, it is quite appropriate to refer it to optimal pH and optimal substrate concentration. With respect to the latter, optimal is to be considered as that substrate concentration at which the initial rate of reaction is at its maximum; this will imply reaction rate at substrate saturation for an enzyme following typical Michaelis-Menten kinetics or the highest initial reaction rate value in the case of inhibition at high substrate concentrations (see Chapter 3). With respect to pH, it is straightforward to determine the value at which the initial rate of reaction is at its maximum. This value will be the true operational optimum in most cases, since that pH will lie within the region of maximum stability. However, the opposite holds for temperature where enzymes are usually quite unstable at the temperatures in which higher initial reaction rates are obtained; actually the concept of "optimum" temperature, as the one that maximizes initial reaction rate, is quite misleading since that value usually reflects nothing more than the departure of the linear "p" versus "t" relationship for the time of assay. For the definition of IU it is then more appropriate to refer to it as a "standard" and not as an "optimal" temperature. Actually, it is quite difficult to define the right temperature to assay enzyme activity. Most probably that value will differ from the one at which the enzymatic process will be conducted; it is advisable then to obtain a mathematical expression for the effect of temperature on the initial rate of reaction to be able to transform the units of activity according to the temperature of operation (Illanes et al. 2000).

It is not always possible to express enzyme activity in IU; this is the case of enzymes catalyzing reactions that are not chemically well defined, as it occurs with depolymerizing enzymes, whose substrates have a varying and often undefined molecular weight and whose products are usually a mixture of different chemical compounds. In that case, units of activity can be defined in terms of mass rather than moles. These enzymes are usually specific for certain types of bonds rather than for a particular chemical structure, so in such cases it is advisable to express activity in terms of equivalents of bonds broken.

The choice of the substrate to perform the enzyme assay is by no means trivial. When using an enzyme as process catalyst, the substrate can be different from that employed in its assay that is usually a model substrate or an analogue. One has to be cautious to use an assay that is not only simple, accurate and reproducible, but also significant. An example that illustrates this point is the case of the enzyme glucoamylase (exo-1,4-α-glucosidase; EC 3.2.1.1): this enzyme is widely used in the production of glucose syrups from starch, either as a final product or as an intermediate for the production of high-fructose syrups (Carasik and Carroll 1983). The industrial substrate for glucoamylase is a mixture of oligosaccharides produced by the enzymatic liquefaction of starch with α-amylase (1,4-α-D-glucan glucanohydrolase; EC 3.2.1.1). Several substrates have been used for assaying enzyme activity including high molecular weight starch, small molecular weight oligosaccharides, maltose and maltose synthetic analogues (Barton et al. 1972; Sabin and Wasserman

1987; Goto et al. 1998). None of them probably reflects properly the enzyme activity over the real substrate, so it will be a matter of judgment and experience to select the most pertinent assay with respect to the actual use of the enzyme. Hydrolases are currently assayed with respect to their hydrolytic activities; however, the increasing use of hydrolases to perform reactions of synthesis in non-aqueous media make this type of assay not quite adequate to evaluate the synthetic potential of such enzymes. For instance, the protease subtilisin has been used as a catalyst for a transesterification reaction that produces thiophenol as one of the products (Han et al. 2004); in this case, a method based on a reaction leading to a fluorescent adduct of thiophenol is a good system to assess the transesterification potential of such proteases and is to be preferred to a conventional protease assay based on the hydrolysis of a protein (Gupta et al. 1999; Priolo et al. 2000) or a model peptide (Klein et al. 1989).

1.4 Enzyme Classes. Properties and Technological Significance

Enzymes are classified according to the guidelines of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB) (Anonymous 1984) into six families, based on the type of chemical reaction catalyzed. A four digit number is assigned to each enzyme by the Enzyme Commission (EC) of the IUBMB: the first one denotes the family, the second denotes the subclass within a family and is related to the type of chemical group upon which it acts, the third denotes a subgroup within a subclass and is related to the particular chemical groups involved in the reaction and the forth is the correlative number of identification within a subgroup.

The six families are:

1. Oxidoreductases. Enzymes catalyzing oxidation/reduction reactions that involve the transfer of electrons, hydrogen or oxygen atoms. There are 22 subclasses of oxido-reductases and among them there are several of technological significance, such as the dehydrogenases that oxidize a substrate by transferring hydrogen atoms to a coenzyme (NAD^+, $NADP^+$, FAD^+, FMN) that acts as an acceptor. Oxidoreductases are involved in the central metabolic pathways of the cell; they require coenzymes and are strictly intracellular.
2. Transferases. Enzymes catalyzing the transfer of a functional group from a donor to a suitable acceptor. There are nine subgroups of transferases according to the chemical nature of the group being transferred. These enzymes play a crucial role in cell metabolism; among them, methyltransferases, acyltransferases, transaminases, phosphotransferases and glycosyltransferases are particularly relevant. Transferases require coenzymes and are strictly intracellular. No large-scale applications of transferases exist but some of them are commercial enzymes of relevance in research. A prominent example is *Taq* DNA polymerase (DNA nucleotidyltransferase RNA-directed or reverse transcriptase; EC 2.7.7.49), a

thermostable enzyme from the thermophilic bacterium *Thermus aquaticus* (Tindall and Kunkel 1988) which is a key enzyme in PCR amplification of genetic material (Bartlett and Stirling 2003).

3. Hydrolases. Enzymes catalyzing reactions of hydrolysis, this is, the cleavage of a chemical bond by the action of water. There are 12 subgroups of hydrolases according to the type of susceptible bond. These enzymes are relevant for catabolism by supplying assimilable nutrients to the cell. Most of the enzymes of technological relevance belong to this family: esterases, proteases and glycosidases are prominent. Most hydrolases do not require coenzymes, many are extracellular and robust enough to withstand harsh process conditions. Under proper conditions, hydrolases can catalyze the reverse reactions of bond formation with water elimination; this type of reactions is of considerable technological potential.
4. Lyases. Enzymes catalyzing reactions of non-hydrolytic and non-oxidative cleavage of chemical bonds. They are divided into seven subgroups, according to the type of susceptible bond: C−C; C−O; C−N; C−S; C−X (halides); P−O and other bonds. Enzymes belonging to this family perform different metabolic functions associated not only with cell catabolism but also with biosynthesis by acting in reverse. Prominent among lyases are aldolases, usually acting in reverse reactions of C−C bond formation (see section 6.5), carboxylases and decarboxylases, hydratases and dehydratases. Most, but not all lyases are intracellular and some do not require coenzymes. For instance, pectate lyase ($(1 \rightarrow 4)$-α-D-galacturonan lyase; EC 4.2.2.2) from different sources is extracellular and requires Ca^{++} as cofactor but has no coenzyme requirement (Nasser et al. 1990; Brühlmann 1995; Castang et al. 2004). These properties make lyases potential candidates for technological applications. In fact, nitrile hydratase (nitrile hydrolyase; EC 4.2.1.84) is a prominent enzyme of this group that has acquired technological relevance in the industrial production of acrylamide from acrylonitrile with a market exceeding 30,000 tons/year (Yamada and Kobayashi 1996; Miller and Nagarajan 2000). Aside from the production of high-fructose corn syrup, this might be the large enzymatic process to date and surely the most relevant industrial application of enzymes in organic synthesis. Other relevant biocatalytic processes with lyases are the production of L-aspartate (a building block for aspartame) with aspartase (aspartate ammonia-lyase; EC 4.3.1.1) (Chibata et al. 1974; Fusee 1987; Gill et al. 1996), the production of fumarate with fumarase (fumarate hydratase; EC 4.2.1.2) (Furui et al. 1988; Bélafi-Bakó et al. 2004) and the production of urocanic acid with histidase (L-histidine ammonia lyase; EC 4.3.1.3) (Shibatani et al. 1974). Lyases have been also studied for asymmetric synthesis of optically active organic compounds (van der Werf et al. 1994; Vidal et al. 2005).
5. Isomerases. Enzymes catalyzing reactions of conversion of a substrate into an isomer, this is, a substance with the same number and types of atoms. There are six subgroups of isomerases depending on the type of isomer produced: racemases and epimerases; *cis–trans*-isomerases, intramolecular oxidoreductases,

intramolecular transferases (mutases), intramolecular lyases and other isomerases. Most isomerases are intracellular and some of them require cofactors but nor organic coenzymes. Very few isomerases are being exploited technologically; however, the case of glucose isomerase (actually xylose isomerase; D-xylose aldose–ketose-isomerase EC 5.3.1.5) is paradigmatic, being the largest application of enzyme technology up to now. The enzyme is used in the production of high-fructose syrups (HFS), mostly from cornstarch (Carasik and Carroll 1983), with an estimated output of over 10 million tons. HFS has replaced the industrial use of sugar (sucrose) to a considerable extent: in 2001 HFS from corn accounted for 55% of the sweetener market and annual production of HFS is still growing at a rate of 3–4% (Bhosale et al. 1996).

6. Ligases. Enzymes catalyzing reactions of covalent linkage of two molecules. These are the enzymes responsible for cell anabolism and as such perform an essential role in the reactions of synthesis inside the cell (sometimes they are named synthetases). There are six subgroups of ligases according to the type of bond formed: C−O, C−S, C−N, C−C, phosphoric esters and C-metal. Ligases are complex high molecular weight strictly intracellular coenzyme requiring enzymes; the reaction of synthesis is frequently coupled with the hydrolysis of an energy rich bond, as in ATP or other energy rich containing triphosphates. Despite its metabolic relevance, there are no current technological applications of these complex and unstable enzymes at large scale. Some of them are, however, commercialized at a high price as specialty enzymes for research applications. This is the case of T4 DNA ligase (polydeoxyribonucleotide synthase; EC 6.5.1.1) routinely used in genetic engineering protocols (Aslanidis and de Jong 1990; Brenner et al. 2000).

In summary, from the six families of enzymes only hydrolases have had technological significance. The reason underlying is that these enzymes are well endowed to perform as biocatalysts since they are robust, rather simple proteins not requiring coenzymes being many of them extracellular. Production is therefore rather simple (see Chapter 2), enzyme costs are low and they perform well under harsh process conditions. For the rest of the enzymes, some ligases (Thomas et al. 2002) and isomerases (Crabb and Shetty 1999) have been used on large scale processes and more recently, some dehydrogenases as well (Hummel 1999; Leuchtenberger et al. 2005), even though in this later case the technology is much more complex involving cofactor retention and regeneration (van der Donk and Zhao 2003; Woodyer et al. 2006). Several new applications for non-hydrolytic enzymes in organic synthesis will bloom in the following decades (García-Junceda et al. 2004; Pollard and Woodley 2006; Thayer 2006); however the use of cheap and robust hydrolases acting in reverse is at present foreseen as a better option for biocatalysis in organic synthesis (Davis and Boyer 2001).

A detailed presentation of enzyme nomenclature and classification can be obtained from the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology at the website http://www.chem.qmul.ac.uk/iubmb/enzyme/.

1.5 Applications of Enzymes. Enzyme as Process Catalysts

Enzymes were used long before their nature and properties were known. Some digestive enzymes and pepsin were reported in the 1830s, but the onset of industrial enzymes can be traced back to the end of the nineteenth century when Takamine (1894) obtained the first patent for an enzymatic process: a diastase from mold, designated as Takadiastase which notably has remained in the marked up to present time. Enzymes from animal organs played an important role in the early 1900s when Röhm (1908) developed the first normalized pancreatin as a bating agent in leather manufacture and later introduced the use of such enzyme for detergent formulations. Some early microbial enzyme preparations belong to that period, like an amylase product from *Bacillus subtilis* used for textile sizing (Boidin and Effront 1917) and fungal proteases for leather bating produced by semi-solid fermentation by Röhm and Haas. A complete review on the early patents in enzyme technology was published by Neidleman (1991). Enzyme applications bloomed after World War II, associated to the development of industrial microbiology and biochemical engineering. The first fully enzymatic industrial process was developed in the mid-1960s for the conversion of starch into glucose syrup and included starch thinning with bacterial α-amylase and saccharification with fungal glucoamylase (Michael-Sinclair 1965); then the process was extended to the production of high-fructose syrup that was made possible because of the advances in biocatalyst production by enzyme immobilization (Hemmingsen 1979; Carasik and Carroll 1983). Genetic engineering and protein engineering tools have been major contributors to widen the spectrum of enzyme uses in the last two decades (Tucker 1995; Alberghina 2000); it is estimated that no less than 50% of the enzymes marketed today come from genetically manipulated organisms by genetic and protein engineering techniques. Traditional areas of application like, food, feed, laundry, textiles and tanning (Uhlig 1998) have been extended in recent years to the pharmaceutical and fine-chemicals industry (Lauwers and Scharpé 1997; Huisman and Gray 2002; Aehle 2003; Pollard and Woodley 2006). In fact, enzyme applications in organic synthesis represent now the most promising and challenging area for enzyme technology development (Asano 2002; Schoemaker et al. 2003; García-Junceda et al. 2004), as will be analyzed in the next section.

Industrial applications represent more than 80% of the global market of enzymes. A distinction should be made between those cases in which the enzymatic conversion of the raw material into the product is the key operation and those in which the enzyme is used as an additive to modify certain functional property of the product. In the first case the enzymatic reaction is carried out in a controlled environment at optimized conditions with respect to the catalytic potential of the enzyme, while in the second case conditions for enzyme action are not specified to optimize its activity and sometimes not even controlled. Examples of the first case are the production of high-fructose syrups with immobilized glucose isomerase and the production of 6-aminopenicillanic acid from penicillin G with immobilized penicillin acylase; examples of the second case are the use of fungal proteases in dough making and the use of pancreatin in leather bating. Most conventional uses of enzymes refer to

the use of hydrolases as process catalysts or additives for the food, feed, detergent, leather and textile industries and despite the impressive advances in biocatalysis they still represent the major share of the enzyme market. Most relevant applications of those enzymes are summarized in Table 1.2. Food enzymes are by far the most widely used and detailed information on them can be found in books devoted to the subject (Wong 1995; Whitehurst and Law 2002). A review on the subject can be found elsewhere (Illanes 2000).

Fungal α-amylases are extensively used as additives in bakery and brewing, while bacterial α-amylases are used as process biocatalyst in the liquefaction of starch to produce dextrins for subsequent hydrolysis with glucoamylase. Hyperthermostable, acid tolerant and less Ca^{++} dependent α-amylases are highly desirable for glucose production and significant progress has been obtained in recent years through genetic and protein engineering (Joyet et al. 1992; Declerck et al. 1995; Crabb and Mitchinson 1997; Crabb and Shetty 1999). Fungal glucoamylase is predominantly used for glucose production from enzyme thinned starch and also significant progress has been made to tailor the enzyme to match the process requirements. One major problem with glucoamylase is that at the high concentration of substrate and high conversion yield required the enzyme tends to form reversion products (mainly maltose and isomaltose) that decreases yield. By using protein engineering, variants of *Aspergillus awamori* have been obtained where these reverse reactions are severely depressed without affecting the hydrolytic activity over α1–4 linkages (Sierks and Svennson 1994). Increased thermal stability and shift in pH optimum are also relevant characteristics for glucoamylase that have been obtained by genetic and protein engineering (Coutinho and Reilly 1997; Fang and Ford 1998). Glucoamylase is used mainly in soluble form and the industry has been reluctant to move into a continuous process with immobilized enzyme because of the difficulty of obtaining the high conversion yields (96 to 98 dextrose equivalent) required (Maeda et al. 1979) and to avoid the redesign of already well established processes. The former problem has been solved to a great extent by the advances in enzyme immobilization (Bryjak et al. 2007; Kovalenko et al. 2007; Milosavić et al. 2007) so that replacement of the current batch process with soluble enzyme by a continuous process with immobilized enzyme is just a matter of time. Co-immobilization of glucoamylase and pullulanase is a viable option for obtaining very high conversion yields (Roy and Gupta 2004).

Pectinases are actually mixtures of different enzyme activities, mainly pectin methylesterase, polygalacturonase and pectate lyase (Whitaker 1990). They are intensively used for fruit and vegetable juice extraction and also for fruit juice clarification. Wine makers, initially reluctant to use exogenous enzymes, have been slowly but steadily incorporating pectinases (and also β-glucanases) in different stages of the process: maceration, clarification and maturation (van Oort and Canal-Llaubères 2002). Use of pectinases in the fruit processing industry has become quite sophisticated and enzymes are now marketed by the leading companies to suit particular customer needs according to the characteristics of the raw material; it is common to combine pectinases with other hydrolytic enzymes, like cellulases, hemicellulases and β-glucanases. More recently, alkaline pectinases have been developed for the

Table 1.2 Hydrolytic Enzymes of Commercial Relevance

Enzyme	Source	Application
Carbohydrases		
α-Amylase	Mold	Bakery, confectionery
α-Amylase	Bacteria	Starch thinning; detergents; fabrics desizing
Glucoamylase	Mold	Glucose syrup
β-Amylase and pullulanase	Plant, bacteria	Glucose syrup
Pectinase	Mold	Fruit juice extraction and clarification; winemaking
Cellulase	Mold	Fruit juice extraction and clarification; textile stonewashing and bio-polishing, detergents; digestive-aid
Hemicellulase	Mold, bacteria	Bakery, bleaching of wood pulp
Lactase	Yeast, mold	Delactosed milk and dairy products; whey treatment and upgrading
Invertase	Yeast, mold	Confectionery
Phytase	Bacteria	Animal nutrition
β-Glucanase	Mold	Animal nutrition; brewing
Naringinase	Mold	Juice debittering
Proteases		
Papain	Papaya	Yeast and meat extracts; beer chill-proofing; protein hydrolyzates; meat tenderization; leather bating, animal nutrition; digestive-aid; anti-inflammatory
Bromelain	Pineapple	Pharmaceutical: anti-inflammatory; burn debridement; enhancement of drug absorption
Pepsin	Animal	Cheesemaking
Rennin	Animal, recombinant yeasts and molds	Cheesemaking
Neutral protease	Mold, bacteria	Bakery; protein hydrolyzates
Alkaline protease	Bacteria	Detergents; stickwater recovery
Aminopeptidase	Mold, bacteria	Debittering of protein hydrolysates
Other hydrolases		
Pancreatin	Animal	Digestive aid; tannery
Lipase	Animal, mold, yeast, bacteria	Flavor development in milk and meat products; detergents
Aminoacylase	Mold	Food and feed supplementation
Penicillin acylase	Bacteria, mold	β-Lactam precursors for semi-synthetic β-lactam antibiotics
Urease	Bacteria	Removal of urea in alcoholic beverages

retting and degumming of textile fibers, treatment of pectic wastewaters, paper making, and coffee and tea fermentations (Kashyap et al. 2001; Hoondal et al. 2002).

Cellulase is an enzymatic complex composed usually by an exo acting hydrolase (1,4 β-D-glucan cellobiohydrolase) an endo acting hydrolase (1,4 β-D-glucan glucano hydrolase) and a cellobiase (β-D-glucoside glucohydrolase) (Mandels and Reese 1960; Illanes and Rossi 1980; Marsden and Gray 1986). These fractions act synergistically to breakdown the cellulose fibers down to glucose (Ryu and Mandels 1980). Cellulases have many and increasing applications in the food, feed, detergent and textile industries and also in the pharmaceutical industry as digestive-aid. Cellulases are used alone or in combination with pectinases and hemicellulases for the extraction of juices, oils and agar (San Martín et al. 1988; Uhlig 1998; Ovando-Chacón and Waliszewski 2005), for the enzymatic stonewashing of denim and cotton fabric bio-polishing (Foody and Tolan 1999; Anish et al. 2006), as ingredients in detergent formulations (Convents et al. 1995) and in several digestive-aid preparations in combination with other hydrolytic enzymes (Rachman 1997). Beyond these rather small-scale applications for cellulases, a tremendous potential lies in the field of biofuels. Bioethanol reached the impressive levels of 4 billion liters per year in the 1970s in Brazil, but after the oil crisis political interest disappeared and production was severely reduced (Lima et al. 2001). Biofuels are again center stage because of the increasing levels of energy consumption, progressive depletion of oil reservoirs and the threatening of the greenhouse effect (Schubert 2006). In fact, biofuels (mainly bioethanol, but also biodiesel, biogas and biohydrogen) are produced from renewable resources and are CO_2 neutral. Bioethanol is now mainly produced from corn kernels and sugarcane. In the first case, α-amylase and glucoamylase are required prior to fermentation. However, to reach a significant impact on the energy bill more abundant and less demanded feedstocks are required. It is estimated that only lignocellulose derived ethanol can meet this challenge, so considerable effort is now being spent to overcome the technological limitations still prevailing (Wyman 1996; Sheehan and Himmel 1999). Among those, the requirement of more active and more stable cellulases is crucial. The goal is to reduce the cost of using cellulase enzymes by front line technology with an expected reduction from about US$ 0.1 to about US$ 0.015 per liter of bioalcohol. This requires significant increase in specific activity and production efficiency. Optimized combinations of the different cellulase fractions have been successfully employed (Baker et al. 1998) and promising results have been obtained in cellulase improvement by genetic and protein engineering techniques (Godbole et al. 1999; Schülein 2000). These advances go in parallel with those in the field of plant genetic engineering where fast growing species with lower lignin and higher cellulose content, and ligninase self producing species are promising developments (Sticklen 2006). It is estimated that within the next decade massive production of bioalcohol from lignocelluose will be a reality, contributing significantly to fossil fuel replacement (Black and Miller 2006; Gray et al. 2006).

Enzymes have very relevant applications in the pulp and paper industry now threatened by environmental regulations. Hemicellulases are being currently used in wood pulp bleaching to partially substitute chlorine-based bleaching procedures

that produce harmful chlorinated organic compounds and an increasing market for this enzyme has developed in the last decade (van Beilen and Li 2002) Ligninases have a great potential both in wood bleaching and pulping; however, ligninases are quite complex coenzyme requiring enzymes being this complexity a major hurdle for its massive application (Eriksson et al. 1997).

β-Galactosidase (lactase) breaks down lactose into its monosaccharide constituents: glucose and galactose. Monomers are far more soluble, sweet and digestible than lactose, so the enzyme is intensely utilized in the dairy industry. β-galactosidase is a very ubiquitous enzyme but industrial sources come mainly from yeasts (neutral β-galactosidase) and mold (acid β-galactosidase). Lactose hydrolysis in milk and dairy products and in sweet whey is then obtained with yeast β-galactosidase, while mold β-galactosidase are preferred for acid whey treatment (Illanes et al. 1993). Applications of enzymes to the dairy industry have been thoroughly analyzed (Greenberg and Mahoney 1981; Gekas and López-Leiva 1985). Lactose intolerance due to lactose deficiency is an ethnic related deficiency that tends to be more severe in infants and children and affect many million people worldwide (Heyman 2006). Low-lactose milk produced by enzymatic treatment has a striking commercial success and can be found in almost any supermarket today. Several process strategies for lactose hydrolysis in milk have been developed mainly based on membrane enzymatic reactors and process innovations appear every year (Petzelbauer et al. 2002; Novalin et al. 2005; Neuhaus et al. 2006). β-Galactosidase tablets are also sold in pharmacies over the counter (Law 2002). Reduction of lactose in dairy products is beneficial by avoiding lactose crystallization in *dulce de leche* and ice-cream (Trzecieski 1983; Martínez et al. 1990; Monte 1999) and by improving fermentation in yoghurt products; additional benefits are increased sweetness and color development. Upgrading of cheese whey by lactose hydrolysis is another relevant application of lactase. Hydrolyzed whey can be used as a feed supplement, as medium for alcohol production and as a starting material for the production of syrups (Sprössler and Plainer 1983; Gekas and López-Leiva 1985; van Griethuysen-Dilber et al. 1988; Illanes et al. 1990). Actually, hydrolyzed and isomerized whey has a sweetening power similar to that of sucrose. However, the main point for whey reclamation is environmental protection (Marwaha and Kennedy 1988).

Enzymes are increasingly being used in monogastric animal nutrition, since non-starch polysaccharides in diets have an antinutritive activity (Bedford 2000). Microbial enzymes targeting these polymers, mainly phytase and β-glucanase, are beneficial to enhance feed to animal weight ratio and to abate pollution (Walsh et al. 1993). Phytase breaks down the undigestible phytic acid and release digestible phosphorus; in this way digestibility increases and excess phosphate in the diet is avoided so reducing phosphate output in the manure (Cooperband and Good 2002; Vohra and Satyanarayana 2003). It has been reported that addition of recombinant phytase to animal feed reduces the addition of extra phosphorus by 20% and the release of phosphate through manure by 25–30% (http://www.efb-central.org/topics/genetic/menu4_5.htm). By the end of the past century the market for phytase was already US$ 500 million (Abelson 1999) but in the last decade it must have increased significantly because of the increasing costs of grain and

concern about phosphate pollution. No food grade phytase is already in the market, but phytases from yeast are very good candidates (Kaur et al. 2007). Genetic engineering has contributed to increase the levels of expression and in this way increase productivity (van Dijk 1999). Grains used for animal nutrition contain considerable amount of β-glucans that coat proteins and starch so reducing its nutritional value. Added β-glucanases have shown to produce a significant increase in nutrient assimilation (Walsh et al. 1993; Choct et al. 1995; Józefiak et al. 2006). A major concern in enzymes for animal nutrition is that feed pellets involve processing at elevated temperatures and harsh conditions that the enzyme must withstand; therefore, considerable effort has been devoted to develop more resistant enzymes by genetic manipulation (Pasamontes et al. 1997; Lucca et al. 2001).

Protease degrading enzymes constitute the largest category of industrial enzymes, its application covering relevant industrial sectors like food and beverages, detergents, leather and pharmaceuticals. Acid and neutral proteases are relevant to the food industry and, among them, rennin and its substitutes are of paramount importance in cheesemaking; its evolution and present status is analyzed in depth in section 2.1. Plant and animal acid and neutral proteases are still important, especially in pharmaceutical products and some food applications. They roughly represent 15% of all protease market, but microbial proteases are now more relevant for the production of protein hydrolyzates (Barzana and García-Garibay 1994; Nielsen and Olsen 2002) and other applications in the food sector (see section 2.1). Protein and genetic engineering of neutral proteases have been devoted to produce more potent and stable enzyme preparations (Imanaka et al. 1986). Alkaline proteases are of paramount importance in detergent formulation and considerable progress has been made since its introduction in the 1960s (Maurer 2004). The case of alkaline proteases is actually one of the best examples of successful application of genetic and protein engineering techniques for industrial enzyme production (Teplyakov et al. 1992; Gupta et al. 2002; Tjalsma et al. 2004). Besides detergents, alkaline proteases are used in the tanning (Varela et al. 1997; Tang et al. 2004; Thanikaivelan et al. 2004) and fish-meal industries (Schaffeld et al. 1989; Aguilera 1994). Pancreatin is a multi-enzyme extract from animal pancreas containing proteolytic, lipolytic and amylolytic activities that has been used traditionally in the tanning industry (Outtrup and Boyce 1990) and also as a digestive-aid (Greenberg 1996).

Lipases are defined by its capacity of hydrolyzing esters from fatty acids and as such several traditional applications of lipases emerged in the food sector (i.e. flavor improvement in dairy and meat products) and cleansing of glass surfaces (Scoville and Novicova 1999). More recently lipases have been incorporated into detergents to aid in the stain removal of oily stains; technological development was not easy because lipases were required that withstand the harsh conditions of laundry: high pH, moderately high temperature and the presence of oxidizing agents (Rathi et al. 2001; Gulati et al. 2005). Lipases have also industrial application for the control of pitch in paper and pulp manufacturing (Gutiérrez et al. 2001). However, lipases are now being considered as the most important group of biocatalysts because of the enormous potential of lipases for organic synthesis as will be analyzed in section 1.6 and further considered as a case study in section 6.3. A comprehensive review on

industrial lipase applications and potentials has been recently published (Hasan et al. 2006). The chimioselective esterification of wood sterols with lipases will be analyzed as a case study in section 6.3.

Aminoacylase is another hydrolytic enzyme of industrial impact used in the multiton process for the production of L-amino acids for human and animal nutrition from the corresponding racemates. The process is based on the enantiospecificity of the enzyme to selectively hydrolyze the L-enantiomer of the previously acylated racemate so that the L-amino acid is easily separated from the acylated D-amino acid that, after racemization, is recycled back into the enzyme stage (Sato and Tosa 1993a). This process has the historical record of being the first large scale process conducted with immobilized enzyme (Chibata et al. 1987).

Penicillin acylase is an extremely important enzyme for the industrial production of 6-aminopenicillanic acid and 7-amino 3-desacetoxicephalosporanic, as key intermediates of semi-synthetic β-lactam antibiotics (Parmar et al. 2000). These precursors are now industrially produced mainly by hydrolysis of penicillin G and cephalosporin G with immobilized penicillin acylase, which have replaced the former cumbersome chemical processes almost completely (Bruggink 2001; Kallenberg et al. 2005), representing one of the most successful cases of industrial application of hydrolytic enzymes in bioprocesses.

Urease is industrially used to remove urea from alcoholic beverages in Japan (Kodama 1996). Removal of urea precludes the formation of toxic ethylcarbamate during fermentation, which is particularly relevant in the production of sake. The continuous process with immobilized *Lactobacillus fermentum* urease has been developed and optimized (Matsumoto 1993).

Beyond hydrolytic enzymes there are some other enzymes of significant industrial impact. Some of the most relevant are listed in Table 1.3.

Glucose isomerase (actually xylose isomerase) is undoubtedly the most important and successful application of enzyme technology. Glucose isomerization by glucose isomerase was developed in the late 1960s but it was not until the mid-1970s when the process acquired industrial significance as a consequence of the

Table 1.3 Non-hydrolytic Enzymes of Commercial Relevance

Enzyme	Source	Application
Glucose isomerase	Bacteria, actinomycetes	Production of high-fructose syrups
Glucose oxidase	Mold	Food and beverage preservation
Catalase	Bacteria	Food preservation, peroxide removal in milk
Nitrile hydratase	Bacteria	Acrylamide
Lactamase – αamino-εcaprolactam racemase	Bacteria	Production of L-lysine
Aspartate ammonia lyase	Bacteria	Production of L-aspartic acid

development of immobilized glucose isomerase biocatalysts. High-fructose syrup (HFS) is a multimillion ton business with producing plants in many places all over the world (Cheetham 1994; Crabb and Mitchinson 1997). Annual production of HFS from cornstarch in the US alone is estimated to be close to 10 million tons, representing about 40% of the caloric sweetener market (Olsen 2002). Replacement of sugar by HFS in soft drinks is the trend, and it has contributed to the greatest extent to expand the market for glucose isomerase despite the controversy about potential health problems associated to fructose consumption (Melanson et al. 2007). HFS with 55% of fructose has the equivalent sweetness of sucrose, although temperature and pH have influence on the sweetness perception of HFS. However, reaction of isomerization is reversible and at conditions compatible with enzyme activity and stability the equilibrium constant is close to 1 (Illanes et al. 1992) so in practice a syrup with 42% fructose is produced at the enzyme reactor outlet. This syrup can be enriched by fructose-glucose separation using ion-exclusion chromatography (http://www.ameridia.com/html/ic.html), so that 55% and 90% fructose syrup are produced. A detailed description of the process for HFS production has been reported by Buchholz et al. (2005). Production of HFS with immobilized glucose isomerase is a mature technology; however, advances in the field are still occurring. Glucose isomerases with lower pH optimum and more stable in the presence of Ca^{++} have been screened (Lee et al. 1990) with the purpose of a one-pot saccharification and isomerization of hydrolyzed starch (Mishra and Debnath 2002). Several strategies of genetic improvement of producing strains have also developed, like site-directed mutagenesis to improve thermal stability and shift pH optimum (Quax et al. 1991; Bhosale et al. 1996), and cloning of thermostable glucose isomerase genes into a mesophilic hosts (Liu et al. 1996). Increasing temperature of isomerization is very important because of the positive effect on equilibrium. As is usual in a low added value process, optimization of reaction conditions is necessary to keep competitive, so enzyme reactor design has been modeled and optimized considering both enzyme inactivation and mass transfer limitations (Illanes et al. 1992; Abu-Reesh and Faqir 1996; Faqir and Abu-Reesh 1998; Illanes et al. 1999). Besides its main application for HFS production, glucose (xylose) isomerase is also used for bioethanol production from hemicellulose derived xylose that is converted to xylulose and so metabolized by conventional yeasts such as *Saccharomyces cerevisiae* or *Saccharomyces pombe* (Bhosale et al. 1996).

Glucose oxidase has miscellaneous applications related to the food industry. It is used as a substitute of chemical oxidants in baking (Si and Drost-Lustenberger 2002), to reduce oxidative damage in brewing (Schmedding and van Gestel 2002) and in preservation of foods prone to oxidative damage (by depleting oxygen), like mayonnaise, or to reduce color fouling (by depleting glucose), like in commercial dried egg white and egg batter (Uhlig 1998). May be its main application is in the analytical field, together with peroxidase, for glucose determination (Tramper 1994). Hydrogen peroxide formed by glucose oxidase activity can be removed by catalase.

Production of acrylamide from acrylonitrile by nitrile hydratase (nitrile hydrolyase) is now, together with HFS production with glucose isomerase, the largest scale enzymatic process. Enzymatic production of acrylamide in Japan exceeded

30,000 tons/year a few years ago, representing 40% of the total world market (Yamada and Kobayashi 1996; Miller and Nagarajan 2000). Production should have increased further because of the advantages of the bioprocess over the conventional chemical process in terms of environmental protection and energy consumption. The enzyme process has several advantages over the chemical process, associated to the high conversion efficiency obtained under moderate conditions (Ashina and Suto 1993). The former enzymatic process used resting cells of *Pseudomonas clororaphis* (Nagasawa and Yamada 1990) but the enzyme required organic coenzymes and was psychrophilic requiring very low operating temperatures. The process now is conducted with nitrile hydratase-rich cells of *Rhodococcus rhodocrous*; the enzyme is mesophilic and requires no organic coenzymes (Buchholz et al. 2005; Liese et al. 2006). Cloning of the *R. rhodocrous* nitrile hydrates genes into *E. coli* was unsuccessful because the enzyme was produced as inclusion bodies almost devoid of activity (Kobayashi et al. 1995). Other sources of nitrile hydratase have been tested and selected in terms of thermal stability (Padmakumar and Oriel 1999). Besides acrylamide production, nitrile hydratase is also being used in waste water treatment and bioremediation (Okamoto and Eltis 2007).

There are some other non-hydrolytic enzymes, like α-amino-ε-caprolactam racemase and aspartate ammonia lyase that have been reported as industrially relevant (Cheetham 1994). The first process was developed in Japan with cells containing the enzyme caprolactamase, that breaks down DL-α-amino-ε-caprolactam into L-lysine while the unreacted D-α-amino-ε-caprolactam is racemized with α-amino-ε-caprolactam racemase to the DL form and recycled back. It is uncertain if this process can compete with the well established fermentation process with *Corynebacterium glutamicum* (Demain 1968; Tryfona and Bustard 2005). The industrial production of aspartate from fumarate using immobilized cells containing aspartate ammonia lyase in Japan goes back to the 1960s (Sato and Tosa 1993b) and more recently demand has been strongly stimulated because aspartate is a raw material for the production of aspartame, the leading non-caloric sweetener (Cheetham 1994).

Enzymes are increasingly being used for environmental management in waste treatment and bioremediation. Biological waste treatment is based on aerobic and anaerobic processes where microbial consortia bring about the degradation of the organic contaminants. In this context, enzymes are being used in the removal of specific chemicals from complex industrial wastes or highly diluted effluents to remove particularly recalcitrant or insoluble pollutants (López et al. 2004), as polishing agents in municipal or industrial waste water treatment to meet specific environmental discharge regulations (Aitken 1993), and also to reinforce the hydrolytic potential of the microbial populations (Leal et al. 2002; Cammarota and Freire 2006). The subject has been reviewed by Karma and Nicell (1997). The enzymatic treatment of recalcitrant pollutants is analyzed as a case study in section 6.6. Enzymes are increasingly being used in bioremediation strategies (Sutherland et al. 2004), where some advantages over chemical or microbial remediation strategies are the lower toxicity of side-products, the biodegradable nature of the catalyst and the higher tolerance than microorganisms to organic co-solvents. Some of the enzymes used in

bioremediation are mono and di-oxygenases, dehalogenases and lignin-degrading enzymes, like laccase and manganese peroxidase. High production costs of enzymes remain a hurdle for widespread application of enzymatic remediation (Alcalde et al. 2006).

Limited open information about industrial enzyme market level and its evolution is available so that it is not an easy task to give a market overview. Available data refers to enzyme sales and does not include all countries worldwide. Significant amounts of enzymes are being produced now in countries like China and India, which have to be considered now as very relevant enzyme producers; however market information is scarce in the case of China and rather recent in the case of India. The trend has been for some time for the consumers of enzymes to develop their own production facilities or establish joint ventures with enzyme producers to supply them; therefore, an increasingly higher proportion of the enzymes simply do not go into the open market. This is to say that the figures about enzyme market have to be considered in that context and do not reflect its economic impact. A total estimate of enzyme sales in 1970 was around US$ 50 million and by 1988 estimates were close to US$ 570 million (Cianci 1986; Uhlig 1998). By the mid-1990s an estimate of US$ 800 million to 1 billion was suggested (Katchalsky-Katzir 1993; Hodgson 1994; Koskinen and Klibanov 1996) even though a figure as high as US$ 1.4 billion was claimed (Cowan 1996). At the end of the decade a figure higher than US$ 1.5 billion was estimated (van Beilen and Li 2002). More recent information gives estimates of US$ 2 billion for 2004 and a projection to US$ 2.35 billion for 2009. These latter figures consider so-called technical enzymes with a market share of 52%, food enzymes with a share of 37% and animal feed enzymes with a share of 11% (Hasan et al. 2006). Forecast for the average growth rate in the next decade is about 3%/year. This figure can be higher if novel applications of specialty enzymes in the fine-chemicals and pharmaceutical industries develop (Wrotnowsky 1997; Schmid et al. 2001)

Beyond industrial applications, there is an ever-increasing use of enzymes in other fields, like chemical and clinical analysis, biomedicine and research.

Enzymes are potent analytical tools because of its specificity and sensibility that allows them to quantify substances at very low concentrations with minimal interference (van Brunt 1987). The analyte is the substrate (or the coenzyme) of the enzyme that converts it into a measurable signal (light absorption or emission, heat, hydrogen ion...). The low stability and high cost of enzymes was an asset for using them as analytical tools (Price 1983); however, this problem has been circumvented by the use of robust immobilized enzymes that increase the useful life of the analytical device and by the development of flow injection analysis (Bowers 1986; Gorton et al. 1991; Schwedt and Stein 1994; Weigel et al. 1996). Very robust enzyme electrodes are used for chemical analysis in several areas like the fermentation (Enfors and Molin 1978; Nilsson et al. 1978; Verduyn et al. 1984; Schügerl 2001) and food industries (Mason 1983; Mandenius et al. 1985; Richter 1993). The analyte is sensed by the immobilized enzyme within the electrode and the product formed is detected by a conventional (pH, dissolved oxygen) or ion-specific electrode. The system is then quite versatile, allowing the

determination of a myriad of organic substances. Immobilized enzyme thermistors based on calorimetry have been also used as analytical devices for organic compounds (Danielsson 1987; Lawung et al. 2001). Development in this area has paralleled that in enzyme immobilization (Xu et al. 2007) so that now very robust and micro-fluidic analytical systems have been developed with immobilized enzymes (Hanbin et al. 2002). More information about enzyme electrodes can be found in: http://www-biol.paisley.ac.uk/marco/Enzyme_Electrode/htm. Future perspectives include the development of enzyme analytical devices within the context of nanobiotechnology (Scouten et al. 1995; Laval et al. 2000; Jianrong et al. 2004; Trojanowicz 2006). Immobilized enzymes are extensively used in clinical analysis (Endo et al. 1979; Bhargava et al. 1999; Yamamoto et al. 2000) and as detectors in immunoassay (Wisdom 1976) where an antibody is immobilized onto a solid support that selectively extracts the antigen and then the captured antigen is exposed to an antibody–enzyme complex with which reacts, its presence being revealed by an assay for the enzyme (Yakovleva et al. 2002). Enzymes are also used as tracers in diagnostic kits (Lowe 1989), like the pregnancy test based on human choriogonadotropin (Christensen et al. 1990) and the fertility test based on the luteinizing hormone and follicle stimulating hormone (http://monobind.com).

Therapeutic use of enzymes is not new and several hydrolases, mainly from plants and animal organs, have been traditionally used as digestive aids or topically as anti-inflammatory, in burn-healing and caries prevention (Christie 1980). Besides enzymes have a great potential in clinical medicine in the treatment of congenital metabolic deficiencies, where the exogenous enzyme subsidizes it, the elimination of toxic metabolites accumulated by organ malfunction and the selective nutritional depletion of malignant cells. Relevant cases of potential applications of enzyme in medical therapy are listed in Table 1.4. Applications may be extracorporeal (ex vivo) or intracorporeal (in vivo). In all cases enzyme immobilization to solid particles or confinement within semipermeable membranes is highly desirable (Klein and Langer 1986).

Ex-vivo applications imply blood perfusion through an outer device where the enzyme removes the unwanted metabolite. A striking example is the enzymatic artificial kidney in which the dialyzate containing the urea is passed through a removal chamber composed of immobilized urease and absorbents for the products of hydrolysis; in this way the concentration of urea in the dialyzate is maintained at a very low value increasing its flow through the membrane and in this way reducing perfusion time (Chen et al. 1994; Caridis and Papathanasiou 1995). Several systems considering immobilized urease have been tested (Arica 2000; Liang et al. 2000; Ayhan et al. 2002).

Intracorporeal applications are far more complex: the enzyme should be directed to its target within the patient's body and avoid the immune response. Immobilization to biocompatible supports may reduce the immune response significantly (Klein and Langer 1986). Several systems for enzyme delivery have been envisaged: microencapsulation, liposome entrapment (Chen and Wang 1998; Fonseca et al. 2003), microencapsulation (Dai et al. 2005) and artificial red blood cell ghosts (Serafini et al. 2004). An updated review on the subject has been published

Table 1.4 Enzymes of Potential Application in Medical Therapy

Enzyme	Source	Mode of Action and Application	Reference
Asparaginase	Bacteria	Selective depletion of nutrients for tumor cells; treatment of leukemia	Duval et al. (2002)
Bilirubin oxidase	Mold	Oxidation of bilirubin to biliverdin; jaundice treatment	Soltys et al. (1992) and Chen and Wang (1998)
Carboxypeptidase	Bacteria	Folate metabolism inhibitor; methotrexate removal in cancer therapy	Krackhardt et al. (2000)
α-Glucosidase	Mold	Glycogen breakdown; assist in glycogen removal from cells (Pompe's disease)	van den Hout et al. (2000)
α-Galactosidase	Plant	Renoval of galactose from globotriaosylceramide; avoids ceramide accumulation (Fabry's disease)	Ioannou et al. (2001) and Pastores and Thadhani (2001)
Heparinase	Bacteria	Inactivates heparin by sugar removal; residual heparin removal after extracorporeal blood circulation	Yang et al. (1986) and Comfort et al. (1989)
Phenylalanine ammonia lyase	Yeast	Converts phenylanine to *trans*-cinnamic acid and ammonia; reduction of blood phenylalanine in phenylketonurics	Sarkissian et al. (1999) and Ikeda et al. (2005)
Streptokinase	Actynomycete	Activates plasminogen to plasmin; dissolution of blood clots in thromboembolic patients	Hoffmeister et al. (1998)
Superoxide dismutase		Convert oxygen free radicals into oxygen and hydrogen peroxide; wound healing, potential treatment of several diseases	Noor et al. (2002) and Paramonov et al. (2005)
Urease	Bacterial	Urea removal from blood (enzymatic artificial kidney)	Qin and Cabral (2002)
Uricase	Animal	Oxidation of uric acid to allantoin and CO_2	Wortmann (2005)
Urokinase	Urine recombinant bacteria	Activates plasminogen to plasmin; dissolution of blood clots in thromboembolic patients	Winkler et al. (1985); Maksimenko (1998); and Ouriel et al. (1998)

by Swi (2007). Targeting of the enzyme to the corresponding site of action within the body is a crucial aspect of in-vivo applications and a variety of different strategies are now available (Lübbe et al. 2001). A prominent example of intracorporeal application is asparaginase, which might be the most promising enzymatic chemotherapeutic agent, now considered clinically acceptable especially for the treatment of lymphoblastic leukemia. An updated review on the present status of asparaginase as chemotherapeutic agent has been published recently (Verma et al. 2007). Despite the great potential of enzymes in clinical medicine, the bright future envisaged twenty years ago (Klein and Langer 1986) has not come to reality yet, despite the impressive advances in the field. Based on that, it is tempting to forecast that enzyme technology will contribute significantly to the advances in biomedicine in the forthcoming decades and will complement some other technological developments, like gene therapy (Verma and Somia 1997).

Enzymes are fundamental tools for research and development in several areas of biotechnology. Enzymes are essential components of the toolbox of different areas of biotechnology being especially relevant in molecular biology and genetic engineering. Restriction endonucleases, which are essential for gene splicing (Pingoud 2004), and thermostable DNA polymerases, which are essential for amplification of genetic material (Bartlett and Stirling 2003; Weissensteiner et al. 2003), are relevant examples that illustrate this. Enzymes for research and development, even though produced at small scale, are usually required in a high degree of purity and therefore represent a significant and increasing sector of the enzyme market (Polisson 1992), which is becoming highly sophisticated and competitive (http://www.theinfoshop.com/study/tk18690_dna_pcr_toc.html; http://www.gene2drug.com/about/archives.asp?newsId = 183).

1.6 Enzyme Processes: the Evolution from Degradation to Synthesis. Biocatalysis in Aqueous and Non-conventional Media

Most industrial enzymatic processes refer to reactions conducted by hydrolases in aqueous medium for the degradation of complex molecules (often polymers) into simpler molecules in conventional processes with limited added value (Neidelman 1991). Reasons underlying are clear since hydrolases are robust, usually extracellular and have no coenzyme requirements, which makes them ideal process biocatalysts. Enzyme immobilization widened the scope of application allowing less stable, intracellular and non-hydrolytic enzymes to be developed as process biocatalysts (Poulsen 1984; D'Souza 1999), as illustrated by the paradigmatic case of glucose isomerase for the production of HFS (Carasik and Carroll 1983) and the production of acrylamide from acrylonitrile by nitrile hydratase (Yamada and Kobayashi 1996).

The trend is now to develop enzymatic processes of organic synthesis where the potential added value is much higher. There is an impressive number of reactions of organic synthesis of technological relevance that have been studied using enzymes

or enzyme-containing cell biocatalysts (Roberts 1998, 2000; Sugai 1999; Davis and Boyer 2001; Koeller and Wong 2001; Drauz and Waldmann 2002). However, the use of enzyme catalysts for organic synthesis, despite its enormous potential, has been confronted with several drawbacks that mainly stems from an industry not sufficiently acquainted to deal with biological material. The case of lipases with respect to its use in the oleochemical industry illustrates this point (see section 6.3). Enzyme catalysts, within the context of organic synthesis, have been considered too expensive, not easily available, too unstable, only acting properly on their natural aqueous habitat and on their natural substrates with very narrow substrate specificity and requiring complex cofactors (Bommarius and Riebel 2004). Some of these appreciations can be clearly refuted while others are being solved by recent technological developments. Enzyme prices have dropped consistently in the last decades and an increasing number of suppliers are now at hand, many of them willing to satisfy their customers' needs on an individual basis. Advances in genetic and protein engineering and also in screening of novel enzyme sources have contributed significantly to increase the range of enzymes available and cutoff prices. Enzymes are indeed unstable catalysts, but enzyme stabilization has been a major concern in biocatalysis and advances in the field are impressive (Ó Fágáin 1997, 2003; Illanes 1999) so that now enzymes stable enough even under stringent conditions are available. The fact that enzymes act properly only in aqueous environment is now contradicted by a mass of information gathered on enzyme catalysis in non-conventional media since the pioneering work of Klibanov (Klibanov 1977; Zaks and Klibanov 1985, 1988a). Because of its relevance, this aspect will be analyzed in-depth ahead. Enzymes, especially those acting on small to medium molecular size substrates, have often a broad specificity and can act over non-natural substrates as well. Nowadays, enzyme can be modified and acquire new functionalities by using site-directed mutagenesis (Svendsen 2000; Terao et al. 2006). Cofactor requirements may be a problem especially in the case of coenzymes, but now this is possible to circumvent as will be further analyzed.

Despite these drawbacks, enzymes are quite attractive catalysts for performing organic synthesis and have been considered to match the fundamental principles of environmentally benign manufacturing, sustainable development and green chemistry (Bommarius and Riebel 2004), which represents a bonus of increasing significance as environmental pollution becomes one of the most serious threats to mankind. Selectivity is maybe the most attractive property of an enzyme biocatalyst for performing organic synthesis. Enzymes are highly regioselective and enantioselective, which are very valuable attributes for the synthesis of pharmaceuticals and other biologically active compounds. The leading non-caloric sweetener (aspartame) is a relevant example: the enzymatic synthesis with thermolysin only requires the protection of the amino group in aspartic acid because it is regioselective with respect to the carboxyl group adjacent to the amino group, and cheap racemic phenylalanine methyl ester can be used because thermolysin is enantioselective with respect to L-phenylalanine methyl ester. There is an increasing pressure to the pharmaceutical industry to produce chiral drugs as pure enantiomers, which is not attainable by chemical synthesis. On the contrary, enzymes are chiral catalysts so that any

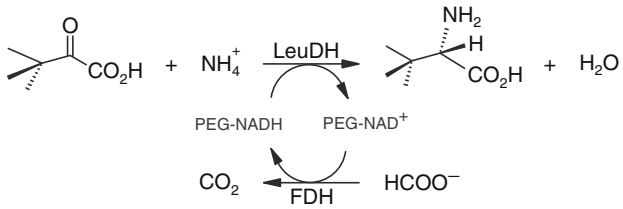


Fig. 1.6 Reaction scheme for the enzymatic production of L-tert-leucine (L-tert). Leu DH: leucine dehydrogenase; FDH: formate dehydrogenase; PEG: polyethylene gycol

chirality present in the substrate molecule is recognized upon the formation of the enzyme–substrate complex and in this way both enantiomers of a racemic substrate may react at quite different rates affording a kinetic resolution. In this way, a high proportion of the drug can be produced as the required enantiomer (*eutomer)* and the potential adverse effects of the unwanted enantiomer (*distomer*) be avoided (Faber 1997). Selectivity has then a profound influence in process economics by reducing the number of steps and protective reactions and reducing downstream operations for product purification. Natural enantioselectivity cannot be modified easily, but inversion by protein engineering has been reported (Ijima et al. 2005).

Enzymes are active at mild environmental conditions, which is also a valuable attribute especially for the production of labile compounds; it also represents an advantage in terms of energy consumption and reactor design. Enzymes can catalyze quite complex reactions that are difficult to perform by chemical synthesis, like Baeyer–Villiger oxidation of ketones (Ottolina et al. 2005; Rehdorf et al. 2007) and the ring expansion of the penicillin nucleus (Cho et al. 1998).

Enzymes, whose physiological role is the catalysis of reactions of synthesis are, by contrast to most industrial enzymes, labile intracellular proteins requiring coenzymes. Therefore, its application as process catalysts is confronted with technological difficulties: enzymes have to be stabilized (usually by immobilization) and coenzymes retained (usually by co-immobilization or derivatization) and recycled (usually by coupled auxiliary enzymatic reactions). Impressive technological advances are now opening up the option of developing such processes to industrial level as elegantly illustrated by the synthesis of L-tert-leucine, an important building-block for the synthesis of pharmaceuticals. The reaction scheme is depicted in Fig. 1.6: leucine dehydrogenase catalyzes the reductive amination of trimethyl pyruvate in a membrane reactor that confines the enzyme and the pegylated coenzyme (NADH), while coenzyme regeneration is produced by the reduction of formate to carbon dioxide that, being highly volatile, leads to a favorable shift of the equilibrium and does not contaminate the product. Very high coenzyme turnover numbers have been obtained (Adlercreutz et al. 1994).

More technologically promising is the use of hydrolytic enzymes in reverse reactions of synthesis. Hydrolases that catalyze the breakdown of a certain chemical bond can, under certain conditions, catalyze its formation so that, in principle, any hydrolase can catalyze the reverse reaction of synthesis. In this way proteases and

acylases can catalyze the formation of a peptide bond instead of its cleavage (Björup et al. 1999; Wegman et al. 2001), carbohydrases can catalyze the formation of glycosidic linkages (Stevenson et al. 1993; Bucke 1996) and lipases can catalyze esterification, interesterification and transesterification reactions instead of hydrolyzing ester bonds in lipid substrates (Coulon and Ghoul 1998; Undurraga et al. 2001). As said above, hydrolases are ideal process biocatalysts for being robust, not coenzyme requiring and easily accessible at moderate prices. Additionally, they have a rather broad substrate specificity accepting as such different synthetic substrate analogs, being highly stereoselective even for those unnatural substrates. In summary, hydrolases have a tremendous potential for organic synthesis (Bornscheuer and Kazlauskas 1999). However, to fully exploit the synthetic capacity of hydrolases and depress the competing reactions of hydrolysis, non-conventional (non-aqueous) media are required (Ballesteros et al. 1995; Hari Krishna 2002). Intense research on enzyme biocatalysis in such media has been conducted in the last three decades considering: gases (Lamare and Legoy 1993), supercritical fluids (Kamat et al. 1995; Marty et al. 2004), organic solvents (Klibanov 2001; Ru et al. 2002), ionic liquids (Park and Kazlauskas 2003; van Rantwijk et al. 2003; Ulbert et al. 2005; Durand et al. 2007) and semi-solid systems (Erbeldinger et al. 1998; Ulijn et al. 2001).

Enzyme catalysis in gaseous phase has been studied for reactions involving gaseous or highly volatile compounds (Barzana et al. 1987, 1989). Some potential benefits are the easy recovery of the biocatalyst, the high mass transfer rates, the removal of volatile inhibitors, the high thermal stability of the enzyme at low water activity and the asepsis of the operation. However, it is limited to highly volatile substrates and products, temperature and pressure of operation are sometimes incompatible with enzyme activity and control of water activity, a critical variable in gas-phase biocatalysis, is difficult (Barton et al. 1997; Trivedi et al. 2006). Biocatalysis in gas phase has been proposed as a viable alternative for decontamination of industrial effluents from particularly toxic pollutants (Dravis et al. 2000).

Supercritical fluids (SCF) have several potential advantages as reaction media for enzyme catalysis when compared to gas or liquid systems (Garcia et al. 2004). At conditions above the critical pressure (P_c) and critical temperature (T_c), molecular thermal energy exceeds the attractive forces between molecules and a gas-like state exists so that the properties of SCF are somewhere in between those of liquids and gases. In fact, density of SCF is only slightly lower than liquids but orders of magnitude higher than gases, which makes them very good solvents, viscosity of SCF is somewhat higher than gases but lower than liquids and diffusivity in SCF is lower than in gases but orders of magnitude higher than in liquids so that mass transfer rates are much higher than in liquids. SCF also share the potential benefits of gaseous systems in terms of easy recovery of the biocatalyst and the product of reaction (Russell and Beckman 1991; Mesiano et al. 1999). From the many fluids available, only a few exhibit values of P_c and T_c compatible with enzyme activity, as can be appreciated in Table 1.5. Among those, carbon dioxide has been used in most cases since it has several advantages like non-toxicity, non-flammability and availability in high purity (Olsen et al. 2006; Laudani et al. 2007). However, biocatalysis in SCF requires of special equipment, operation costs are high and the effect of critical variables, like pressure and water activity, a key operational parameter, is

Table 1.5 Critical Temperatures and Critical Pressures of Different Fluids

Solvent	T_c (°C)	P_c(bar)
Ammonia	133	111
Argon	−122	48
Carbon dioxide	31	73
Chloro-trifluoromethane	29	39
Ethane	33	48
Ethylene	9	51
Fluoroform	26	48
Helium	−268	2
Hydrogen sulfide	100	89
Methanol	239	81
Neon	−229	27
Nitrogen	−147	34
Propane	96.7	42.5
Propene	91.7	46.0
Oxygen	−127	50
Sulfur dioxide	158	78
Sulfur hexafluoride	46	37
Sulfur trioxide	218	84
Water	374	221

not predictable (Russell et al. 1994; Mori et al. 1998; Knez et al. 2007). Recently, biphasic liquid-supercritical carbon dioxide systems have been used for esterification with lipase, the enzyme being in the liquid phase while the product is extracted to the supercritical carbon dioxide phase (Reetz and Wiesenhöfer 2004).

Enzyme catalysis in ionic liquids has had a tremendous development in recent years. Ionic liquids are highly polar solvents composed usually by a rather simple anion and a complex organic cation (van Rantwijk et al. 2003). Ionic liquids have negligible vapor pressure which is quite important from an environmental perspective. In addition, they are less harmful than organic solvents and form biphasic systems with them, being attractive for lipase catalyzed reactions (Mori et al. 2005). Increased enzyme enantioselectivity (Liu et al. 2005; Durand et al. 2007) and stability (Machado and Saraiva 2005; Ulbert et al. 2005) have also been reported in ionic liquid medium when compared to aqueous or organic media. However, biocatalysis in ionic liquids presents several challenges: difficulty in purifying them and controlling water activity and pH, high viscosity and problematic product recovery. Despite this, its potential is significant especially within the framework of green chemistry (Park and Kazlauskas 2003). Complete and updated information on ionic liquids can be obtained in Ionic Liquids Today (www.iolitec.de).

Enzyme catalysis in nearly solid or semi-solid systems has been thoroughly studied (Ulijn et al. 2003). In this system the reaction mixture consists of solid reactants suspended in a comparatively small volume of liquid phase, either aqueous (van Langen et al. 1999, 2000) or organic (Basso et al. 2000) that becomes saturated; reaction ensues and the product formed precipitates out from that liquid phase. This

precipitation driven system has been studied mainly for peptide synthesis with proteases (Ulijn et al. 2002a; Chaiwut et al. 2007) but with other enzymes as well (Cao et al. 1997; Ulijn et al. 2002b; Youshko and Švedas 2002; Basso et al. 2006). Prediction about feasibility of precipitation driven reactions has been made based on thermodynamic considerations (Ulijn et al. 2001). It is concluded that higher conversion yields are obtained when both substrate and product solubilities in the solvent phase are lower (Ulijn et al. 2002a). An obvious advantage of solid systems is the extremely high volumetric productivity since at the end of the reaction virtually the whole content of the reactor is product. Other salient features are its environmental acceptance, high conversion yields in reversal of hydrolytic reactions and high stability. However, mass transfer limitations and mixing problems are to be addressed especially for scale up purposes (Erbeldinger et al. 1998).

Enzyme catalysis in organic solvents has been a major subject of research in biocatalysis in the last 25 years and represents the most important non-aqueous system, despite its environmental constraints (Koskinen and Klibanov 1996; Gupta and Roy 2004). The replacement of water by an organic solvent has several potential advantages that apply differently according to each particular system: reversal of hydrolytic reactions with hydrolases are feasible, thermodynamically unfavorable reactions in water are possible, poorly water soluble substrates can be efficiently used, ionic state of reactants can be altered in a positive direction, recovery of biocatalyst and product is simpler, conditions of asepsis are less stringent, thermal stability is increased, product inhibition effects can be reduced and even affinity and specificity of substrate can be different (Brink et al. 1988). Increased thermal stability and the option of favoring synthetic capacity of hydrolytic enzymes indeed represent major technological breakthroughs. The presence of an organic solvent has a profound effect on enzyme structure (Clark 2004; Quiroga et al. 2007) and, as a consequence, on their properties (Barberis and Illanes 1996). As compared to aqueous media, enzymes are considerably less active, but can be significantly more stable; substrate selectivity, (enantio, regio and chemoselectivity) may differ significantly and molecular memory is a very distinctive property of enzymes in organic media (Klibanov 2001). Lipases outstand as enzymes particularly well suited to perform, in non-aqueous media since they have evolved to perform in cellular ambient of low water activity where they exhibit a very high regio and enantioselectivity (Jaeger and Eggert 2002; Petkar et al. 2006). Lipases are also quite versatile enzymes that have been used in a myriad of reactions of organic synthesis (Bornscheuer and Kazlauskas 1999).

There are basically two forms of biocatalysis in organic medium: homogeneous systems, which are mixtures of water and water miscible solvents (Castro and Knubovets 2003) and heterogeneous systems (Krieger et al. 2004) in which a second phase is produced by the presence of a water-immiscible solvent.

Heterogeneous systems can be divided into macroheterogeneous, in which two immiscible liquid phases are apparent, and microheterogeneous, in which one of the phases (usually the aqueous phase that surrounds the enzyme) is not visible to the naked eye. Both homogeneous and heterogeneous systems can perform with the enzyme dissolved in the medium or insolubilized in it, be it because the enzyme

protein is itself insoluble in that medium or because it is immobilized in a solid carrier (Guzmán et al. 2007).

Homogeneous systems are composed by a mixture of water and a water-miscible solvent (cosolvent) in which the enzyme is dissolved (Torres and Castro 2004). Cosolvents are usually detrimental for enzyme activity at moderately high concentrations, because they tend to penetrate the aqueous microenvironment that surrounds the enzyme molecules, altering the pattern of interaction of the enzyme with the solvent and distorting its three-dimensional structure (Klibanov 1986; Laane et al. 1987; Deetz and Rozzell 1988; Zaks and Klibanov 1988a,b; Mozhaev et al. 1989; Levitsky et al. 1999). Polyols and glymes are, however, notable exceptions among cosolvents (Castro 2000; Illanes and Fajardo 2001; Gorbhel et al. 2003). Inactivation can be reduced by immobilization, being usual that enzymes are used in immobilized form when performing in homogeneous liquid media (Castro 1999; Illanes et al. 2004). In general, both the activity and stability of enzymes in cosolvents is impaired with the exceptions noted above. Macroheterogeneous or biphasic systems are composed by water and a hydrophobic solvent (Kuhl and Jakubke 1990). Substrates can be dissolved in the organic or in the aqueous phase but biocatalysis will always occur in the aqueous phase where the enzyme is. The product formed can be partitioned to the organic phase, which can be highly desirable to avoid inhibition or unwanted hydrolysis (Barberis et al. 2002; Bordusa 2002). The main drawback of biphasic systems is the presence of an interface that can impose diffusional restrictions to the substrates and in this way reduce the reaction rate; this can be alleviated by intense agitation, but will in turn promote enzyme inactivation (Barros et al. 2000).

Suspension of nearly anhydrous enzymes in hydrophobic solvents can be considered as a microheterogeneous system in the sense that the liquid phase appears as homogeneous to the naked eye; however, the system is microscopically heterogeneous since the solid enzyme is covered by a water shell tightly bound to the enzyme and a bulk hydrophobic organic solvent surrounding it. The enzyme is usually protected from the aggressive hydrophobic solvent by a layer of denatured enzyme (Kanerva and Klibanov 1989; Klibanov 1989). This is maybe the most simple and most promising strategy for enzymatic synthesis since it exploits to the highest extent the advantages of working in non-conventional medium (Clark 2004), namely high thermostability (Zaks and Klibanov 1984; Klibanov 2001), potentially favorable changes in substrate specificity (Westcott and Klibanov 1994; Ebert et al. 1996; Kawashiro et al. 1997; Carrea and Riva 2000) and easiness of biocatalyst and product recovery (Dickinson and Fletcher 1989). The enzyme biocatalyst is simply an acetone precipitated enzyme powder or a lyophilized preparation that is suspended in the organic medium in which it is completely insoluble (Zaks and Klibanov 1988a); immobilization is then unnecessary since the enzyme is already insolubilized in the reaction medium, even though it might help by offering an increased surface of contact with the substrate and providing additional stabilization. Best results are obtained with highly hydrophobic solvents ($\log P > 4$; where P stands for the partition coefficient between *n*-octanol and water) since the intrusion of solvent in the water shell is hindered and so the enzyme is better protected from

the direct contact with the organic solvent molecules (Zaks and Klibanov 1985). The role of water is critical in this system since it is present in minute amounts that are determinant for enzyme behavior (Halling 2004). Enzymes require variable amounts of water to conform the minimal hydration level required for expressing activity and this may vary significantly from one enzyme to another. Up to now, enzyme biocatalysis has not been demonstrated in the absolute absence of water even though it has been claimed that there is no fundamental reason for that since the extremely rigid nature of the protein and the absence of water do not preclude catalysis per se as long as a catalytically competent conformation is attained and a stable transition state is achieved (Xu and Ding 2007). It has been claimed, however, that a minimal level of hydration is necessary for the enzyme molecule to acquire the required flexibility to perform catalysis, while too much water is detrimental by favoring conformational distortion; according to this, an optimum water activity exists in each case where enzyme activity and stability are optimally counterbalanced. Despite its advantages, this strategy has important drawbacks, being the most relevant the dramatic decrease in activity that enzymes usually express in this kind of media (Klibanov 1997; Quiroga et al. 2005). However, impressive advances have been reported in the design of enzyme biocatalysts well suited to perform in such aggressive conditions (Hari Krishna 2002). They refer to enzyme protection by suitable additives (van Unen et al. 2001; Lee and Dordick 2002), chemical modification (Salleh et al. 2002; Davis 2003; Hudson et al. 2005), immobilization (Yan et al. 2002; Cao et al. 2003; Wilson et al. 2004a; Mateo et al. 2007), aggregation (Wilson et al. 2004b; Amorim-Fernandes et al. 2005; Wilson et al. 2006; Sheldon et al. 2007), solubilization (Akbar et al. 2007), directed evolution (Moore and Arnold 1996; Arnold and Moore 1997; Gupta and Roy 2004) and molecular redesign by protein engineering (Adamczak and Hari Krishna 2004). Another system that can be considered as microheterogeneous is reverse micelles, which are spontaneously formed when small amounts of water are added to a hydrophobic solvent in the presence of a surfactant under agitation (Gómez-Puyou and Gómez-Puyou 1998). It has been claimed that the microenvironment in the internal cavity of the micelles is more natural to the enzyme than the bulk aqueous medium, which leads in some cases to an enhanced expression of its catalytic potential (Castro and Cabral 1989). Reverse micelles have, however, several drawbacks: they are mechanically week, there are no rational methods for its optimization and the surfactant impairs the recovery and purification of products (Bordusa 2002).

Biocatalysis has evolved from traditional processes in aqueous media, where the substrates, and usually the enzyme as well, are dissolved, to processes of synthesis with different forms of biocatalysts performing in different kinds of nonconventional media. As a consequence, a broad spectrum of opportunities for performing organic synthesis has been created in the last decades pushed by the impressive advances both in biocatalyst and medium engineering. In 2000, it was claimed that about 100 processes of organic synthesis were conducted by biocatalysis at varying levels of industrial production, mainly for the synthesis of pharmaceutical and agrochemical precursors (Wandrey et al. 2000). At least three of those processes are conducted in organic medium with production levels exceeding

100 tons/year: aspartame with thermolysin, and cocoa butter analogues and 3,4-dihydroxy-L-phenylalanine (L-DOPA) with lipases (Powell et al. 2001). It is tempting to forecast that an increasing number of new products of biocatalysis will reach the market within the next decade and a large portfolio of novel applications will be available.

Nomenclature

A_λ	absorbance (optical density) at wavelength λ
a	enzyme activity
c	analyte concentration
coe, coe′	coenzyme molar concentration
I	intensity of transmitted light
I_0	intensity of incident light
l	optical path length
p	product molar concentration
s	substrate molar concentration
t	time
v	initial rate of reaction
z	coupled product molar concentration
α	optical rotation
α_0	specific optical rotation
ε	extinction coefficient

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Chapter 2
Enzyme Production

Andrés Illanes

2.1 Enzyme Sources

Enzymes are the catalysts responsible for cell metabolism. Cells from different sources have been, are, and will continue to be the main source of enzymes. Enzymes can be produced from any living organism, either by extracting them from their cells or by recovering them from cell exudates.

Plant tissues and animal organs were the most important sources of enzymes at the onset of enzyme biotechnology; in 1960, about 70% of the enzymes were extracted from plant tissues or exudates and animal organs. Twenty years later the situation had reversed and most industrial enzymes were produced from microbial sources (Lambert and Meers 1983). Nowadays, enzymes from plants and animals, mostly proteases, are still in the market and some of them are of commercial relevance. Catalase (EC 1.11.1.6) from liver (Yildiz et al. 2004), lipase (EC 3.1.1.3), chymotrypsin (EC 3.4.21.1), and trypsin (EC 3.4.21.4) from pancreas (Underkofler et al. 1958) and rennin from calf abomasus are the most relevant animal enzymes, widely used in the food and leather industries (Oberg et al. 1992; Kosikowski and Mistry 1997). Animal derived enzymes represent about 10% of the total enzyme market. Plant derived enzymes, such as papain and bromelain, are still industrially relevant (Balandrin et al. 1985; Tucker and Woods 1995; Uhlig 1998) and they roughly represent 5% of total market. Papain (EC 3.4.22.2), a cysteine protease obtained from the latex of papaya (*Carica papaya; Carica candamarcensis*), is the most prominent plant enzyme marketed today. It is widely used in meat tenderization (Ashie et al. 2002), beer clarification (Monsan et al. 1978), yeast extract production, stain removal (Gebreselassie et al. 2002), and, in highly purified form, in several cosmetic and medical applications (Craig 1975; Pendzhiev 2002). Bromelain, a complex of cysteine proteases extracted from pineapple stems (Rowan et al. 1990) is another relevant plant protease with different applications, mainly in

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the medical area (Taussig and Batkin 1988; Gregory and Kelly 1996) as a wound-healing, anti-inflammatory, digestive-aid and appetite suppressant agent. Plant and animal proteases have been usual components of digestive-aids (Bilton 1984); recently a crude protease extract from kiwifruit has had a considerable market success as a digestive-aid (see http://www.vitalfoods.co.nz/ourproducts/zylax/). Proteases from animals and plants have also been evaluated in the synthesis of peptides in non-conventional media (Riechmann and Kasche 1985; Adlercreutz et al. 1990; Adlercreutz and Clapes 1991; Clapes et al. 1995; Hansler et al. 1995). Some animal enzymes, like urokinase and tissue plasminogen activator are being produced from in-vitro cultivation of established cell lines (Mizrahi 1986; Avgerinos et al. 1990; Zang et al. 1995; Altamirano et al. 2006). Some plant enzymes, like invertase (Pressi et al. 2003), neutral protease (Cimino et al. 2006) and acid phosphatase (Su and Arias 2003) are produced by in-vitro plant cell cultivation. Only high value enzymes for sophisticated applications are potential candidates for in-vitro production with animal and plant cell lines, because of the complexities and high costs of production at large scale (Chu and Robinson 2001; Hood 2002). Glycoenzymes, not easily produced as recombinant proteins in microbial hosts (i.e. glutamine synthetase), are good candidates for in-vitro production with established animal cell lines (Barnes et al. 2000).

Since the early 1960s, microbial enzymes have gradually and progressively replaced those from other sources (Lambert and Meers 1983). In a conservative estimate, they might now represent almost 90% of the total market. This is so because microorganisms are excellent cell systems for enzyme production: they are metabolically vigorous, they are quite versatile and easy to propagate on a large scale by submerged or solid-state fermentation, they are simple to manipulate both environmentally and genetically, their nutritional requirements are simple and their supply is not conditioned by seasonal fluctuations (Blanch and Clark 1997). These attributes have profound technological implications, making the production of microbial enzymes more reliable, simpler and cheaper, independent from side-products and produced irrespectively of the season with reliable supply of raw materials of constant composition, usually free of any harmful substance. Most enzymes are produced by mesophilic organisms; however, the search for enzymes well suited to perform under extreme conditions has prompted an active research on enzymes from extremophiles under the hypothesis that organisms that can thrive in extreme environments have an enzyme machinery adapted to perform under such conditions; enzymes that are stable at high temperatures and those active at low temperatures are now being screened and tested as potential industrial enzymes (Niehaus et al. 1999; Gerday et al. 2000; Vieille and Zeikus 2001; de Miguel Bouzas et al. 2006). Even though such extremophiles are difficult to grow under laboratory conditions, their genes can be cloned into suitable mesophilic hosts as illustrated by the cloning of thermophilic enzyme genes from archaea and bacteria (Bertoldo and Antranikian 2002), and psychrophilic enzyme genes into mesophilic hosts (Feller et al. 1998). In fact, the advances in molecular genetics and genetic engineering in the last decades have made possible to clone and express virtually any gene into a suitable microbial host, so that now enzymes from other microorganisms and also from higher

organisms can be produced in convenient microbial hosts like bacteria, yeasts and fungi. This fact has contributed significantly to increase the number of enzymes that can be produced by microbial fermentation and also to increase the productivity of the fermentation and the quality of the enzyme product. In 1994 it was estimated that about 50% of the industrial enzymes (on a mass basis) were produced from genetically engineered organisms (Hodgson 1994). This proportion must have increased significantly in the last decade because of the advances in recombinant DNA technology and protein engineering and also because of the increasing production of specialty enzymes for the pharmaceutical and fine-chemicals industries (McCoy 2001; Rasor and Voss 2001; Schmid et al. 2001; Thomas et al. 2002).

The evolution of sources for enzyme production has been fast during the last decades. An example that illustrates this evolution is the case of chymosin (EC 3.4.23.4). Chymosin is a very specific aspartic acid protease (the active enzyme is formed from its zymogen, prochymosin, by proteolytic cleavage of the N-terminal 42-residue propeptide) that hydolyzes the Phe_{105}–Met_{106} bond of κ-casein, and, to a lower extent, some other four peptide bonds (Foltmann 1981; Kim et al. 2004); it is the hydrolysis of the 105–106 peptide bond that triggers the clotting of casein in the presence of calcium ions to yield the curd (Budtz 1994). No other enzyme is so specific for milk clotting and chymosin is therefore the best choice for cheese making, because of high yields of curding and proper flavor development in aged cheeses (Duxbury 1989). The traditional source of chymosin is calf rennet, an extract from the inner mucosa of the fourth stomach (abomasus) of suckling calves obtained as a by-product of veal production. Crude rennet contains three chymosin variants: A, B, and C, as well as pepsin which produce a less specific action (Kim et al. 2004). Shortage of rennet as source of chymosin for cheese making became critical (Green 1977) and a continuous biotechnological development has occurred during the last decades to substitute rennet as its source, which is illustrative of the dynamics of knowledge in the field of enzyme production. A first approach to solve the crisis was the partial substitution of chymosin for pepsin; therefore, rennets from varying chymosin: pepsin ratios began to be used despite the sacrifice in quality of the cheeses so produced (Emmons and Binns 1990). In the early 1960s, chymosin activity in microbial sources was being actively pursued (Neelakantan et al. 1999); most successful were the proteases obtained from *Mucor miehei* (Bailey and Siika-aho 1988) and *Mucor pusillus* (Hiramatsu et al. 1989). The so-called microbial rennets were introduced in the market in the 1960s and still represent a significant share of the chymosin market (Duxbury 1989). However, they are not as acceptable as calf chymosin because curd yields are lower and not so pleasant flavor is developed upon maturation. An additional drawback is that microbial rennets are more thermostable and its inactivation at high temperature impairs the texture of the product so that the enzyme requires being chemically modified (van den Berg et al. 1987). With the advent of genetic engineering, the logical option was to express the chymosin gene into a suitable microbial host (Pitcher 1986). Much information accumulated since then on the expression and production of chymosin in microbial hosts (Johnson and Lucey 2006). Initially (pro)chymosin was expressed in *Escherichia coli*; however the enzyme was produced in the form of insoluble inclusion bodies that required

post-transcriptional processing and a complex process of denaturation and renaturation (Emtage et al. 1983; Marston et al. 1984). Better hosts for chymosin expression were then eukaryotic microorganisms, namely fungi and yeasts. Filamentous fungi, such as *Trichoderma* (Harkki et al. 1989) and *Neurospora* (Stuart et al. 1997) have been reported as hosts, but *Aspergilli* have been claimed as the best hosts for chymosin production, since the enzyme can be excreted in higher amounts in active form by using the secretory control regions of some extracellular enzyme coupled to pro-chymosin cDNA (Cullen et al. 1987; Lamsa and Bloebaum 1990; Ward et al. 1990; Tsuchiya et al. 1993). The enzyme from *Aspergillus oryzae* has been in the market for over a decade (Dunn-Coleman et al. 1991). Chymosin has also been successfully synthesized in yeast hosts. Originally, *Saccharomyces cerevisiae* was the organism of choice and the calf chymosin cDNA gene was cloned and expressed, prochymosin being synthesized at a level of 5% of the total yeast cell protein (Dobson et al. 1983). However, the enzyme was poorly excreted in an active form, so that *Kluyveromyces lactis*, a yeast capable of synthesizing and excreting fully active prochymosin, was soon considered as the best option (van den Berg et al. 1990; Morris and Anderson 1991) because of its impressive secretory capacity, its excellent fermentation characteristics at large scale, its food grade status and the availability of both episomal and integrative expression vectors (Swinkels et al. 1993; Bonekamp and Oosterom 1994). The recombinant chymosin from *K. lactis* has been in the market for more than 20 years now (Tramper 1994; van Dijck 1999) with great commercial success and it is probably the most widely used enzyme in cheese-making. Protein engineering has also been applied to improve chymosin performance by conferring the enzyme an increased specificity and better pH profile (Mantafounis and Pitts 1990).

The above example illustrates the evolution of the field of enzyme production. Chymosin is just one case; others exist that are also quite significant, like the production of alkaline proteases for detergents which has made extensive use of the tools of genetic and protein engineering to tailor-make proteases specifically designed to act efficiently under the harsh conditions of laundering (Estell et al. 1985; Aehle et al. 1993; Bryan 2000; Maurer 2004). The rational modification of enzyme structure and function by modern techniques, like directed evolution and site-directed mutagenesis, and the high throughput screening methods for finding novel or improved activities in nature's diversity will certainly impact enzyme biotechnology in the forthcoming years (Ogawa and Shimizu 1999; Panke and Wubbolts 2002).

2.2 Production of Enzymes

Enzymes are now produced for a variety of applications, going from bulk high tonnage processes in which the enzymes are considered as commodities (Hodgson 1994) to small-scale applications for refined uses and research where enzymes are considered specialties (Thomas et al. 2002). Level of production and type of application define the kind of process for its production. Specialty enzymes to be used in medicine and health-care products are usually required in high levels of purity

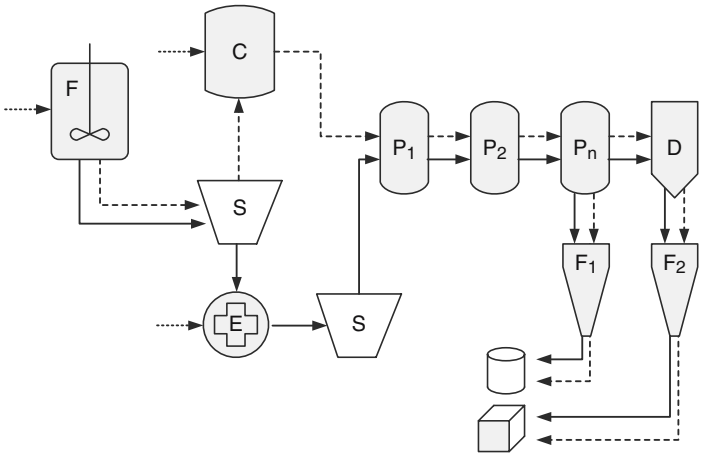

Fig. 2.1 Scheme for the production of enzymes. F: fermentation; S: solid–liquid separation; E: cell extraction; C: concentration; P_i: operations of purification; D: drying; F_i: formulation; - - - - ▶: extracellular enzyme; ——▶: intracellular enzyme; cell tissue or fluid - - - - ▶

and in rather small quantities, while enzymes used in the bulk production of food, feed, fabrics and fuel are usually produced as rather crude preparations in high tonnage (Headon and Walsh 1994). The increasing use of immobilized enzymes for large-scale processes has increased the demand for purer enzyme preparations (Cao 2005). The production process will depend on the source and localization of the enzyme. Enzymes from plant and animal origin will simply be extracted from the corresponding tissues or recovered from the corresponding fluids; microbial enzymes will be produced by fermentation and recovered either from the spent fermentation medium (extracellular enzymes) or from the cell paste after extraction by cell rupture or permeabilization (Aehle 2003). A generalized scheme for enzyme production is presented in Fig. 2.1.

The production process can be divided into four stages:

- Enzyme synthesis: it represents the propagation stage of the producing cells.
- Enzyme recovery: it represents the extraction of the enzyme from the producing cell system and involves solid–liquid separations, cell extraction and/or concentration.
- Enzyme purification: it represents a series of operations after enzyme recovery aiming to remove unwanted contaminants (mainly accompanying proteins).
- Enzyme product formulation: it consists in different operations aimed to give the enzyme product its final presentation; it includes final polishing operations, stabilization and standardization.

2.2.1 Enzyme Synthesis

Plant enzymes from tissues or exudates and enzymes from animal organs or fluids are synthesized as a part of the agricultural processes of plant growth and animal

breeding, so that the subject is outside of the scope of this book. These enzymes are usually byproducts of a main product to whose market is the enzyme production tightly bound.

Microbial enzymes produced by fermentation under controlled conditions constitute now the most relevant option for enzyme synthesis. Microbial strains can produce not only the enzyme proteins coded by their own genetic information, but also those produced by the expression of foreign genes as recombinant proteins. As analyzed before, microorganisms are ideal hosts for enzyme synthesis and only those glycoenzymes which cannot be properly produced in microbial hosts are to be produced in plant (Ma et al. 2003) or animal cell culture (Altamirano et al. 2004; Wurm 2004).

Microbial enzymes are produced mainly by submerged fermentation under tightly controlled environmental conditions (Rose 1980). However, solid-state fermentation (SSF) has also a good potential for the production of enzymes (Raimbault 1998; Pandey et al. 1999), especially those from filamentous organisms that are particularly suited for surface growth. Some enzymes, particularly those related to lignocellulose degradation, are currently being produced by SSF (Dueñas et al. 1995; Pandey et al. 2000; Kanga et al. 2004), but other hydrolases, namely amylases (Bogar et al. 2002) proteases (George et al. 1997) and phytase (Bogar et al. 2003; Vohra and Satyanarayana 2003; Roopesh et al. 2006) are being produced by SSF as well. SSF compares favorably with submerged fermentation in terms of energy requirements, volumetric productivity and product recovery; it represents a good option when production costs should be reduced as is the case of the microbial enrichment of agricultural residues or the production of bulk inexpensive enzymes (Illanes et al. 1992; Pandey et al. 2000). A complete treatment of the subject of solid-state fermentation has been recently published (Mitchell et al. 2006). In most cases, submerged fermentation is the technology of choice for microbial enzyme production (Lambert and Meers 1983; Barredo 2005). Submerged fermentation was vigorously developed after World War II for the industrial production of antibiotics (Mateles 1998) and since then it has represented the most relevant area of bioprocess engineering. Comprehensive reviews on the subject can be found in several textbooks (Aiba et al. 1973; Bailey and Ollis 1977; Wang et al. 1979; Quintero-Ramírez 1981; Blanch and Clark 1997; Nielsen et al. 2003; Acevedo et al. 2004; Bommarius and Riebel 2004). The technology is highly developed and automated and nowadays utilized for the production of most industrial enzymes (El-Mansi et al. 2007).

Submerged fermentation can be conducted in different modes of operation. The most traditional is batch fermentation, in which the bioreactor is filled with medium, inoculated and incubated under controlled conditions to the point in which the product (enzyme) has been synthesized to (or nearly to) its maximum level; then the cells are harvested for enzyme recovery, if intracellular, or else discarded to recover the medium containing the enzyme, if extracellular. Fed-batch fermentation is a variant of the former in which, after certain time of batch cultivation, the bioreactor is fed with nutrients according to a controlled rate profile and up to a final volume and the product is then recovered as above. This mode of cultivation is particularly appealing for the production of enzymes because it allows the control of the metabolic

responses of the producing cells and operation is rather simple (Moon and Parulekar 1991; Acevedo and Gentina 1996; Bojorge et al. 1999; Cereghino et al. 2002). The third mode is continuous culture, in which the medium is fed continuously to the bioreactor and the fermented broth continuously removed at the same rate. Steady state will eventually be obtained, so that the theory of the chemostat is applicable to describe this operation (Smith and Waltman 1995). Continuous culture has been extensively used as a tool to study enzyme regulation (Wiersma and Harder 1978; Egli et al. 1980); however, despite its obvious advantages of higher productivity and better control of operating conditions, the industry has been reluctant to adopt it, mainly because of the hazards of contamination and mutation that can washout the producing strain (Wang et al. 1979; Acevedo et al. 2004).

Some relevant aspects to be considered for developing a fermentation process for enzyme production are now analyzed.

Enzyme localization with respect to the producing microorganism is a key aspect in enzyme production. The enzyme can be properly intracellular, periplasmic or excreted into the medium during fermentation and this will define the downstream operations for its production. In principle, enzyme excretion is an asset as will be analyzed in the forthcoming sections. Most enzymes are intracellular but among extracellular enzymes there are many of technological significance; actually, a significant part of the commodity enzymes are extracellular. There are enzymes that are intracellular in one organism and extracellular in another; for instance, invertase is mainly intracellular in *Saccahromyces* (Illanes and Gorgollón 1986), while a significant portion of it is excreted in *Candida* (Dworschack and Wickerham 1961) and *Streptomyces* (Kaur and Sharma 2005); β-galactosidase is extracellular in molds (Park et al. 1979) while intracellular in yeasts (Mahoney et al. 1974). Intracellular enzymes can be made extracellular by genetic engineering and protein engineering techniques (Becerra et al. 1997).

Specific activity (units of enzyme activity per unit mass of microorganism) is a very relevant parameter for enzyme production by fermentation and much effort has been devoted to increase it by both genetic and environmental manipulations (Parekh et al. 2000). Conventional mutation and selection, genetic engineering, site-directed mutagenesis and directed evolution are powerful genetic tools to obtain high producing microbial strains (White et al. 1984; Arbige and Pitcher 1989; Verdoes et al. 1995; Reetz and Jaeger 1999; Chen 2001; Morley and Kazlauskas 2005; Kaur and Sharma 2006); in some cases, a substantial portion of the total protein synthesized by the organism corresponds to the enzyme. High specific activity not only reduces the cost of fermentation but also the cost of downstream operations. Significant increase in enzyme specific activity can be obtained by adequate environmental manipulations, mainly through medium design and optimization of relevant operation parameters like temperature, pH, agitation and aeration rates (Acevedo and Cooney 1973; Illanes et al. 1994; Barberis and Gentina 1998). Enzyme synthesis is subjected to different types of control by the producing strain (see ahead), so by proper medium design the biological signals that trigger such mechanisms can be put under our control. Most of the present applications of enzymes relate to hydrolases, which are enzymes mainly associated to cell catabolism;

therefore, their synthesis is controlled by induction (Jacob and Monod 1961; Clarke and Brammar 1964) and catabolite repression (Moses and Prevost 1966; Epstein et al. 1975; Shinmyo et al. 1978). Both controls are exerted at the level of transcription; the former requires the presence of the inducer to block the repressor protein and allow the transcription of the structural gene coding the enzyme; the later allows the cell to establish a hierarchy of substrate utilization by repressing the expression of the genes coding the catabolic enzymes of one substrate while the other (supposedly the better) is being consumed. Catabolite repression is related to the substrate consumption rate and not to the substrate structure. In Gram-negative bacteria, control is associated to the level of cyclic AMP that acts as a positive modulator by preventing the blockage by a repressor protein of the structural genes coding the enzymes (Demain 1968; Pastan and Adya 1976; Nandakumar et al. 1999). This is not a universal mechanism: in some enterobacteria cyclic AMP has proven to have no significant effect (Wanner et al. 1978) and in Gram-positive bacteria, yeasts and moulds other signal molecules, like cyclic GMP and polyphosphorylated nucleotides, are involved (Karaolis et al. 2005; Traxler et al. 2006). These control mechanisms have profound influence on enzyme synthesis. Culture medium should have adequate levels of inducer, which can be the substrate, a substrate analogue or the product of the enzyme reaction (Rosenfeld and Feigelson 1969; Kurasawa et al. 1992). Substrate analogues are more potent inducers than the substrate itself because they are not acted upon by the enzymes they induce; in the case of depolymerases, inducers are usually intermediate or end products of hydrolysis, since the substrate as such cannot enter the cell to trigger the mechanism (Illanes and Rossi 1981; Illanes et al. 1988a). Level and time of addition of the inducer affects the level of enzyme synthesized and are operation parameters that should be optimized (Illanes et al. 1994).

Specific growth rate of the producing strain is also a relevant parameter for enzyme production by fermentation. Many enzymes are synthesized as growth-associated metabolites so that cell specific growth rate has a direct impact on enzyme specific rate of synthesis as shown by the non-structured model of Luedeking and Piret (1959):

$$\frac{dp}{dt} = \alpha \cdot \frac{dX}{dt} + \beta \cdot X \tag{2.1}$$

$$q_P = \frac{1}{X}\frac{dp}{dt} = \alpha \cdot \mu + \beta \tag{2.2}$$

where α prevails over β for growth associated metabolites. Conditions that maximize the specific cell growth rate are often in compromise with those that maximize the specific rate of enzyme production (Gordillo et al. 1998). In fact, it is usual that pH (Illanes et al. 1988a; McDermid et al. 1988), temperature (Akinrefon 1969; Feller et al. 1994) and the level of dissolved oxygen (García-Garibay et al. 1987; Barberis and Gentina 1998) optimal for growth differ from the corresponding optima for enzyme production. Compromise values are often used, but impressive increases in enzyme productivity have been reported by profiling these variables during cell culture (Mukhopadhyay and Malik 1980; Mukhopadhyay 1981;

Ioniţă et al. 2001). In the case of enzymes whose synthesis is non-growth associated, a two stage culture can be envisaged and the variables optimized for each stage.

Genetic stability and safety of the producing microbial strain are also relevant aspects to be considered in enzyme production. This is particularly so in the case of recombinant enzyme proteins because of structural and segregational instability of the cloning vector (Vehmaanperä and Korhola 1986; Impoolsup et al. 1989; Murooka and Imanaka 1994; Ansorge and Kula 2000). Depending on the use of the enzyme, the producing strain must be considered safe for its application. For instance, enzymes used in the food industry in USA should have the GRAS (generally recognized as safe) status conferred by the Food and Drug Administration (FDA). A list of GRAS enzymes and the corresponding producing strains can be found in http://www.cfsan.fda.gov/∼dms/opa-enzy.html. To obtain a GRAS status for an organism is costly and time-consuming so that sometimes it is a better option to clone the enzyme structural gene into a GRAS host (White et al. 1984; Domínguez et al. 1998).

Morphological and rheological properties of the producing strain are also relevant for enzyme production, especially for the case of mycelial microorganisms (Jayanta et al. 2001; Kim et al. 2003). Viscosity increase and non-Newtonian rheology may reduce oxygen transfer rates and enzyme synthesis is usually related to one particular growth morphology (Olsvik et al. 1993; Bhargava et al. 2005).

2.2.2 Enzyme Recovery

Once the enzyme has been synthesized by the producing organism, it must be recovered, which implies its separation from the cell system (see Fig. 2.1).

2.2.2.1 Solid–Liquid Separation

First downstream operation is the solid–liquid separation of the cells from the spent fermentation medium. Separation can be done conventionally by centrifugation or dead-end filtration. Filtration is more adequate for multicellular organisms of filamentous morphology, like molds and actynomycetes, but filter-aid should be used because of the compressible nature of the cell cake formed. Plate and frame and rotary filters are frequently used. Centrifugation is more adequate for unicellular organisms, like bacteria and yeasts, but usually prior flocculation is required because of the small size of the individual cells. Tubular or disk-type centrifuges are the most used. These operations, though widely employed in bioprocesses, suffer from some drawbacks because microbial cells are small, compressible, and their density is similar to that of the spent medium. Therefore, alternatives to these operations have been developed. Among them, cross-flow microfiltration is outstanding: flow is tangential to the membrane which severely reduces cake formation and membrane clogging, operating costs are lower and the operation is

modular and scalable (Quirk and Woodrow 1983; Le and Atkinson 1985; Nagata et al. 1989). Variables in cross-flow microfiltration for enzyme recovery have been thoroughly analyzed (Kroner et al. 1984). Improvements of the original design of microfilters have been developed and proven useful for enzyme recovery (Lee et al. 1995; Frenander and Jönsson 1996). A comprehensive review on conventional solid–liquid separation operations for biological products has been published by Medronho (2003).

If the enzyme is excreted during cell growth, recovery will proceed from the liquid phase; if the enzymes remains associated or within the cell, recovery will proceed from the solid phase (see Fig. 2.1). Excretion is desirable from a process perspective, so efforts have been made for genetically engineered cells to export otherwise intracellular enzymes (Hatti-Kaul and Mattiasson 2003a).

2.2.2.2 Concentration of Extracellular Enzyme Crude Broths

Excretion of the enzyme is favorable because of cell membrane selectivity that acts as a powerful purification step, since only few proteins are excreted. Therefore, the clarified fermentation broth has a rather high degree of purity that in many cases suffices the requirements for a particular enzyme use so that further purification steps are not required. The main concern here is the low protein concentration. This represents the main drawback of extracellular enzymes, since concentration of the starting material has a profound impact on final production cost (Knight 1989). Even under dense culture conditions, protein concentration rarely exceeds a few grams per liter (Liu et al. 2000) so that the enzyme broth has to be concentrated at least by one order of magnitude prior to further purification steps or formulation.

Many of the operations suitable for enzyme concentration have some potential for protein fractionation (purification). Only those mainly devoted to concentration will be analyzed here; the others will be considered in the section 2.2.3.

The most obvious operation for carrying this out is vacuum evaporation: technology is conventional and has been traditionally applied for the concentration of foodstuffs (Conrad et al. 1993; Singh and Heldman 2001) and pharmaceuticals (Manzatu et al. 1999; Shire et al. 2004). Enzymes are labile molecules so that evaporation must be conducted under rather high vacuum to prevent denaturation (Lambert and Meers 1983; Schaffeld et al. 1988). Other gentler methods of concentration are becoming more relevant. Concentration by water freezing has also been considered (Darbyshire 1981; Whitaker 1994) and high throughput crystallyzers have been designed (Janson et al. 1974). The method is not competitive with evaporation in economic terms and protein occlusion within the ice-water crystals reduces yield so that it is not a viable option except for the case of very labile enzymes. Water removal by freeze drying is acceptable as a final polishing step for highly purified enzymes, but it is not economically viable at that step (van den Tweel et al. 1994); besides, the presence of dissolved salts produce eutectic mixtures and foaming is a problem because of enzyme denaturation. Concentration by foaming has also been performed taking advantage of the tensoactive properties of proteins (Lalchev and

Exerowa 1981; Ekici et al. 2005). Foaming can be induced by air or inert gas bubbling through the enzyme solution and proteins will accumulate at the gas–liquid interface as predicted by Gibbs law (Thomas and Winkler 1977):

$$-\frac{d\sigma}{dc} = \frac{R \cdot T}{c} \cdot \Gamma \tag{2.3}$$

This technique has also the potential for protein fractionation (Sarkar et al. 1987; Miranda and Berglund 1993; Varley et al. 1996; Brown et al. 1999a,b), but this has not been fully appreciated, maybe because yields of recovery are low due to enzyme inactivation at the gas–liquid interface.

The most relevant operation for enzyme concentration is ultrafiltration. Ultrafiltration is a membrane filtration operation, where pressure difference across the membrane is the driving force. Molecules are separated on the basis of their size in the range from 1,000 to 100,000 Da, so covering most of the enzymes. Despite its potential of fractionation, ultrafiltration has been mainly used to concentrate enzymes by removing solvent (water) and smaller size solutes (Ehsani et al. 1996; Euzenat et al. 1998). Ultrafiltration membranes are made of different materials, among which polyacrilonitrile, polisulphone, polyamide and polyvinylidene fluoride are outstanding in terms of stability, mechanical strength and chemical compatibility. Ultrafiltration membranes are characterized in terms of its molecular weight cutoff and can be isotropic or anisotropic, the latter being best in terms of flowrate and control of operation (van den Tweel et al. 1994). Rejection coefficient, this is, the fraction of the solute (protein) concentration that is retained by the membrane is also used to characterize it. Membranes are composed by a selective skin of about 0.2 μm width supported by a sponge-like structure of about 50 to 150 μm width that confers mechanical strength. Ultrafiltration equipments come in different formats and sizes. Large size units are usually composed by stacked flat sheets or hollow fiber bundles of about 1 mm in diameter. Units are quite compact, exhibiting large area to volume ratios, and are modular so that they can flexibly adapt to process requirements. Operating pressures range from 100 to 500 KPa and capacities range from 10 to $200\,L/m^2 \cdot h$ (Aroca and Zúñiga 2004). Ultrafiltration is a mild operation; therefore, enzyme inactivation is kept to a minimum. Its main problem is the phenomenon of concentration polarization (Sablani et al. 2001), which refers to the buildup of protein near the membrane as a consequence of the flow through it. Increase in protein concentration near the membrane establishes a gradient that promotes back diffusion of the protein away from the membrane surface. At high protein concentration or flux, the membrane can be saturated and transmembrane flow determined by the protein back diffusion rate, which is slow enough to severely affect the operation. A secondary membrane composed by retained solutes (proteins) builds up increasing the hydraulic resistance and reducing membrane selectivity (Goosen et al. 2005). This latter aspect is of major concern when using ultrafiltration for protein fractionation (Ghosh and Cui 1998). To minimize concentration polarization, the judicious selection of the membrane and the flow regime near the membrane are important to reduce the film thickness and to scour deposits; low flux and low protein concentrations are advisable if practical. The effect of

concentration polarization can be managed by offering a large area for filtration as it occurs in the very compact modules now available. Selective removal or replacement of low molecular weight solutes in the enzyme preparation can be done by diafiltration in which ultrafiltration proceeds against a solution of defined composition (Pacheco-Oliver et al. 1990). Ultrafiltration can be considered now as the best option for enzyme concentration in most cases: it is a mild operation that causes no significant losses and the advances in the field of material science and process engineering provide now very flexible options to select the most adequate membrane and equipment for each particular case. A comprehensive review on protein isolation by membrane technology has been reported by Ulber et al. (2003).

2.2.2.3 Extraction of Intracellular Enzymes

Enzyme extraction will be determined to a great extent by the type of container cells and the location of the enzyme within the cell structure (Balasundaram and Pandit 2001). Enzymes from animal cells or tissues are easy to extract since such cells are devoid of cell wall; therefore, osmotic shock and other rather mild extraction methods are quite effective (Shin et al. 1994). Enzymes from plant cells or tissues require more rigorous conditions because they are endowed with a thick cellulose wall; however they have a rigid structure that can be efficiently ruptured by applying shear forces (Pierpoint 2004). Microbial cells, especially bacteria and yeast, are particularly difficult to disrupt because of the resilient nature of their cell envelopes. However, some are periplasmic and can be extracted rather easily by gentle procedures like osmotic shock; actually, a differential release can be obtained in such cases aiding substantially to enzyme purification (Fonseca and Cabral 2002). On the other extreme, some enzymes are bound or contained within membranes or other internal cell structures; in those cases, extraction not only requires cell disruption but also special extractive procedures involving detergents or solvents that can certainly be harmful for the enzyme (van den Tweel et al. 1994; Whitaker 1994). Notwithstanding, most intracellular enzymes are cytoplasmic and their recovery implies cell disruption or permeabilization, which is not an easy task. Methods for intracellular enzyme recovery can be divided into those that produce cell rupture by mechanical forces (Chisti and Moo-Young 1986; Kleinig and Middelberg 1998) and those that produce cell permeabilization by membrane damage (Bansal-Mutalik and Gaikar 2003; Cheng et al. 2006). Some new methodologies have arisen, like the extraction of thermostable enzymes by thermolysis (Ren et al. 2007). The most relevant methods for intracellular enzyme extraction are listed in Table 2.1. Not all of them are amenable for large-scale operation, whether for economical or technological reasons. Only those with potential application for the large-scale recovery of enzymes will be shortly reviewed here. Comprehensive reviews on the subject can be found elsewhere (Asenjo 1990; Doelle 1994; Cumming and Iceton 2001).

Mechanical disruption methods are the most studied and frequently represent the best option in terms of process economics. They are well endowed for large-scale operation, validation is rather simple and conditions of operation can be optimized

Table 2.1 Methods of Extraction of Intracellular Enzymes

Method	Principle	Application at Large Scale
Cell rupture		
Pressure	Compression, shear stress	Moderate
Homogenization	Shear stress, cavitation	Highly feasible
Milling	Compression, shear stress	Highly feasible
Sonication	Cavitation	Moderate
Decompression	Decompressive explosion	Moderate
Freezing–thawing	Shear stress	Unlikely
Dispersion in water	Osmotic shock	Unlikely
Thermolysis	Cell wall rupture	Moderate
Cell permeabilization		
Alkali treatment	Cell wall digestion	Unlikely
Solvent treatment	Membrane digestion	Moderate
Enzymatic lysis	Cell wall digestion and osmotic rupture	Feasible
Autolysis	Cell wall digestion and osmotic rupture	Feasible

(see ahead). Their main drawback is that drastic conditions are required for efficient cell breakage so that enzyme inactivation during operation is a major concern. Depending on the method, temperature may rise significantly and very efficient heat transfer is required, which is not easy to attain especially at large scale of operation (Foster 1995). Among the many options available, the use of homogenizers and bead mills outstand. High-pressure homogenizers are intensively used in the food industry. Actually, equipments similar to those used in milk homogenization can be used for cell disruption and are available in all kind of sizes and design (see www.directindustry.com). They have been extensively used for enzyme and recombinant protein extraction (Chi et al. 1994; Leser and Asenjo 1994; Choi et al. 1997; Fonseca and Cabral 2002) and perform well especially with bacterial cells being also efficient with yeast cells, but not applicable with highly filamentous organisms. Homogenizers work by forcing the cell suspensions under high pressure through a very narrow passage in the valve, which then impinges on a hard-impact ring. Disruption of the cell wall occurs by a combination of compression, highly turbulent eddies and strong shear forces (Kleinig and Middelberg 1998; Middelberg 2000). Most of the studies on mathematical modelling of cell disruption (see ahead) have been performed in homogenizers, presumably because the number of relevant operational variables is small: pressure is the key variable and also temperature and the number of passages through the homogenization valve are relevant. Bead milling is another important method for cell disruption which is considered one of the most efficient techniques for cell disruption (Darbyshire 1981). It works very well with yeast (van Gaver and Huyghebaert 1991; Zúñiga et al. 1992; Garrido et al. 1994; Illanes et al. 1995; Illanes et al. 1996) and is also effective with bacteria (Veide et al. 1983; Santos et al. 1996; Bierau et al. 2001; Singh et al. 2005) and filamentous organisms (Baldwin and Moo-Young 1991a,b). Cells in the form of a paste or slurry are mixed with small size beads (made of glass, ceramic or metal) that act as abrasives, and subjected to intense stirring, rupture being produced by a combination of compression and shear forces. Process can be continuous or discontinuous and temperature

Table 2.2 Components of Microbial Cell Envelopes

Microorganism	Cell Structure	Components
Gram-negative bacteria	Cell wall	Peptidoglycans
	External membrane	Lipopolysaccharides, proteins
	Cytoplasmic membrane	Phospholipids, proteins
Gram-positive bacteria	Cell wall	Peptidoglycans
	Cytoplasmic membrane	Phospholipids, proteins
Yeasts	Cell wall	Mannanoproteins, β-glucans
	Cytoplasmic membrane	Phospholipids, proteins
Molds	Cell wall	Cellulose, chitin, β-glucans, proteins
	Cytoplasmic membrane	Phospholipids, proteins

control can be exerted by an outer jacket or by recycling through a heat exchanger. There are a number of equipments that have been specifically deigned for cell disintegration and are available in different sizes and designs (see: www.glenmills.com). Mathematical modelling of this type of operation has been hampered by the large number of variables involved (see ahead).

Digestion of cell envelopes is a more selective technique for the release of intracellular compounds. Contrary to mechanical methods, cell rupture is not required and in most cases cells are merely pemeabilized. The system of choice should be dictated by the chemical composition of the cell envelopes. Among cell permeabilization methods, those involving alkali (Wiseman 1995) or organic solvent treatment (Decleire et al. 1987; Bachhawat et al. 1996; Panesar et al. 2007) are usually too harmful, costly and non-specific, so that their use is limited.

Cell permeabilization by selective enzyme digestion is a more promising technology that can be of interest for selectively recovering labile and expensive intracellular enzymes. Lysozyme has been routinely used for disrupting bacterial cell wall peptidoglycans (Chen and Chen 1996), though it has been postulated that can also act as a an activator of pre-existing autolytic wall enzymes (Wecke et al. 1982); it has also been used in combination with β-glucanase for the degradation of yeast cell wall (Knorr et al. 1979). Helicase from *Helix pomatia* has been routinely used for cell wall degradation of yeasts (Koch and Rademacher 1980) and molds (Anné et al. 1974) The chemical composition of the cell envelopes, shown in Table 2.2 (Aroca and Zúñiga 2004), can be used to judiciously select the appropriate enzyme or enzyme cocktail.

Former lytic enzyme preparations were expensive, non-specific and not readily available in large quantities (Kula and Schütte 1987), but more specific microbial lytic enzymes have been developed that can be produced more economically on a large scale (Asenjo and Andrews 1990). β-Glucanases from *Cytophaga* and *Oerskovia* have proven to be quite effective in yeast cell wall degradation (Hunter and Asenjo 1987a). Selective extraction of recombinant proteins and enzymes have been performed with such enzymes with considerable success; in many cases, no more than 20% of the intracellular protein has been released which is a major saving in terms of further purification requirements (Huang et al. 1991; Asenjo et al. 1993). Actually, the potential of this method relies very much on that selectivity,

but enzyme cost is still a major restriction that can be overcome by screening better lytic enzymes by directed evolution (García et al. 1988; Salazar et al. 2006) and protein engineering (Salazar et al. 1999). Kinetics of extraction with lytic enzymes has been modelled and the process optimized (Hunter and Asenjo 1987b; Liu et al. 1988). The subject of enzymatic lysis of microbial cells has been recently reviewed (Salazar and Asenjo 2007).

Autolysis is a very appealing method for enzyme extraction in those organisms prone to autolysis, like yeasts (Knorr et al. 1979; Kollar et al. 1991) and bacilli (Svarachorn et al. 1991). Under certain stress conditions (i.e. drying, high organic solvent or electrolyte concentration) lytic enzymes (proteases, nucleases and glucanases) are induced that partially digest its own cell wall being then the intracellular content easily extracted by osmotic shock (Reed and Peppler 1973; Breddam and Beenfeldt 1991; Kollar et al. 1993). Autolysis has been used for long in the extraction of cellular proteins; it is the basis for commercial production of some types of yeast extracts (Nagodawithana 1992; de Palma Revillion et al. 2003) and it has been used efficiently to extract yeast enzymes (Amrane and Prigent 1998). Invertase, both periplasmic and cytoplasmic, was efficiently extracted from bakers' yeast cells subjected to autolysis by drying. At a critical moisture content autolysis was triggered, the efficiency of protein extraction and enzyme recovery depending on the time that the cells remained below such critical moisture (Illanes and Gorgollón 1986). The process was scaled up to pilot level and the crude extract was used as raw material for the production of a biocatalyst employed in the continuous inversion of sucrose syrup (Illanes et al. 1988b). The process of autolysis has been optimized, the addition of exogenous papain notably increasing yield and productivity by reinforcing the autolytic effect (Gutiérrez 1993). It is an interesting alternative for extraction of yeast enzymes: it is cheap, simple and readily scalable, the main drawback being its low productivity as a consequence of the prolonged time required for autolysis.

There are many efficient methods for disrupting cells for the release of its intracellular content. The problem with enzymes is that the method must be sufficiently rough to disrupt or distort the cell envelopes, but gentle enough to preserve activity. This poses a compromise so that the process can be optimized. A suitable objective function for optimization is the amount of active enzyme recovered:

$$E = p_R \cdot a \tag{2.4}$$

Protein extraction and enzyme inactivation follow their own kinetics so that:

$$p_R = f(t) \tag{2.5}$$

$$a = \varphi(t) \tag{2.6}$$

$$E = f(t) \cdot \varphi(t) \tag{2.7}$$

and the optimum condition will be given by:

$$\frac{dE}{dt} = f(t) \cdot \frac{d[\varphi(t)]}{dt} + \varphi(t) \cdot \frac{d[f(t)]}{dt} = 0 \tag{2.8}$$

In principle, any extraction method can be optimized accordingly as long as suitable and validated expressions for protein release and enzyme inactivation rates are available. In practice Eqs. 2.5 and 2.6 can be complex and depend on many operational variables (Currie et al. 1972). If both protein release and enzyme inactivation are assumed to proceed according to first order kinetics:

$$\frac{dp_R}{dt} = k_R \cdot (p_T - p_R) \tag{2.9}$$

$$-\frac{da}{dt} = k_D \cdot a \tag{2.10}$$

From Eqs. 2.8 to 2.10, the optimum $(^*)$ conditions for extraction are obtained (Illanes 1994):

$$p_R^* = \frac{k_R}{k_R + k_D} \cdot p_T \tag{2.11}$$

$$a^* = a_0 \cdot \left(\frac{k_R + k_D}{k_D}\right)^{-\frac{k_D}{k_R}} \tag{2.12}$$

$$t^* = \frac{\ln \frac{k_R + k_D}{k_D}}{k_R} \tag{2.13}$$

$$E^* = a_0 \cdot p_T \cdot \frac{k_R}{k_R + k_D} \left(\frac{k_R + k_D}{k_D}\right)^{-\frac{k_D}{k_R}} \tag{2.14}$$

Protein release by bakers' yeast and *Bacillus brevis* cell disruption in an industrial homogenizer has been carried out and modelled. The most relevant variable was pressure drop across the homogenizer valve; k_R depended on the pressure at exponents of 2.9 and 1.8 for the yeast and the bacteria respectively (Follows et al. 1971; Augenstein et al. 1974). In the latter case, the recovery of a labile intracellular enzyme was optimized by combining protein release and enzyme inactivation kinetics. Protein release was also modelled in an industrial bead mill, but in this case k_R depended on too many variables: bead size, cell concentration, beads to cell paste volumetric ratio, temperature, agitation speed, agitator design, and recycling ratio (Schütte et al. 1983).

2.2.2.4 Removal of Cell Debris

After extraction, the enzyme preparation is contaminated with undisrupted cells and cell debris that have to be removed before purification. Common operations of solid–liquid separation, like centrifugation and dead-end filtration, can be used but the drawbacks already mentioned with respect to cell separation are augmented by the very small size of the cell fragments. Microfiltration is a better option that has been used successfully (Vaks et al. 1984; Chan et al. 1991).

Aqueous two-phase extraction is the most promising operation for cell debris removal that, despite its potential for protein fractionation (Marcos et al. 1999;

Mohamadi and Omidinia 2007), will be revised in this section. It is basically a liquid–liquid extraction system that considers two aqueous phases comprising either two polymers or a polymer and a salt (Kroner and Kula 1978; Kula 1979; Hustedt et al. 1985; Abbott et al. 1990). Phase separation is produced due to the phenomenon of polymer incompatibility (Albertsson et al. 1990) that produces two-phases by mutual exclusion. However, each phase is aqueous in nature, so in principle not detrimental for enzyme activity as in conventional water-solvent two-phase systems (Andersson and Hahn-Hagerdal 1990). Other advantages are the variety of polymers that can be used, its environmental benignity and the availability and suitability of equipment for liquid–liquid extraction, which is a conventional operation in the chemical and pharmaceutical industry (Podbielniak et al. 1970; Raghavarao et al. 2003). The most common systems are polyethyleneglycol-dextran and polyethyleneglycol-salt. The former is expensive, the medium is highly viscous and the process is hard to validate when crude dextran fractions are used so the latter is preferred especially for large-scale operation, as long as the enzyme withstands the high ionic strength required in the salt phase (Gupta et al. 1999; Banik et al. 2003). Several other biphasic systems have been tried with the purpose of using less expensive an more environmentally benign polymers (Tjerneld et al. 1986; Andersson and Hahn-Hagerdal 1990; Miranda and Berglund 1990; Kepka et al. 2003; Raghavarao et al. 2003; Sarubbo et al. 2004; Rosso et al. 2005; Bezerra et al. 2006), but the polyethyleneglycol-salt system is still the most used (Sarmento et al. 1997; Iwamoto and Shiraiwa 2005; Dolia and Gaikar 2006; Jaw et al. 2007). Two-phase partitioning can be smoothly integrated to the extraction step (Zhou et al. 1997; Su and Feng 1999; Chang and Su 2005) and can also be improved by combining with affinity ligation (affinity partition) (Xu et al. 2003; Lam et al. 2004; Teotia and Gupta 2004; Castell et al. 2006).

The two-phase system can be represented by a phase diagram, as the one shown in Fig. 2.2 for the polyethylene glycol (MW 4000)-dextran (T500) system where the equilibrium is represented by the curved line.

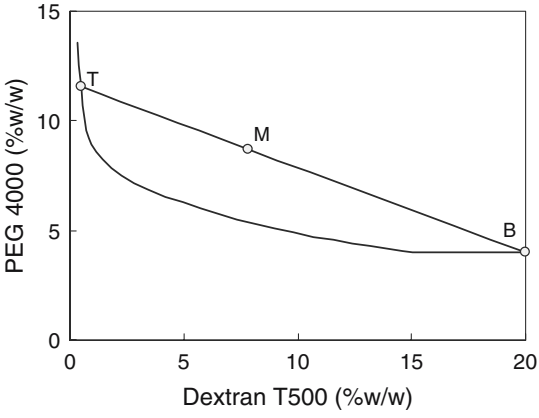

Fig. 2.2 Phase diagram of the polyethylene glycol 4000-dextran T500 system

All mixtures corresponding to points over the equilibrium curve (M) separate into two phases whose composition is defined by points T and B, corresponding to the top and bottom phases respectively. The volume ratio of top phase to bottom phase will be given by the relative values of the traces BM and MT over the corresponding tie-line:

$$\frac{V_T}{V_F} = \frac{\overline{BM}}{\overline{MT}} \tag{2.15}$$

Proteins and cell debris are distributed between the two phases according to their partition coefficient. For a specific protein, its partition coefficient is:

$$K_P = \frac{c_T}{c_B} \tag{2.16}$$

When used for cell debris removal, proteins invariably concentrate in the top phase (PEG in the case of PEG/dextran and PEG/salt), while fragments do it in the bottom phase. Therefore yield of protein recovery is:

$$Y_T = \frac{V_T \cdot c_T}{V_T \cdot c_T + V_B \cdot c_B} = \frac{1}{1 + \frac{V_B}{V_T} \cdot \frac{1}{K_p}} \tag{2.17}$$

Yield will obviously increase with K_P, but also with the top to bottom volume ratio. K_P depends on many factors related both to the biphasic system (i.e. molecular weight of polymers, concentration of polymers and salts, pH, temperature, concentration of cell fragments), and to the target protein (i.e. hydrophobic amino acid residues, number of carboxyl and amino side chains). General rules on how to increase or decrease K_P have been set up by Huddleston et al. (1991).

Most large-scale equipment for liquid–liquid extraction is available for two-phase extraction and usually multistage systems have to be used for obtaining high yields. Kühni type columns, Podbielniak centrifugal extractors and disk and bowl centrifuges have been used (Kula et al. 1981; Veide et al. 1983; Cunha and Aires-Barros 2002).

2.2.3 Enzyme Purification

The crude extract or clarified broth containing the enzyme is then subjected to purification, conceived as a sequence of operations (see Fig. 2.1) aimed to remove all contaminants that can interfere with its intended use. Purification can also serve to the purpose of increasing the specific activity of the biocatalyst in the case of enzyme immobilization. The situation will be radically different for an intracellular than for an extracellular enzyme. In the first case, the enzyme extract is a complex mixture of proteins, nucleic acids and other cell constituents, while in the latter the enzyme concentrate contains only some extracellular proteins and small molecular weight solutes, since the cell membrane acts as a powerful barrier to retain most of the cell constituents aiding powerfully to purification. Many extracellular hydrolases

sold as commodities are in fact rather crude preparations, hardly subjected to any purification step. In the case of intracellular enzymes, the extract is heavily contaminated not only with other proteins but also with nucleic acids, so that a previous step for nucleic acid removal is customary. Several options exist, including nuclease treatment and precipitation with different agents (Harve and Bajpai 2000). Ammonium sulfate is effective in removing nucleic acids but precipitates protein as well, so more specific precipitants are required. They are usually positively-charged materials which form complexes with the negatively-charged phosphate residues of the nucleic acids. These include polyethyleneimine, the cationic detergent cetyltrimethyl ammonium bromide, streptomycin sulfate and protamine sulfate (Burgess 1969; Yang et al. 1987; Cordes et al. 1990). All these procedures are expensive and may have some detrimental effect upon enzymes so they are used at a large scale only when contamination of the enzyme product is unacceptable. Treatment with bovine pancreatic nucleases has been considered as the most cost-effective method of nucleic acid removal (http://www.lsbu.ac.uk/biology/enztech/nucleicic.html).

Small molecular weight solutes, particularly ions, might interfere with the initial purification steps and in such case they should be previously removed either by diafiltration or size exclusion chromatography. This is not usual, so that removal of ions (desalination) is rather used as a final polishing step.

Enzyme purification is aimed mainly to the removal of contaminant proteins; therefore, enzyme purification is in essence a series of operations of protein fractionation. A compromise exists between purification and yield of recovery. Each operation intended for purification produces an increase in purity (conveniently expressed in terms of specific activity: units of activity per unit mass of protein) but inevitably some enzyme activity is lost so that yield of recovery is lower than 100%. Purification factors and yields for a given operation (i) and global values for N consecutive operations are then:

$$(\mathrm{PF})_i = \frac{a_i}{a_{i-1}} \tag{2.18}$$

$$\mathrm{PF} = \frac{a_N}{a_0} = \prod_{i=1}^{N} (\mathrm{PF})_i \tag{2.19}$$

$$Y_i = \frac{E_i}{E_{i-1}} \tag{2.20}$$

$$Y = \frac{E_N}{E_0} = \prod_{i=1}^{N} Y_i \tag{2.21}$$

There is clearly a compromise between purification and yield of recovery, since increasing levels of purity require a higher number of operations, each of them having its own yield of recovery. Even if the yield of recovery per operation is high (i.e. 80%), if purification requires several (i.e. five) consecutive operations, global yield of recovery will be low (i.e. only 33%). If average purification factor is 2.5 per operation, after five operations it will be 98. This can be appreciated in Fig. 2.3.

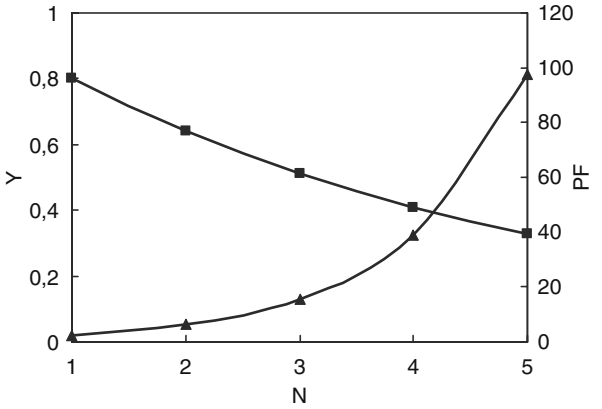

Fig. 2.3 Purification factor (PF) and yield of recovery (Y) as a function of the number of purification steps (N)

For the case of industrial enzymes, which are produced at a large scale as commodities, the criterion of purification is the minimum compatible with its intended use. This is so, because purification at large scale is cumbersome and expensive and usually not compensated for the benefit of producing a purer protein. In such cases, yield of enzyme recovery rather than enzyme purity is the objective function. The situation is different for the case of specialty enzymes, where purity cannot be sacrificed for yield considerations. However, there are increasing incentives for purification even for bulk enzymes, especially in the case of immobilized enzymes where purity might have a significant impact in process economics and also because of recent advances in the field of protein purification on a large scale (Ladisch et al. 1998). Besides yield of recovery and resolution, capacity and speed are relevant characteristics that can be considered when selecting a given operation of purification.

In principle, any method intended for protein fractionation can be used for enzyme purification. However, the methods applicable for production purposes are restricted to those amenable for scale-up at a reasonable cost. Only those will be reviewed here. Comprehensive reviews on protein purification can be found elsewhere (Asenjo 1990; Janson and Rydén 1998; Roe 2001; Hatti-Kaul and Mattiasson 2003b; Rosenberg 2004) and handbooks on the subject are also available (http://www.biochem.uiowa.edu/donelson/Database%20items/protein_purification_handbook.pdf).

Some general guidelines can be helpful when designing a purification system (Wheelwright 1987). Operations based on physical properties (i.e. molecular size, solubility, surface charge distribution) or biological properties (ligand specificity) should be selected to fully exploit those in which marked differences exist between the target enzyme and the rest of the contaminant proteins (Watanabe et al. 1994). Each operation should be based on a different property. More bulky operations should be conducted first to early reduce the processing volume; more expensive operations should be performed last to act on a reduced volume of product stream.

Damaging contaminants, like proteases, should be early removed. All operations developed at laboratory scale should be judiciously analyzed for its scalability. Number of operations should be as few as possible. Use of additives should be minimized to avoid further purification steps. The famous KISS rule (keep it simple, stupid – recalling Nobel laureate William Shockley) certainly applies.

Rational design and optimization of protein purification processes have been developed and optimal operation sequences determined by using expert system (Asenjo et al. 1991; Leser and Asenjo 1992; Mao and Hearn 1996; Lienqueo et al. 1999; Vásquez-Alvarez et al. 2001; Simeonidis et al. 2005).

Most reported protocols for protein purification at laboratory scale are hardly applicable for production purpose since a large number or complex operations are considered and very low yields of recovery (usually below 10%) and productivities are obtained at that scale (Saha 2004; Hanson et al. 2005; Sian et al. 2005; Patel et al. 2006). Only highly priced recombinant proteins for therapeutic use that are produced on a small scale can be obtained with such low yields since in such cases very high purification factors are required that demand many and complex operations (Schaap and Parker 1990; Hua 1997; Bond et al. 1998).

Some of the most relevant methods of enzyme purification by protein fractionation applicable at large scale will be reviewed. Such methods can be roughly divided into those based on fractional precipitation and those based on differential retention in a solid matrix.

The formers are based on the reduction in solubility as a consequence of the addition of salts (*salting-out*), organic solvents or polymers. These methods have been traditionally used as initial purification steps because they are simple and readily scalable. However, because of its low selectivity, purification factors are modest so they are used mostly as an initial concentration step that will facilitate subsequent more selective operations. The solubility of proteins varies with the ionic strength and hence with the salt concentration in the solution. At low concentrations of salt, the solubility of the protein increases with salt concentration (*salting-in*), but as the salt concentration increases further, the solubility of the protein begins to decrease and protein precipitates from solution (*salting-out*). Salting-out is a traditional operation for protein fractionation, usually performed first (Coulon et al. 2004), which is based on the decrease in protein solubility produced at high concentrations of dissolved salts. The ability of a salt as precipitating agent is well correlated to the Hoffmeister series (Hoffmeister 1887). In general, the more effective are the salts of univalent cations and multivalent anions, which are more antichaotropic and increase hydrophobicity promoting protein aggregation (Erson et al. 1998). Protein precipitation at high salt concentration has been explained by ion solvation that reduces the availability of water for protein dissolution (Dixon and Webb 1961). Salting-out has been described by Cohn's equation:

$$\log S = \beta_S - K_S \cdot \mu' \tag{2.22}$$

where:

$$\mu' = \frac{1}{2} \sum m_i \cdot Z_i^2 \tag{2.23}$$

β_S represents the logarithm of the solubility of the protein at zero ionic strength and is a strong function of temperature and pH. K_S is the slope of the solubility curve and depends on the nature of the protein and the salt, but not on pH or temperature. The magnitude of K_S defines the range of ionic strength at which the protein precipitates; then, a high K_S value is convenient in terms of purification because the protein will precipitate in a narrow range of ionic strength. β_S defines the magnitude of ionic strength at which protein precipitation starts so that a low value of β_S is convenient because the lower its value the lower the ionic strength at which the protein precipitates. Cohn's equation does not contain any protein concentration dependent term and in fact, the precipitation curve will not change with protein concentration, its value simply determining the ionic strength threshold value for precipitation (Illanes 1974). For any given salt, K_S may vary significantly according to the size of the molecule, being larger for large asymmetric proteins. Ammonium sulfate, even though not being the best salt according to the Hoffmeister series, is usually the choice since it is harmless to the enzyme (it is actually used for enzyme preservation) and quite soluble allowing ionic strengths high enough to precipitate almost any protein. Salting-out precipitation is very fast, equilibrium being reached after a few minutes, but particle size is small (from 0.5 to 5 μm) which hinders recovery since high centrifugal forces are required to obtain a good separation (Foster et al. 1976). As any precipitation method, salting out is not very selective and purification factors below 10 are obtained even under optimized conditions (Illanes 1974). It is however quite useful as an early concentration step since the precipitated protein can be readily dissolved in a small amount of water.

Water-miscible organic solvent precipitation has been also used traditionally in the early steps of enzyme purification (Drapeau et al. 1972; Omar et al. 1987; Iizumi et al. 1990). Some drawbacks of them are that operation must be conducted at low temperatures (near or below 0 °C) because these solvents are protein denaturants at room temperature, and explosion-proof motors should be used. Ethanol and acetone have been the most used solvents; ranges of precipitation are narrower with acetone but it is quite harmful, so very low temperatures have to be used. Despite this, organic solvent precipitation has some advantages over salting-out, since it is more selective and strictly based on the physicochemical properties of the protein (Askonas 1951); additionally, it produces less amorphous precipitates, easier to recover by conventional centrifugation (Illanes 1974). Precipitation is promoted by protein–protein Coulombic interactions at low ionic strength, which are magnified by the decrease in dielectric constants promoted by the water-miscible solvent. An empirical correlation, analogous to the salting-out equation of Cohn, has been found useful for evaluating protein precipitation by water-miscible organic solvents:

$$\log S = \log S_0 - K'_S \cdot \varepsilon^{-2} \tag{2.24}$$

The explanation above has been questioned since when working with ethanol at very low temperatures, reduction of dielectric constant is not significant with respect to water; in that case it has been demonstrated that van der Waals forces of attraction are enough to promote protein aggregation (van Oss 1989). Protein solubility has proven to correlate well with solvent polarity and the Hildebrand solubility

parameter has been used to predict protein precipitation by organic solvents (Hwang et al. 2007). Salt and solvent precipitation have been combined in a three phase partition system where the precipitated enzyme concentrates in the water-solvent interface obtaining high purification factors at acceptable yields of recovery (Sharma et al. 2000; Sharma and Gupta 2002).

Non-ionic soluble polymers have been used also as precipitants in enzyme purification (Gupta et al. 1994; Yu et al. 1994; Gupta et al. 1997). Protein–protein interactions promoted by the polymers have been explained in terms of the volume-exclusion potential of Asakura and Oosawa (Mahadevan and Hall 1990), whereby the depletion of solvent between the protein molecules causes attractive forces that are primarily responsible for protein aggregation and phase separation (Vlachy et al. 1993). As opposed to organic solvents, polymers like polyethylene glycol are harmless to the enzyme structure and can be used at room temperature without any detrimental effect on enzyme structure (Haire et al. 1984; Gupta et al. 1997). Some difficulties associated to polymer precipitation are the increase in viscosity and the complex removal of the polymer after precipitation. Solubility of proteins decays sharply with polymer concentration and it has been modelled according to an exponential decay equation (Atha and Ingham 1981), which is analogous to the Cohn's salting-out equation:

$$\log S = \log S'_0 - K''_S \cdot c_P \tag{2.25}$$

Some other techniques of protein precipitation, like differential inactivation by temperature or pH, have been occasionally used for those cases where abnormal stability allows the enzyme to retain its functional structure while contaminant proteins are precipitated by irreversible unfolding (Prado et al. 1999; Harris 2001).

The low selectivity of most methods of fractionation by precipitation represents its main drawback. The addition of specific ligands to polymers can be a powerful tool for purification (affinity precipitation) and "smart" polymers (i.e. those whose solubility changes dramatically with small changes in the environment) are increasingly being used for protein purification, since it allows to include a highly selective operation at the very beginning of the purification process (Galaev and Mattiasson 2002; Kumar et al. 2003).

Liquid chromatography is a very powerful system of enzyme purification by protein fractionation. It consists in the passage of a mixture of solutes (proteins in this case) dissolved in a mobile phase through a stationary phase (usually a solid matrix) with which the solutes interact at varying strengths so that a differential retardation is produced that allow their separation by a suitable eluent. Chromatography is a very powerful analytical system, but preparative chromatography has been extensively used for laboratory scale enzyme purification. The most common configuration is column chromatography, in which the stationary phase is packed into a column through which the mobile phase containing the protein and later on the eluent are pumped. Scale-up of chromatographic methods for enzyme production is cumbersome since the system must be robust, have a reasonably high throughput and a reasonable cost, which is by no means an easy task. Liquid chromatography for protein fractionation has been extensively treated and only the most relevant options for enzyme purification will be shortly reviewed here with especial consideration to

its application at a productive scale. A comprehensive coverage of the subject can be found in several publications (Janson and Rydén 1998; Kastner 2000; Ahuja 2003; Cutler 2004).

Several types of chromatography are available for protein fractionation, among which the most relevant are, according to the principle of separation involved: size-exclusion, ion exchange, hydrophobic and affinity chromatography.

Size exclusion chromatography (also not so correctly termed gel filtration or gel permeation), separates proteins according to their molecular size. Smaller molecules enter the porous structure of the matrix and are the most retarded, while larger molecules are progressively excluded from the stationary phase and leave the column earlier. Resolution is high (it is used for molecular mass determination) but throughput is low and therefore is commonly used at a later stage of purification or as a final polishing step (Singh et al. 2007). Elution is simple and does not require gradients of any kind. Several types of matrices are available among which cross-linked dextran, polyacrylamide, polymethacrylate, polyvynil alcohol and agarose gels are prominent. Several particle sizes and degrees of cross-linking produce different resolutions in different molecular size ranges, allowing protein fractionation as well as desalting for which size-exclusion chromatography is very effective and widely used. The range of molecular size fractionation is closely related to the rehydration value of the gel which is a very relevant property that should be considered when choosing the appropriate stationary phase. When scaling-up the column, height to diameter ratio is decreased to reduce the pressure drop and hence bed compaction. This usually implies a sacrifice in resolution, which is roughly proportional to the square root of the bed height. Resolution is also decreased if concentrated protein solutions are fed to the column. Small particle size gels produce a higher resolution but also a higher pressure drop. Stacked columns are frequently used on a large scale to avoid that problem. As seen, scaling up to production level may severely reduce resolution (Simpson 1994). Complete information on operating procedures, types of supports and columns for size-exclusion chromatography is available in the format of handbooks (Anonymous 1999; Wu 1999).

Ion-exchange chromatography has been extensively used for the purification of enzymes of commercial significance (Yang et al. 1987; Chauthaiwale and Rao 1994; Falco et al. 2000; Aguilara et al. 2006; Liu and Xi 2006). The amphoteric nature of proteins means that they will be cationic or anionic depending on the pH of the solution and separation is based on the reversible ionic interaction between charged amino acid residues in the protein molecule and a chromatographic support (often an ion exchange resin) of opposite charge. As they pass through the column, proteins bind differentially according to their surface charge distribution at the pH of operation. When above its isoelectric point, proteins exhibit a net negative charge and bind to anion exchangers; when below, they exhibit a net positive charge and bind to cation exchangers. The retained proteins are eluted differentially by titration, decreasing the pH to approach the isoelectric point in the first case, and increasing the pH to approach the isoelectric point in the second case. In both cases differential elution can be performed by increasing ionic strength, which is sometimes preferred for not submitting the enzyme to deleterious pH values (see Fig. 2.4).

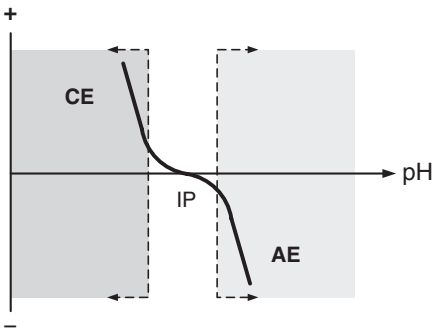

Fig. 2.4 Principle of ion-exchange chromatography. Above the isoelectric point (IP) proteins bind to anion exchangers (AE) and are eluted by a decreasing pH, and below it they bind to cation exchangers and are eluted by a increasing pH. In both cases proteins can be eluted by increasing ionic strength

Protein fractionation is produced in the elution step where a pH or salt gradient is applied; gradient can be performed stepwise or continuously. Ion-exchange chromatography is usually designed to retain the target protein, but it can be designed to retain impurities as well. Most common matrices and ion exchange groups for ionic exchange chromatography are shown in Table 2.3. Exchange capacity of the support is determinant for column performance and can be as high as 5 meq/g (Hostettmann et al. 1998). Ion-exchange chromatography allows higher throughputs than size exclusion chromatography and can be an early operation in the purification process, provided that the ionic strength of the crude enzyme preparation is low or has been reduced by previous desalting. Ion-exchange chromatography is a very powerful operation for enzyme purification and even proteins of similar isoelectric points can be conveniently separated because interaction with the support is determined by the surface charge distribution of the protein rather than by its net charge (Simpson 1994). Scale-up considerations for ion-exchange chromatography are similar to those mentioned for size-exclusion chromatography; however it is a more flexible operation allowing higher throughputs with similar or even better

Table 2.3 Commonly Used Ion Exchange Chromatographic Systems

	Ion exchanger		
Support	Anionic	Cationic	Supplier
Cellulose	DEAE; TEAE; QAE	CM; P	Amersham-Pharmacia; Bio-Rad; Whatman
Dextran	DEAE; TEAE; QAE	CM; P; SP	Amersham-Pharmacia
Agarose	DEAE; PEI	CM	Amersham-Pharmacia; Bio-Rad;
Acrylic copolymers	DEAE	CM; SP	Pall Ind. Biologique Française
Polymer-based Toyopearl and TSK-GEL	DEAE	CM; SP	TosoHaas

DEAE: diethylaminoethyl; TEAE: triethylaminoethyl; QAE: quaternaryaminoethyl; PEI: polyethyleneimine; CM: carboxymethyl; P: phospho; SP: sulfopropyl

resolutions, so that it can be an early or final step of the purification process. Complete information on operating procedures, types of supports and columns for ion exchange chromatography is available in the format of manuals (Westerlund 2004; Wu 1999).

Hydrophobic chromatography has gained increasing importance for the purification of enzymes (Gupta et al. 2002; Aehle 2003; Coulon et al. 2004; Lee et al. 2006). It is based on the interaction of the hydrophobic regions of the protein with a hydrophobic gel matrix; however, the principle underlying protein separation is not clear. Most common matrices are gels coated with hydrophobic aryl or alkyl (frequently butyl, octyl or phenyl) groups. For efficient fractionation ionic strength must be high, since it increases hydrophobic interactions among protein molecules; therefore, this operation is ideal when a previous salting-out operation is considered. Elution is achieved by changing the pH or the ionic strength or by modifying the dielectric constant of the eluent. High throughputs can be obtained but resolution is somewhat lower than in ion-exchange chromatography. The method is, however, quite useful because it is based on a completely different mechanism and can be efficiently combined with other chromatographic operations (Simpson 1994). Detailed information on hydrophobic chromatography is available (Anonymous 1999; Simpson and O'Farrell 2004).

Affinity chromatography is probably the most powerful purification technique in terms of resolution since it is based in the functional rather than the physicochemical properties of the protein (Brocklehurst et al. 2004). Nowadays, this operation is considered in most protocols of recombinant protein purification (Gottstein and Forde 2002; Sahina et al. 2005). Affinity chromatography is especially pertinent for enzymes, that can be purified quite specifically based on their catalytic properties and also, as any other protein, on their immunogenic properties. In many cases a single operation can bring the enzyme to the final desired level of purity (Ito et al. 2004; Mendu et al. 2005; Melissis et al. 2007). This is highly desirable since yield of recovery is expected to be high. However, scale-up to production level is in most cases precluded by its high cost (Robinson et al. 1974; Yang and Tsao 1982), so it becomes relevant mostly for the small-scale production of high value specialty enzymes (Clonis 1987). As materials and operational costs are lowered and more experience is gained in the large-scale operation, affinity chromatography is becoming more relevant for enzyme production. A support for affinity chromatography is composed of a ground matrix which is (usually) linked to a space arm that in turn is linked to the ligand that interacts specifically with the protein. Ground matrix is a polymer that should not interact with the protein to permit a highly specific retardation by the ligand (agarose gel is extensively used). Space arm serves to the purpose of projecting the ligand away from the matrix surface to favor ligand-protein interaction; aliphatic hydrocarbons are usually employed with chain lengths from five to seven carbon atoms, long enough to be effective but not so long to become fragile or favor non-specific interactions (i.e. hydrophobic). Biospecific ligands for enzymes can be any molecule that interacts specifically with the enzyme structure: substrates, coenzymes or cofactors, and the corresponding analogues. The degree of specificity is variable: substrates, inhibitors and their corresponding analogues

are expected to be highly specific for a particular enzyme while coenzymes, cofactors and their corresponding analogues are specific for a group rather than a specific enzyme (i.e. NAD^+ for dehydrogenases; ATP for kinases). Judicious selection of the ligand is crucial for a successful operation. The ligand should bind to the enzyme strongly enough to allow its efficient retardation in the chromatographic column, but not as strongly to preclude elution; values of dissociation constants between 10^{-4} and 10^{-8} M are recommended (Fonseca and Cabral 1996; Wu and Liu 1996). From this viewpoint and also because of economic considerations analogues are often preferred. Substrates are not recommended as ligands, since non-reactive conditions are required to avoid enzyme detachment; some inhibitors can be too weak (high dissociation constant) to effectively retard the enzyme. Cofactor and cofactor analogues, despite their lower selectivity, have been intensively used as ligands for meeting the above criteria (Mosbach et al. 1972); in the latter case, analogue chemicals (i.e. commercial dyes) have the advantage of lower costs (Clonis 2006). Commercial triazine dyes (i.e. Procion red; Cibacron blue) have been used for the purification of several NAD^+ and FAD^+ dependent dehydrogenases (Schneider et al. 1983). In the case of Cibacron blue, ligation is less specific and other classes of enzymes have been purified as well (Subramanian 1984; Koch et al. 1998). Biomimetic analogues of industrial triazine dyes have been successfully used for the purification of the FADH dependent formate dehydrogenase (Labrou et al. 1995). Special affinity chromatography matrices for His-tagged recombinant proteins have been developed and successfully applied for the one-step purification of industrially relevant enzymes (Zheng et al. 2007). Many eukaryotic enzymes are glycoproteins and in this case lectin affinity chromatography has been used where a ligand specific for carbohydrates (i.e. Concavalin A) is used (Mislovičová et al. 1996; Mateescu et al. 2002; Bahar and Tuncel 2004). Immunoaffinity chromatography has also been used for enzyme purification although on a rather small scale, mainly for research and therapeutic enzymes (Ehle and Horn 1990; Thompson et al. 1990). The enzyme retained by its interaction with the ligand is then recovered by elution, which can be specific (gradient of ligand or ligand analogue) or non-specific (gradient of pH or ionic strength). Affinity chromatography is suggested as an intermediate operation within the purification process, but it can be an early (and even the only one) operation (Simpson 1994; Anonymous 1999). A review on practical and fundamental aspects of affinity chromatography can be found elsewhere (Harakas 1994; Hostettmann et al. 1998; Prado et al. 1999). Purification of recombinant proteins for therapeutic use by affinity chromatography has been reviewed by Hage (1999). The same author has recently published a comprehensive review on the subject (Hage 2006).

The principles of affinity chromatography can be combined with other operations of purification to improve them (Labrou and Clonis 1994). Affinity partition combines the selectivity of affinity ligation with aqueous two-phase extraction (Kamihira et al. 1992; Köhler et al. 1991) and has been successfully employed in enzyme recovery and purification (Johansson and Tjerneld 1989; Schustolla et al. 1992) obtaining impressive increases in the partition coefficient (Eq. 2.16) and therefore in yield of enzyme recovery (Eq. 2.17). Affinity partition has also been combined with membrane separation (affinity ultrafiltration), where a soluble

polymeric biospecific ligand is bound to the enzyme prior to its separation by ultrafiltration (Galaev 1999; Romero and Zydney 2002). Several enzymes of commercial relevance have been purified by affinity ultrafiltration (Male et al. 1990; Filippusson and Sigmundsson 1992). Continuous affinity recycle extraction is based on the combination of affinity chromatography with molecular filtration. The system is simple, scalable and useful as a front or final purification step and has been used for the purification of β-galactosidase (Pungor et al. 1987) and the process modelled and optimized (Gordon et al. 1990; Sun et al. 1995; Dechechi et al. 1997).

Other protein fractionation methods are available for enzyme purification when a high degree of purity is required. These techniques are mostly oriented to the preparation of highly pure protein samples for characterization, and are usually difficult or extremely costly to scale-up, its application being restricted to specialty proteins and peptides for therapeutic use, clinical diagnosis or research. High pressure liquid chromatography (HPLC) is a high resolution system mostly used for analytical purposes; however, it has been used at a preparative scale for the purification of recombinant proteins and enzymes (Smith et al. 1999). Reverse phase HPLC, where proteins are differentially retarded by hydrophobic interaction with the carrier and then eluted by increasing the organic solvent concentration in the mobile phase, is particularly suitable for protein fractionation. However, protein unfolding during HPLC purification is a potential problem to deal with. Preparative HPLC will have increasing importance as equipments suitable for robust large-scale operation are evolved and their costs reduced (Hostettmann et al. 1998). Electrophoresis is another technique mostly used for analytical purposes that has been developed to preparative scale but mostly for characterizing pure enzymes (Fountoulakis and Juranville 2003; Gul-Guven et al. 2007).

2.2.4 Enzyme Formulation

Once the enzyme has been purified to the required level, the preparation must be formulated according to its intended use. Formulation of enzyme preparations is very much like an art and details are kept secret by enzyme producers or revealed to the customer under an agreement of confidentiality. Even though not much attention is given to this production stage in the open literature, formulation is a crucial step in enzyme production especially in the case of industrial enzymes, since it is usually this step of production that confers the producer the competitive edge (Chaplin and Bucke 1990). However, formulation is a main issue not only for bulk commodity enzymes but also for specialty enzymes that must comply with stringent regulations. Regulations will be determined to a great extent by the end use of the enzyme. For instance, enzymes used in detergents, textiles, leather, pulp and paper are regulated as any other chemical used in the manufacturing process; enzymes used in the food and pharmaceutical sectors are regulated by the corresponding agencies (Food and Drug Administration in the case of the USA and the corresponding agencies in other countries). Therefore, regulations vary throughout the world and even between community countries. Even though enzymes are by their very own nature non-toxic and

non-hazardous, they can be allergenic and the producing organisms must be labelled as safe to be approved as producing strains. The risk of enzyme allergy in the detergent industry is well evaluated now since the critical episodes at the end of the 1960s that threatened the detergent industry and wipe off the use of detergent enzymes for several years in the USA (Vanhanen et al. 2000); allergenic problems have also been found for the case of amylases used in bakery (Baur et al. 1994). A subject of major concern now is the production of enzymes from genetically-engineered organisms. There is no consensus on that issue: while some countries prohibit or strictly regulate the production of such enzymes, others require only that production complies with good manufacturing practices (Uhlig 1998; Aehle 2003). Health and safety aspects related to enzyme production and use was revised by Flindt (1978). A comprehensive review on safety regulation for food enzymes have been published recently (Spök 2006). General guidelines for regulatory and safety aspects on enzyme production have been put forward by Chaplin and Bucke (1990).

Enzyme formulation includes final polishing operations, stabilization and standardization (see Fig. 2.1). Final polishing refers to the elimination of contaminants not previously removed. For the case of small-scale production of specialty enzymes for medical applications, polishing includes several key operations for the removal of trace contaminants like pyrogens, endotoxins, nucleic acids and viruses (Anonymous 1999). For the case of bulk industrial enzymes, final polishing usually considers the removal of salts and adjustment of pH if the enzyme is produced in liquid form and drying if produced as a solid preparation. The choice between solid or liquid is by no means trivial. Solid preparations have the benefit of easy handling and transportation and a comparatively higher shelf-life; however, the problem of dust formation in the production facilities is a serious problem so that containment is necessary to reduce risks of allergy among workers. Liquid preparations have also some benefits: containment is simpler, final drying is avoided and dosage can be easier. Each producing company has its own philosophy in this respect, but in general it is the end-use of the enzyme and the customer needs that prevail. Enzymes that will be a part of a solid product (i.e. detergent enzymes) are usually produced as solids, while for application to liquid products (i.e. enzyme saccharification and isomerization for high-fructose syrup production) a concentrated liquid enzyme preparation is more adequate. Vacuum drying and spray drying are the most used operation for producing solid preparations of robust enzymes (Werner et al. 1993; Fickers et al. 2006) while freeze-drying, a more expensive operation, is used for more labile specialty enzymes (Lambert and Meers 1983). With the increasing use of enzymes in non-aqueous media, the requirement of lyophylized enzymes that will act as insoluble catalysts in such media is increasing (Clark 2004; Gupta and Roy 2004). In some cases, special operations are required, as in the case of detergent proteases where encapsulation of the granulated enzyme powder is required to avoid dust formation during production and prevent direct contact of the enzyme with the end user (Maurer 2004). Liquid enzyme preparations are quite common and enzyme concentrates may require some polishing operations like desalting or final concentration. Desalting can be performed by diafiltration (Lambert and Meers 1983; Charcosset 2006) or size exclusion chromatography (Mischitz et al. 1995; Sajedi et al. 2005).

Enzymes are sold in terms of its specific activity so that the producer must ensure certain minimal value to its customer; it is quite common that the labelled specific activity is lower than the actual value obtained at the point of use because of the margin of security with which the enzyme producers work. Stabilization is then a major concern when producing enzymes, since the enzyme product must withstand storage and transportation conditions without significant activity losses. Liquid enzyme products may lose 10–20% of its activity over a period of 4–6 months at room temperature; therefore, refrigerated storage is recommended to increase its shelf-life for a period of a year or longer. Several additives and strategies are used to improve enzyme storage stability. They are intended for preventing microbial contamination and/or preserving enzyme structure. Microbial contamination is a problem in liquid enzyme products since wild proteases can degrade the enzyme to a considerable extent. Absolute filtration and the addition of accepted preservatives (microbicides or microbistats) are the alternatives to ensure the microbiological quality of the enzyme preparation and to prevent spoilage. However, the key issue for enzyme storage stabilization is the preservation of enzyme conformation to prevent aggregation, unfolding or any deleterious change in its native three-dimensional structure. Several strategies are intended for such goal. Proteins are more stable in concentrated solutions and at high ionic strength. Concentration is then not only a way of removing inert material but also an aid in preservation. Some neutral salts can act as stabilizers by promoting hydrophobic interactions in the enzyme molecule, while others can promote chaotropic effects that destabilize the protein structure so that a judicious choice of the salt is required (Lecker and Khan 1998). Hoffmeister series is a good guide to select salt preservants: multivalent anions (like citrate or phosphate) and monovalent cations (like ammonium or alkaline metal ions) are adequate (Chaplin and Bucke 1990). Some cations that are part of the active site can act as stabilizers; for instance: Mg^{++} or Co^{++} in the case of glucose isomerase (Tashpulatova and Davranova 1992), and Ca^{++} in the case of α-amylase (Yutaki et al. 1969; Ogasahara et al. 1970). Low molecular weight polyols, like glycerol and sorbitol (Larreta-Garde et al. 1988; Breccia et al. 1998), and sugars (Chaniotakis 2004) can also act as stabilizers by reducing water activity and preventing protein unfolding (Gianfreda and Scarfi 1991; Joo et al. 2005). Some hydrophilic polymers like polivinyl alcohol, polyvinylpyrrolidone and hydroxypropylcelluloses can stabilize enzymes by substituting the enzyme–enzyme and enzyme–water interactions by less potentially denaturing enzyme-polymer interactions (Chaplin and Bucke 1990). Synthetic polymers are good enzyme stabilizers (Alfani et al. 1984; Bryjak 1995) and are common in enzyme formulation. They may also act by stabilizing the hydrophobic effects within the enzyme molecules. Viscous glass-forming compounds, like trehalose or glycerol, can also exert protection by enveloping the protein structure (Soles et al. 2006). Glycerol may also be used to protect enzymes against denaturation due to ice-crystal formation at sub-zero temperatures. Substrates and inhibitors are used as specific enzyme preservatives by conferring protection to the active site; actually this is a common practice that requires the enzyme preparation to be dialyzed prior to use. Enzymes that have oxidizable amino acid residues in the active site require special protection to avoid inactivation. This is the case of cysteine proteases like

papain (Sanner and Pihl 1963; Klein and Kirsch 1969) and other industrially relevant enzymes like yeast β-galactosidase (Mahoney and Whitaker 1978; Mahoney 1980; Illanes et al. 1998). Thiol compounds, if allowed, may be used as protective agents or else special precautions should be taken to reduce the level of dissolved oxygen in the enzyme product. Some of the structure-stabilizing agents also confer protection against microbial contamination. Stabilization can be obtained by immobilization, but its potential goes beyond the field of enzyme product stabilization, because of its strong impact on enzyme process economics; therefore, enzyme immobilization will be thoroughly reviewed in section 4.1. A recent publication by Springer gives a complete and updated review on enzyme immobilization (Guisán 2006).

The last step in enzyme production is standardization and is a very relevant aspect since the producer must ensure an enzyme product of uniform quality to its customers. Enzymes are produced from biological systems so that variations among production batches are inevitable. Enzymes produced by extraction from natural products are expected to have significant variations from one batch to another. Microbial enzymes produced by fermentation under tightly controlled conditions are expected to have less variation, but differences in raw materials for media formulation may also produce significant variations. In the validation of a pilot process for the production of β-galactosidase from *K. marxianus* we observed variations of $\pm 10\%$ in volumetric activity among 80 production batches (Illanes et al. 1996). To solve this problem, enzymes are diluted with varying amounts of excipients to absorb such variations. Excipients can be inert materials or the same substances used for enzyme preservation or activity enhancement (Schoemaker et al. 2003; Liao et al. 2004). Enzyme products should have a product sheet and a certificate of analysis containing the mot relevant information for the end-user. Specific activity should be expressed in units of activity (desirably international units) per unit mass or unit volume of enzyme product. Enzyme producers usually have their own way of measuring activity that may be irrelevant for the intended use or sometimes not clearly reproducible. A relevant assay of enzyme activity should be developed by the end-user, but enough information on enzyme activity assay should be provided by the producer to check the quality of the enzyme. Storage stability is also relevant to the end-user and must be clearly specified by the producer, desirably in terms of half-life at specified storage conditions. Information of physical properties of the enzyme product like appearance, solubility, water content, swell factor (if solid) are usually provided. However, information on enzyme product composition and excipients is not frequently provided, despite its relevance to the end-user and the fact that in most industrial enzyme preparations the bulk of the product is represented by other proteins, stabilisers, preservatives, salts and inert diluents for standardization.

Any enzyme product released into the market must fulfil the requirements of quality and compatibility with its intended use and these should be granted to the corresponding regulatory agencies by the producer. Current standards consider the validation not only of the product, but also of the manufacturing process. Production should be made according to good manufacturing practices (GMP) and most enzyme producing companies have their corresponding ISO certificates. A leading

company in enzyme production has all its processes certified according to the ISO 9001:2000 standard.

Nomenclature

a	enzyme activity per unit mass of protein
a_0	enzyme activity per unit mass of protein in the intact cell
c	solute concentration
c_B	concentration of protein in the bottom phase
c_T	concentration of protein in the top phase
c_P	polymer concentration
E	enzyme activity recovered
K_P	partition coefficient for protein
K_S	salting-out constant
K'_S	organic solvent precipitation constant
K''_S	polymer precipitation constant
k_R	First order rate constant for protein release
k_D	First order rate constant of enzyme inactivation
m_i	molarity of ions
q_p	specific productivity
p	product molar concentration
p_R	released protein
p_T	total cell protein
PF	purification factor (ratio of outlet to inlet specific activity)
R	universal gas-law constant
S	protein solubility
S_0	protein solubility at infinite dielectric constant
S'_0	protein solubility at zero precipitant concentration
t	time
T	absolute temperature
V_T	volume of top phase
V_B	volume of bottom phase
X	cell concentration
Y	yield of recovery (ratio of outlet to inlet enzyme activity)
Y_T	yield of recovery at the top phase
Z_i	valence of ions
α	growth associated coefficient in Eq. 2.1
β	non-growth associated coefficient in Eq. 2.1
β_S	coefficient in Eq. 2.22
ε	dielectric constant
μ	specific cell growth rate
μ'	ionic strength
σ	surface tension
Γ	excess protein at interface

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Chapter 3
Homogeneous Enzyme Kinetics

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3.1 General Aspects

The study of enzyme kinetics is important from a fundamental scientific perspective, since it allows the formulation of molecular models for enzyme action, and also for technological reasons, since it allows the formulation of kinetic models for the design and evaluation of reactor performance (Messing 1975; Chaplin and Bucke 1990). The second aspect is emphasized in this chapter, which does not pretend to make an extensive review of enzymology that can be found in excellent textbooks (Laidler and Bunting 1973; Segel 1975; Dixon and Webb 1979; Marangoni 2003). As shown in Fig. 3.1, a model for enzyme reactor design or performance evaluation requires: 1) a kinetic expression of the catalyzed reaction; 2) a material balance over the process; 3) an expression for enzyme inactivation during reactor operation; 4) an expression that accounts for eventual mass transfer constraints. This chapter will analyze the first and third components, while the fourth will be analyzed in Chapter 4. All components will be put together in Chapter 5, being the objective of this chapter to establish the kinetic basis for the analysis, operation and design of enzyme reactors.

Enzyme kinetics refers to the quantitative analysis of all factors that determine the catalytic potential of an enzyme. As presented in section 1.3, enzyme activity represents the maximum catalytic potential of an enzyme that is reflected by the initial rate of the catalyzed reaction. Several factors affect the expression of such potential, being the most important the concentrations of active enzyme, substrates and inhibitors, temperature and pH. In the case of insolubilized enzymes or multiphase systems, other variables that reflect mass transfer constraints must be considered.

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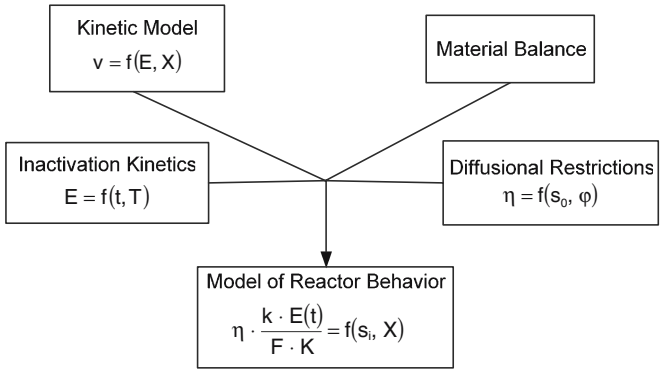


Fig. 3.1 Scheme for the construction of a model for enzyme reactor design and performance evaluation

Enzyme activity depends linearly on enzyme protein concentration, even though in some particular circumstances deviations have been observed (Scott 1987). It is however assumed that enzyme activity is proportional to enzyme protein concentration and this is a fundamental principle of enzyme kinetics. A key variable in enzyme kinetics is substrate concentration and its effect constitutes the basis of the hypothesis for enzyme kinetics.

Conventionally, reaction rates in enzyme kinetics refer always to initial reaction rates where the maximum catalytic potential of the enzyme is expressed and many factors affecting it (i.e. substrate depletion, accumulation of inhibitory products, enzyme inactivation, reverse reaction) are irrelevant (see section 1.3). The quantification of such effects on that maximum catalytic potential is the subject of sections 3.2, 3.3 and 3.4.

3.2 Hypothesis of Enzyme Kinetics. Determination of Kinetic Parameters

3.2.1 Rapid Equilibrium and Steady-State Hypothesis

The effect of substrate concentration on enzyme kinetics was first proposed by Henri at the beginning of the XX century. Making an analogy with reversible chemical reactions between two substrates, Henri proposed that conversion of substrate into product involved a reversible reaction between enzyme and substrate to form an active intermediate that brakes down delivering the product. These ideas were taken a few years later by Michaelis and Menten (1913) who proposed the first formal hypothesis for enzyme catalysis based on two sequential steps, as suggested by Henri: in the first step the substrate is captured in the active site of the enzyme, while in the second step the amino acid residues at that site chemically process the substrate to

transform it into product, which is subsequently released to let the enzyme free and available for the next catalytic round. This is represented by the following reaction scheme for the conversion of substrate S into products (P_i):

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P_i$$

According to the (pseudo) equilibrium proposal of Michaelis and Menten, the substrate binding step is so fast as compared to the rate of enzyme–substrate complex (ES) breakdown into product that the reaction of formation of ES can be considered at equilibrium. This is so in most cases, which validates this hypothesis, even though it is obviously an oversimplification, since no equilibrium is possible as long as ES is being converted into product. According to this:

$$K_{eq} = \frac{k_2}{k_1} = \frac{(e-c)(s-c)}{c} \tag{3.1}$$

However, since $c < e <<< s$, Eq. 3.1 is simplified to:

$$K_{eq} = \frac{k_2}{k_1} = \frac{(e-c)s}{c} \tag{3.2}$$

so that:

$$c = \frac{e \cdot s}{K_{eq} + s} \tag{3.3}$$

According to the reaction scheme:

$$v = k_{cat} \cdot c \tag{3.4}$$

and since $c_{max} = e$

$$V = k_{cat} \cdot e \tag{3.5}$$

$$v = \frac{V \cdot s}{K_{eq} + s} \tag{3.6}$$

Eq. 3.6 represents the parametric expression for the enzymatic reaction rate as a function of substrate concentration.

A few years later, Briggs and Haldane (1925) argued against the validity of the rapid equilibrium hypothesis and proposed a steady-state hypothesis according to which, after a very short transient phase, the ES complex remains constant throughout the whole reaction period, as shown in Fig. 3.2.

Strictly speaking, according to the steady-sate hypothesis:

$$\frac{dp}{dt} = -\frac{ds}{dt} \rangle\rangle\rangle \frac{dc}{dt} \approx 0 \tag{3.7}$$

Then, according to the reaction scheme:

$$\frac{dc}{dt} = k_1(e-c)s - (k_2 + k_{cat})c = 0 \tag{3.8}$$

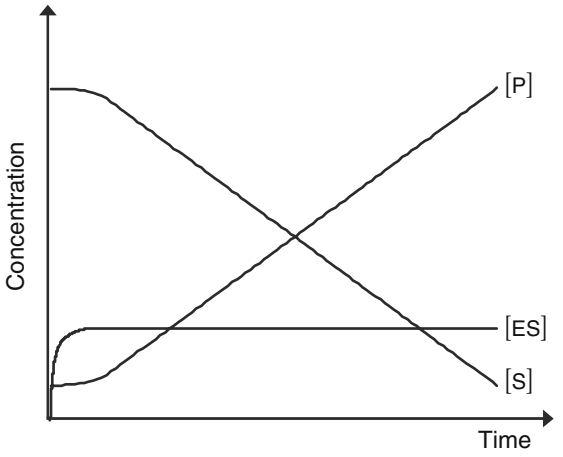


Fig. 3.2 Representation of steady-state hypothesis for enzyme catalyzed conversion of S into P

so that:

$$c = \frac{es}{\frac{k_2 + k_{cat}}{k_1} + s} \tag{3.9}$$

From Eqs. 3.4, 3.5 and 3.9 and defining:

$$K = \frac{k_2 + k_{cat}}{k_1} \tag{3.10}$$

Eq. 3.11 is obtained:

$$v = \frac{V \cdot s}{K + s} \tag{3.11}$$

In parametric terms Eq. 3.11 is analogous to Eq. 3.6, but K (usually termed as Michaelis–Menten constant) is a dissociation constant, while K_{eq} is an equilibrium constant. Of course if $k_{cat} <<<< k_2$ (which is the basis of the fast equilibrium hypothesis) $K = K_{eq}$ and Eq. 3.11 reduces to Eq. 3.6. Then one can conclude that the equilibrium hypothesis is but a particular case of the more generally valid steady-state hypothesis.

As opposite to parameter K (or K_{eq}) and k_{cat}, V is not a fundamental property of the enzyme since it depends on its concentration as indicated by Eq. 3.5. This has to be taken into consideration when determining the kinetic parameters. The catalytic rate constant (k_{cat}) is a fundamental property of the enzyme that can be expressed in different ways and in different units, according to how e is expressed (moles L^{-1}; g L^{-1}; U L^{-1}). If e is expressed in moles L^{-1}, k_{cat} has dimension of T^{-1} (known as turnover number). This requires the knowledge of the molecular weight and the specific activity and number of active centers of the enzyme. Sometimes this information is not available so that k_{cat} is expressed in dimensions of M T^{-1} U^{-1} (mass of substrate converted per unit time and unit of enzyme activity). If U is expressed in international units (IU), then k_{cat} reduces to a dimensionless value of 1, which is to say that it is equivalent to V.

Despite the limitation of the equilibrium hypothesis, it has been proven to be sound in most cases, with the additional advantage of being more easily handled mathematically. In fact, steady-state equations for more complex situations (see sections 3.3. and 3.4) are not easy to solve and usually mathematical algorithms are required (Lam and Priest 1972; Segel 1975). Extensive mathematic manipulation is required to derive parametric equations but software has been developed to handle it (Myers and Palmer 1985). Equilibrium hypothesis was formulated based on the examination of hydrolytic reactions which in aqueous milieu are virtually irreversible. However, for reversible reactions $(S \leftrightarrow P)$ that hypothesis is not applicable and steady-state equations must be derived.

This is the case, for instance, of glucose isomerase, which is a very relevant industrial enzyme that catalyzes the reversible reaction of isomerization of glucose into fructose with a value of the equilibrium constant of the reaction close to 1 at conditions compatible with enzyme activity (Houng et al. 1993).

A minimal reaction scheme for that reaction is:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E + P$$

Then, according to the steady-state hypothesis:

$$\frac{dc}{dt} = k_1(e-c)s + k_4(e-c)p - (k_2+k_3)c = 0 \tag{3.12}$$

$$v = k_3 c - k_4(e-c)p \tag{3.13}$$

Defining the kinetic parameters:

$$K = \frac{k_2+k_3}{k_1}; \quad K_p = \frac{k_2+k_3}{k_4}; \quad V = k_3 \cdot e; \quad V_p = k_2 \cdot e$$

$$v = \frac{\frac{V \cdot s}{K} - \frac{V_p \cdot p}{K_p}}{\frac{s}{K} + \frac{p}{K_p} + 1} \tag{3.14}$$

The global equilibrium constant of the reaction, represents the ratio of p and s at equilibrium, and can be obtained in terms of the kinetic parameters by setting $v = 0$ in Eq. 3.14:

$$K_{EQ} = \frac{V}{V_P} \cdot \frac{K_P}{K} \tag{3.15}$$

Eqs. 3.11 and 3.14 represent the kinetics of irreversible and reversible one substrate reactions respectively. This simple kinetic models are however quite relevant since they represent a good portion of the reactions of industrial relevance. As said before (see section 1.6), most of the traditional enzyme technology refers to hydrolytic reactions performed in aqueous medium with hydrolases. Even though hydrolytic reactions are strictly speaking two substrate reactions, water plays the role of the solvent being in large stoichiometric excess, so its effect on enzyme kinetics can

be neglected. Besides hydrolases, lyases and isomerases also catalyze one substrate reactions that can be properly described by the kinetic expressions above.

3.2.2 Determination of Kinetic Parameters for Irreversible and Reversible One-Substrate Reactions

For simple kinetic mechanisms, like irreversible one-substrate reactions, both rapid equilibrium and steady-state hypothesis lead to rate equations that are formally equal in parametric terms, so when those parameters are experimentally determined, results are the same no matter what hypothesis is considered. Kinetic parameters are to be experimentally determined to obtain validated rate expressions to be used for the design or performance evaluation of enzyme reactors.

There are several methods for the determinations of kinetic parameters, most of them based on initial rate measurements at varying substrate concentrations; alternatively they can be determined from the time course of the enzymatic reaction.

Experimental design for determination based on initial rates consists in a series of experiments in which initial reaction rates are measured at varying substrate concentrations within the zone of linear p versus t relationship.

The equation of Michaelis–Menten is hyperbolic as can be easily deduced. From Eq. 3.11:

$$V \cdot K = (K + s) \cdot (V - v) \tag{3.16}$$

with asymptotes $v = V$ and $s = -K$ as shown in Fig. 3.3. When substrate concentration equals its dissociation constant, initial reaction rate is half of its maximum

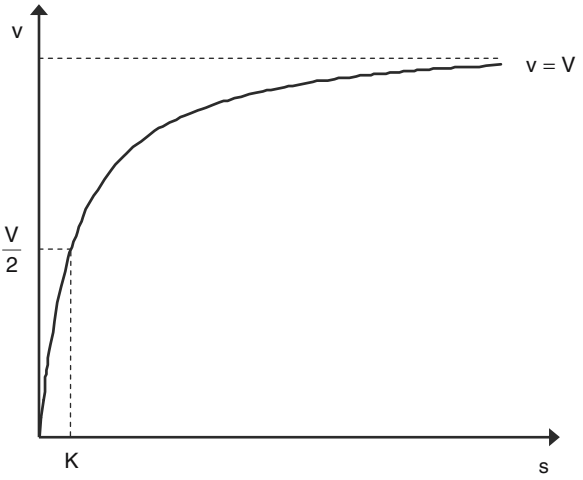


Fig. 3.3 Graphical representation of the Michaelis–Menten equation for the irreversible conversion of substrate S into product P

value, as easily deduced from Eq. 3.11 (see Fig. 3.3):

$$K = s\big|_{v=\frac{V}{2}} \tag{3.17}$$

V should not be determined from that asymptote because considerable error can be introduced. It is worthwhile to remind that V is a limit and certainly not the maximum attainable value of v, since substrate solubility in the reaction medium can be far away from the theoretical saturation value for the enzyme. As an example, the maximum value of v attainable for the hydrolysis of lactose with β-galactosidase is only 88% of V, since the solubility of lactose at the conditions of reaction is about $250\,g\,L^{-1}$ (Illanes et al. 2000). If K is determined from that value of V (substrate concentration at V/2) error is amplified to 27%.

Methods based on linearization of Eq. 3.11 are the most frequently used for the determination of kinetic parameters. Several of such linear correlations have been proposed, being the reciprocal of the equation, known as Lineweaver–Burk plot (Lineweaver and Burk 1934), the most popular. According to it:

$$\frac{1}{v} = \frac{K}{V} \cdot \frac{1}{s} + \frac{1}{V} \tag{3.18}$$

Plotting the reciprocal of initial reaction rate versus initial substrate concentration, V and K can be easily determined as shown in Fig. 3.4: intercept on the Y axis is the reciprocal of V and intercept on the X axis is the negative reciprocal of K. Arguments against this very popular method have been raised because the error is unevenly distributed, being higher for the determination of v at low s values. In fact, at low v values a small error in their determination produces a large error in 1/v, while the opposite holds at high v values. Statistical analysis and differential weighing of data is advisable, as suggested in the original paper by the proponents (Lineweaver and Burk 1934). It has also been argued that distribution of experimental points is uneven over the X axis. This will occur if an arithmetic progression of

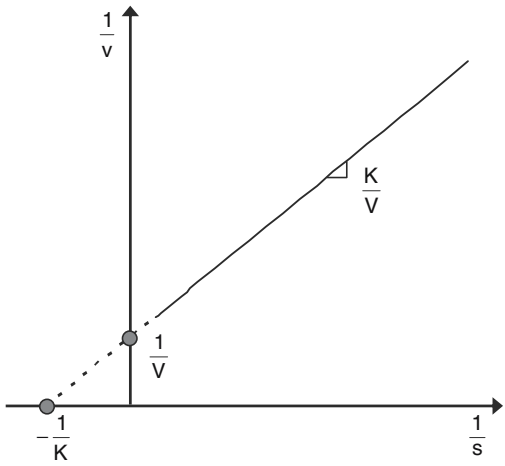


Fig. 3.4 Lineweaver–Burke (double reciprocal) plot for the determination of kinetic parameters

Table 3.1 Determination of Kinetic Parameters by Linearization

Method	Y-axis	X-axis	Intercept in Y-axis	Intercept in X-axis	Slope
Lineweaver–Burke	$\frac{1}{v}$	$\frac{1}{s}$	$\frac{1}{V}$	$-\frac{1}{K}$	$\frac{K}{V}$
Hanes	$\frac{s}{v}$	s	$\frac{K}{V}$	$-K$	$\frac{1}{V}$
Eaddie–Hofstee	v	$\frac{v}{s}$	V	$\frac{V}{K}$	$-K$
Integrated	$\frac{1}{t}\cdot\ln\left(\frac{s_i}{s}\right)$	$\frac{s_i - s}{t}$	$\frac{V}{K}$	V	$-\frac{1}{K}$

s is used; however, if a geometric progression of s is used (which is even more easy to attain in the laboratory by simple sequential dilution of a concentrated substrate stock solution), an even distribution of data points is obtained.

Other methods of linearization of Eq. 3.11 are presented in Table 3.1. The method of Hanes (Hanes 1932) and Eaddie–Hofstee (Hofstee 1959) have been the most used alternatives.

In the method of Hanes, s/v is plotted versus s:

$$\frac{s}{v} = \frac{K}{V} + \frac{1}{V}\cdot s \tag{3.19}$$

In this method, V is determined from the slope (1/V) and K from the intercept in the X axis $(-K)$. Error is more evenly distributed than in Lineweaver–Burk plot and is recalled as a better option (Cornish-Bowden 1995).

In the method of Eaddie–Hofstee, v is plotted versus v/s:

$$v = V - K\cdot\frac{v}{s} \tag{3.20}$$

so that V is determined from the intercept in the Y axis and K from the slope $(-K)$. As in the method of Hanes, error is evenly distributed.

Despite its disadvantages, the double reciprocal plot of Lineweaver–Burk has its merits and is frequently used. It is the only one in which independent and dependent variables are separated. In fact, the Hanes plot contains the independent variable in the Y (dependent) axis, while the Eaddie–Hofstee plot contains the dependent variable in the X (independent) axis. The fact that variables are not separated in these two methods obscure to some extent the interpretation of the data, being the double reciprocal plot quite illustrative to determine kinetic mechanisms, as will be seen in the next section.

A somewhat different method, also based on initial rate measurements, is the one proposed by Eisenthal and Cornish-Bowden (1974). The method is based on tracing straight lines joining the data points $(0, -s_i)$ and $(v_i, 0)$ in a v versus s plot. Such lines intersect at a point whose coordinates are V and K, as can be easily demonstrated. Advantages of such method have been highlighted (Henderson 1978).

Whatever the method of choice, some useful guidelines for the determination of kinetic parameters are:

- The number of data points should be no less than eight and desirably 10 or more.
- Data points should be distributed as evenly as possible.
- The range of substrate concentration should include the K value. Ideally, K should be the half value of that range:

$$K = \frac{s_{max} + s_{min}}{2}$$

If the value of K lies outside, considerable error can be produced, so it is advisable to redefine a new range using the calculated value as its midpoint.

A completely different system for the determination of kinetic parameters is based on the evaluation of substrate concentration through time. In fact, by integration of the Michaelis–Menten equation:

$$v = -\frac{ds}{dt} = \frac{V \cdot s}{K + s} - \int_{s_i}^{s} \frac{K + s}{s} ds = \int_{0}^{t} V \, dt \tag{3.21}$$

The integrated form of Eq. 3.21 is:

$$V \cdot t = K \cdot \ln\left(\frac{s}{s_i}\right) + (s_i - s) \tag{3.22}$$

A linear correlation can be obtained from Eq. 3.22:

$$\frac{1}{t} \cdot \ln\left(\frac{s_i}{s}\right) = \frac{V}{K} - \frac{s_i - s}{K \cdot t} \tag{3.23}$$

that allows the determination of the kinetic parameters from the slope and intercepts of the $1/t \ln(s_i/s)$ versus $(s_i - s)/t$ plot as indicated in Table 3.1. In principle, this system is simpler than the one based on initial rate measurements since only one experiment is needed. However, the conditions of the experiment have to be carefully chosen and controlled so as to discriminate any factor other than substrate depletion that can affect enzyme activity (see section 1.3). Experiment must be conducted then under conditions in which enzyme inactivation, inhibition by product and reversibility of the reaction are negligible. In practice, this means that rather short times of reaction are to be used, especially if the enzyme is unstable or subjected to strong product inhibition.

Determination of kinetic parameters by linearization is summarized in Table 3.1.

The trend now is to determine kinetic parameters by non-linear regression to the rate equation. Non-linear regression is a method of curve fitting to a non-linear estimator of the relationship between dependent and independent variables. Models for non-linear regression can be complex and multi-parameters and there is a vast literature on the subject (Seber and Wild 2003). K and V can be determined directly from the rate equation (Eq. 3.11) and obtain the values that better fit the experimental

data using an appropriate numerical method. The general form of the non-linear regression estimators is:

$$y_i = m\left(x_i, \vec{\alpha}\right) + \varepsilon_i \tag{3.24}$$

where m is a function that depends on parametric vectors $(\vec{\alpha})$ that must be estimated, being ε_i the residuals. The selection of $\vec{\alpha}$ is made by the minimum squares method, determining the one that minimizes the square sum of residuals:

$$\psi\left(\vec{\alpha}\right) = \sum_{i=1}^{n} \left[y_i - m\left(x_i, \vec{\alpha}\right)\right]^2 \tag{3.25}$$

The algorithm to minimize the $\Psi(\vec{\alpha})$ function is an iterative procedure based on the Gauss–Newton method or in even more complex methods, like the Levenberg–Marquard algorithm. The iterative algorithm is started by giving initial values of $\vec{\alpha}(\vec{\alpha}_0)$ and then at each stage "i", a new $\vec{\alpha}_i$ is obtained until convergence according to a predetermined detention criterion. There are several statistical softwares, like SPSS, available to perform non-linear regression, but it can also be done in other type of softwares, like *Mathematics*. Initial values of the parameters are very important to obtain an adequate convergence, so that reasonable values (not very far apart from the real values) must be considered.

3.3 Kinetics of Enzyme Inhibition

3.3.1 Types of Inhibition

In general terms, a *modulator* is any substance that reversibly interacts with the enzyme modifying its kinetic behavior. Most modulators exert a negative effect and are then considered inhibitors. By definition, an *inhibitor* is a substance that reversibly interacts with the enzyme reducing its catalytic potential. Most enzymatic reactions of industrial relevance are subjected to product and/or substrate inhibition, so that kinetics of enzyme inhibition is highly relevant.

Inhibitors are properly classified according to the catalytic step with which they interact. *Competitive* inhibitors are those that affect the first step of catalysis, this is the binding of substrate at the active site of the enzyme, without interfering with the processing of the substrate in it. Competitive inhibition can be total (if the inhibitor binds at the active site completely excluding the substrate) or partial (if the inhibitor interferes with substrate binding without completely excluding it from the active site); partial competitive inhibition is quite infrequent. *Non-competitive* inhibitors are those that affect the second step of catalysis, this is, the chemical processing of the bound substrate in the active site, without interfering with substrate binding. Non-competitive inhibition can be total (if the enzyme–substrate–inhibitor tertiary complex is completely inactive) or partial (if the tertiary complex is partially active). In this case, modulation can be positive (activation) if the tertiary complex is more active than the enzyme–substrate complex; this situation occurs although is rather infrequent. *Mixed-type* inhibitors are those that interfere with both steps of catalysis.

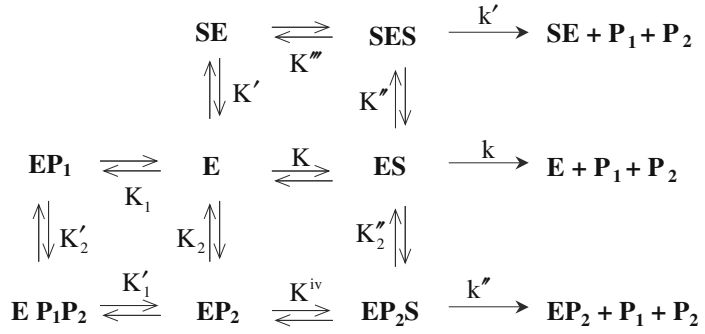
May be most inhibitors are mixed-type; however competitive and non-competitive behaviors are frequently reported when one effect is significantly stronger than the other. Mixed-type inhibition, as non-competitive inhibition, can be partial or total depending on the activity of the tertiary enzyme–substrate–inhibitor complex. A particular case of mixed-type inhibition is *uncompetitive* inhibition; in this case the enzyme has no preformed site for binding the inhibitor, that can only binds to the enzyme after the substrate has bound to it. This situation is not frequent, with the exception of the case when the substrate itself is the inhibitor; in fact, uncompetitive inhibition by high substrate concentration is rather common in enzyme catalyzed reactions.

3.3.2 Development of a Generalized Kinetic Model for One-Substrate Reactions Under Inhibition

Inhibition by products and/or substrates is quite relevant so that a generalized equation will be derived considering most of the situations of inhibition kinetics for one substrate reactions (Siimer 1978). The following reaction scheme will be considered:

$$S \xrightarrow{E} P_1 + P_2$$

where P_1 is a competitive inhibitor product, P_2 is a non-competitive inhibitor product and S is a substrate exerting uncompetitive inhibition at high concentration:



According to the rapid equilibrium hypothesis:

$$E + S \longleftrightarrow ES \qquad K = (e - c - d - f - g - h - i - j) \cdot \frac{s}{c} \tag{3.26}$$

$$E + S \longleftrightarrow SE \qquad K' = (e - c - d - f - g - h - i - j) \cdot \frac{s}{d} \tag{3.27}$$

$$ES + S \longleftrightarrow SES \qquad K'' = \frac{c \cdot s}{f} \tag{3.28}$$

$$SE + S \longleftrightarrow SES \qquad K''' = \frac{d \cdot s}{f} \tag{3.29}$$

$$E + P_1 \longleftrightarrow EP_1 \qquad K_1 = (e - c - d - f - g - h - i - j) \cdot \frac{p_1}{g} \tag{3.30}$$

$$E + P_2 \longleftrightarrow EP_2 \qquad K_2 = (e - c - d - f - g - h - i - j) \cdot \frac{p_2}{h} \tag{3.31}$$

$$EP_1 + P_2 \longleftrightarrow EP_1P_2 \qquad K_2' = \frac{g \cdot p_2}{i} \tag{3.32}$$

$$EP_2 + P_1 \longleftrightarrow EP_1P_2 \qquad K_1' = \frac{h \cdot p_1}{i} \tag{3.33}$$

$$EP_2 + S \longleftrightarrow EP_2S \qquad K^{iv} = \frac{h \cdot s}{j} \tag{3.34}$$

$$ES + P_2 \longleftrightarrow EP_2S \qquad K_2'' = \frac{c \cdot p_2}{j} \tag{3.35}$$

where:

$$K \cdot K'' = K' \cdot K''' \tag{3.36}$$

$$K_1 \cdot K_2' = K_2 \cdot K_1' \tag{3.37}$$

$$K \cdot K_2'' = K_2 \cdot K^{iv} \tag{3.38}$$

$$\begin{aligned} &ES \xrightarrow{k_{cat}} E + P_1 + P_2 \\ &SES \xrightarrow{k_{cat}'} SE + P_1 + P_2 \qquad v = k_{cat} \cdot c + k_{cat}' \cdot f + k_{cat}'' \cdot j \\ &EP_2S \xrightarrow{k_{cat}''} EP_2 + P_1 + P_2 \end{aligned} \tag{3.39}$$

Considering an equimolar reaction:

$$p_1 = p_2 = p \tag{3.40}$$

and solving Eqs. 3.26 to 3.40:

$$v = \frac{k_{cat} \cdot e \cdot s \cdot \left(1 + \frac{k_{cat}' \cdot s}{k_{cat} \cdot K''} + \frac{k_{cat}'' \cdot K \cdot p}{k_{cat} \cdot K_2 \cdot K^{iv}}\right)}{s \cdot \left(1 + \frac{s}{K''} + \frac{K \cdot p}{K_2 \cdot K^{iv}}\right) + K \cdot \left(1 + \frac{s}{K'}\right) + K \cdot p \cdot \left(\frac{1}{K_1} + \frac{1}{K_2} + \frac{p}{K_1 \cdot K_2'}\right)} \tag{3.41}$$

Determination of reaction kinetics for any mechanism involving one-substrate (or two substrates when one is in large excess) can be easily derived from Eq. 3.41 and the above reaction scheme by considering those kinetic constants that have finite values as indicated in Table 3.2.

As an example, in the case of the enzyme β-galactosidase, that catalyzes the hydrolysis of lactose into glucose and galactose, which is inhibited competitively

Table 3.2 Values of Kinetic Parameters for Different Kinetic Models as Derived from the Reaction Scheme

Model	K	K_1	K_2	K'_1	K'_2	K''_2	K'	K''	K'''	K^{iv}	k_{cat}	k'_{cat}	k''_{cat}
Simple M-M	K	∞	∞	–	–	∞	∞	∞	–	–	k	–	–
Comp. inh. by P_1	K	K_1	∞	–	∞	∞	∞	∞	–	–	k	–	–
Total non-comp. inh. by P_2	K	∞	K_2	∞	–	K_2	∞	∞	–	K	k	–	0
Partial non-comp. inh. by P_2	K	∞	K_2	∞	–	K_2	∞	∞	–	–	k	–	k''
Total uncomp. inh. by S	K	∞	∞	–	–	∞	∞	K''	–	–	k	0	–
Partial uncomp. inh. by S	K	∞	∞	–	–	∞	∞	K''	–	–	k	k'	–
Total mixed-type inhibition by P_2	K	∞	K_2	∞	–	K''_2	∞	∞	–	K^{iv}	k	–	0
Partial mixed-type inhibition by P_2	K	∞	K_2	∞	–	K''_2	∞	∞	–	K^{iv}	k	–	k''
Combined inh. By P_1 and P_2 (without EP_1P_2)	K	K_1	K_2	∞	∞	K_2	∞	∞	–	K	k	–	0

by galactose, from Table 3.2 only K, K_1 and k_{cat} have finite values. Then, from Eq. 3.41:

$$v = \frac{k_{cat} \cdot e \cdot s}{s + K + \frac{K \cdot p}{K_1}} = \frac{V \cdot s}{s + K \cdot \left(1 + \frac{p}{K_1}\right)} \tag{3.42}$$

In the case of penicillin acylase, that catalyzes the hydrolysis of penicillin G into 6-aminopenicillanic acid and phenylacetic acid, which is inhibited by 6-aminopenicillanic acid non-competitively and by phenylacetic acid competitively, from Table 3.2 only K, K_1, K_2, K''_2, K^{IV} and k_{cat} have finite values. Then, from Eq. 3.41:

$$v = \frac{k_{cat} \cdot e \cdot s}{s\left(1 + \frac{p}{K''_2}\right) + K + \frac{K \cdot p}{K_1} + \frac{K \cdot p}{K_2}} = \frac{\frac{V \cdot s}{\left(1 + \frac{p}{K''_2}\right)}}{s + K\frac{\left(1 + \frac{p}{K_1} + \frac{p}{K_2}\right)}{\left(1 + \frac{p}{K''_2}\right)}} \tag{3.43}$$

In the case of invertase (β-fructofuranosidase EC 3.2.1.26), that catalyzes the hydrolysis of sucrose into glucose and fructose, which is inhibited uncompetitively by high concentrations of sucrose, from Table 3.2 only K, K'' and k have finite values. Then, from Eq. 3.41:

$$v = \frac{k_{cat} \cdot e \cdot s}{s \cdot \left(1 + \frac{s}{K''}\right) + K} = \frac{\frac{V \cdot s}{(1 + s/K'')}}{s + \frac{K}{(1 + s/K'')}} \tag{3.44}$$

The above analysis was based on the hypothesis of rapid equilibrium. Analysis according to steady-state hypothesis can be quite cumbersome and mathematical algorithms are required to solve the equations. These can be done rather easily by using the algorithm of King and Altmann (Cornish-Bowden 1995) even though compact parametric expressions may be hard to obtain.

3.3.3 Determination of Kinetic Parameters for One-Substrate Reactions Under Inhibition

A convenient way of expressing rate equations for reactions subjected to inhibition is in terms of apparent parameters:

$$v = \frac{V_{AP} \cdot s}{K_{AP} + s} \tag{3.45}$$

where parameters V_{AP} and K_{AP} are functions of the inhibitor concentration.

For competitive inhibition: $K_{AP} > K$ and $V_{AP} = V$

For non-competitive inhibition: $K_{AP} = K$ and $V_{AP} < V$

For mixed-type inhibition there are three cases:

i: $K_{AP} > K$ and $V_{AP} < V$
ii: $K_{AP} > K$ and $V_{AP} > V$
iii: $K_{AP} < K$ and $V_{AP} < V$

In mixed-type inhibition case i, the net effect will be negative irrespectively of the values of K_{AP} and V_{AP}. In the other two cases the net effect could be negative (inhibition) or positive (activation) depending on the values of K_{AP} and V_{AP}. If $K_{AP} < K$ and $V_{AP} > V$ the net effect will be positive (activation) irrespectively of the values of K_{AP} and V_{AP}. Activation by products of reaction is not as frequent as inhibition, but cases have been reported where product activation is significant (Wieloch et al. 1982; Illanes et al. 1990).

Except for the case of uncompetitive inhibition by high substrate concentration (see Eq. 3.44), all mechanisms of inhibition can be represented by Eq. 3.45 (see, for instance, Eqs. 3.42 and 3.43) which is a very convenient expression for determining the kinetic parameters of the corresponding rate equations.

Experimental determination of kinetic parameters for inhibition mechanisms follows the same pattern as in simple Michaelis–Menten kinetics (section 3.2.2). Linearization methods are particularly useful to determine the mechanism of inhibition as a previous step to the quantification of the kinetic parameters. Experimental design consists now in a matrix in which initial rate data are gathered at different substrate and inhibitor concentrations (s and i respectively) as depicted in Table 3.3. Inhibitor is here considered in general terms as any substance exerting enzyme inhibition, be it a product of reaction, as previously considered, or catalytically inert. Of course inhibition by products and/or substrate is more technologically relevant, since catalytically inert inhibitors can be simply kept out from the reaction medium.

If initial rate data are linearized using the double reciprocal plot of Lineweaver–Burke:

$$\frac{1}{v} = \frac{K_{AP}}{V_{AP}} \cdot \frac{1}{s} + \frac{1}{V_{AP}} \tag{3.46}$$

Graphical representation of Eq. 3.46 for all mechanisms is in Fig. 3.5. Lineweaver–Burke plots are quite useful for determining kinetic mechanisms. Competitive inhibition will be represented by straight lines with a common intercept in the Y-axis,

Table 3.3 Experimental Design for the Determination of Kinetic Parameters for Inhibition Mechanisms Based on Initial Rate Measurements

	$i = 0$	i_1	i_2	$\cdots$	i_m
s_1	v_{10}	v_{12}	v_{12}	$\cdots$	v_{1m}
s_2	v_{20}	v_{21}	v_{22}	$\cdots$	v_{2m}
s_3	v_{30}	v_{31}	v_{32}	$\cdots$	v_{3m}
$\vdots$	$\vdots$	$\vdots$	$\vdots$	$\vdots$	$\vdots$
s_n	v_{n0}	v_{n1}	v_{n2}	$\cdots$	v_{nm}
	V	V_{AP1}	V_{AP2}	$\cdots$	V_{APm}
	K	K_{AP1}	K_{AP1}	$\cdots$	K_{APm}

while non-competitive inhibition will be represented by straight lines with a common intercept in the X-axis. Uncompetitive inhibition will be represented by parallel lines (not if the substrate itself is the inhibitor since in that case no straight lines will be obtained). Mixed-type inhibition will be represented by straight lines intersecting somewhere in quadrant II away from both axes. Results have to be judiciously

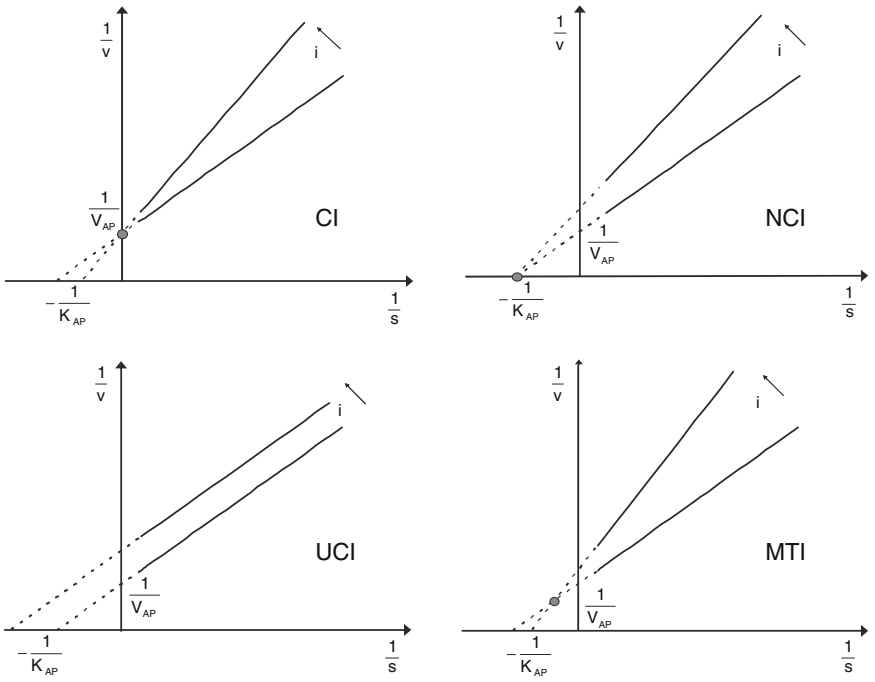


Fig. 3.5 Graphical representation of inhibition mechanisms in Lineweaver–Burke double reciprocal plots. CI: competitive inhibition; NCI: non-competitive inhibition; UCI: uncompetitive inhibition; MTI: mixed-type inhibition

Table 3.4 Values of Apparent Kinetic Parameters for Different Kinetic Models

Mechanism	V_{AP}	K_{AP}	#
Simple M-M	V	K	2
Competitive inhibition	V	$K\left(1+\frac{i}{K_I}\right)$	3
Total non-competitive inhibition	$\frac{V}{1+\frac{i}{K_2}}$	K	3
Partial non-competitive inhibition	$\frac{V+V'\frac{i}{K_2}}{1+\frac{i}{K_2}}$	K	4
Total mixed-type inhibition	$\frac{V}{1+\frac{i}{K_2''}}$	$K\frac{1+\frac{i}{K_2}}{1+\frac{i}{K_2''}}$	4
Partial mixed-type inhibition	$\frac{V+\frac{V'\cdot i}{K_2''}}{1+\frac{i}{K_2''}}$	$K\frac{1+\frac{i}{K_2}}{1+\frac{i}{K_2''}}$	5
Total uncompetitive inhibition	$\frac{V}{1+\frac{i}{K''}}$	$\frac{K}{1+\frac{i}{K''}}$	3
Partial uncompetitive inhibition	$\frac{V+\frac{V'\cdot i}{K''}}{1+\frac{i}{K''}}$	$\frac{K}{1+\frac{i}{K''}}$	4

#: number of kinetic parameters to be determined

analyzed and validated statistically to properly assess the mechanism that more closely represents the experimental data.

Once the mechanism of inhibition has been identified, expressions for the apparent kinetic parameters for each mechanism of inhibition are obtained from Eq. 3.41 and Table 3.2, as indicated in Table 3.4. Their values are experimentally determined from the intercepts of the double reciprocal plots, as shown in Fig. 3.5 and then, by using secondary linear plots, the corresponding kinetic constants can be determined as shown in Fig. 3.6.

Total and partial mechanisms are not easily discriminated in double reciprocal plots. To assess the total or partial nature of the inhibition, a V_{AP} versus i plot is adequate since in the first case V will tend to zero as i increases, while in the second case will tend to a finite value as shown in Fig. 3.7. Actually the parameter V' can be conveniently determined as the limit of V_{AP} as i tends to infinite. In fact:

$$\lim V_{AP}|_{i\to\infty} = \lim \left.\frac{V+\frac{V'\cdot i}{K_I}}{1+\frac{i}{K_I}}\right|_{i\to\infty} = \lim \left.\frac{\frac{V}{i}+\frac{V'}{K_I}}{\frac{1}{i}+\frac{1}{K_I}}\right|_{i\to\infty} = V' \qquad (3.47)$$

where K_I stands for K_2, K_2'' and K'' for non-competitive, mixed-type and uncompetitive inhibition respectively. Non-linear regression of data allows the determination of V' straight from that plot. Error will be higher when the inhibitor is poorly soluble and not so high values of i are attainable.

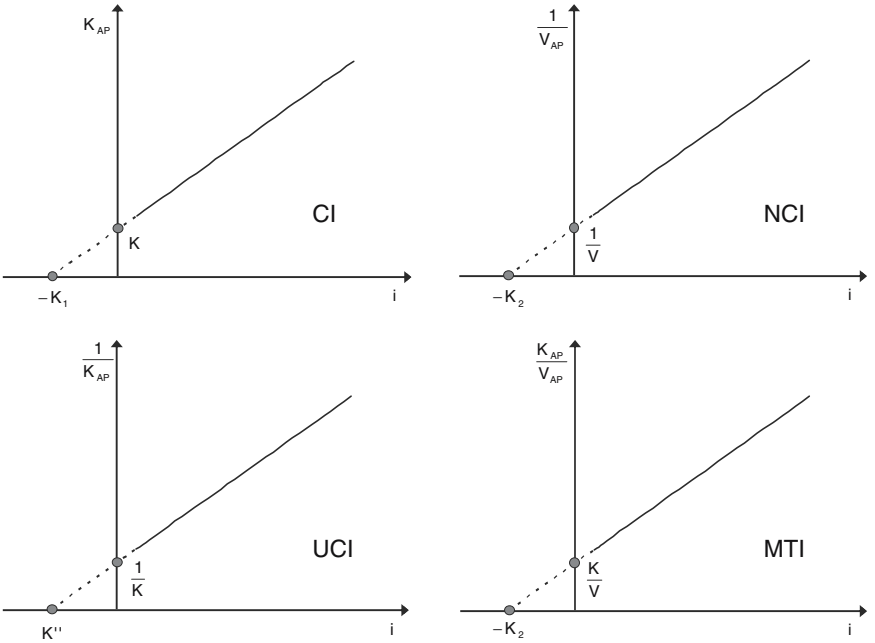


Fig. 3.6 Secondary plots for the determination of kinetic parameters. CI: competitive inhibition; NCI: non-competitive inhibition; UCI: uncompetitive inhibition; MTI: mixed-type inhibition

Same guidelines as those given in section 3.2.2 are applicable for the experimental determination of kinetic parameters of inhibition. Desirably no less than eight substrate concentrations and five initial inhibitor concentrations (including zero) should be considered, data points should be evenly distributed and the range of

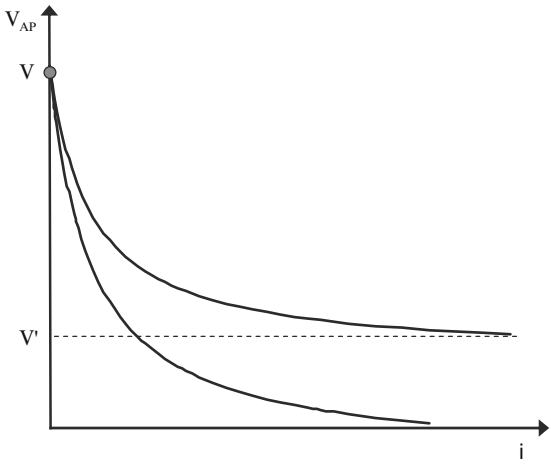


Fig. 3.7 Discrimination between total and partial mechanisms of inhibition

substrate and inhibitor concentrations should contain the values of the Michaelis–Menten and inhibition constants respectively.

Other methods of linearization (i.e. Eaddie–Hofstee; Hanes) can be used, as well as integrated methods, as shown in section 3.2.2 for simple Michaelis–Menten kinetics.

Even though linearization methods are valuable tools for determining the mechanism of inhibition, once determined, kinetic parameters can better be evaluated by non-linear regression to the corresponding rate equations, as presented in section 3.2.2 for simple Michaelis–Menten kinetics.

3.4 Reactions with More than One Substrate

Traditional enzymatic processes are mostly related to hydrolytic reactions than can be conveniently considered as one-substrate reactions. However, the potential of enzyme catalysis is now focused on reactions of organic synthesis in which complex molecules are produced in reactions involving more than one substrate. This is the case of oxidoreductases, transferases and ligases, whose technological potential is increasing, despite the complexity of the processes (see section 1.6). Moreover, robust technologically apt hydrolytic enzymes under suitable reaction conditions can catalyze reverse reactions of synthesis, whose technological impact is impressive (Bornscheuer and Kazlauskas 1999). Therefore enzyme kinetics for reactions involving more than one substrate is quite relevant.

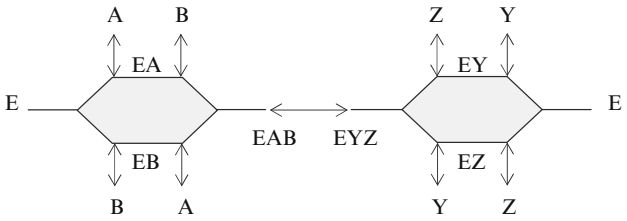
3.4.1 Mechanisms of Reaction

Mechanism of reactions can be divided into two main categories: *sequential* and *ping-pong*. In sequential mechanisms, all substrates must combine with the enzyme before the reaction occurs. Sequential mechanisms are sub-classified into *ordered* or *random* depending on the existence or not of a predetermined sequence of substrate binding to the enzyme (and product release from it). In ping-pong mechanisms, product is formed before all substrates have bound to the enzyme, which means that the enzyme exists in two alternative catalytically active species, each of them recognizing one substrate and transforming it into a product while suffering a conformational change to the other species. Ping-pong mechanisms can also be sub-classified into sequential or ordered but this holds only for reactions involving more than two substrates, which are uncommon. Mechanisms for the bi-bi two-substrate reaction $A + B \xrightarrow{E} Y + Z$ will be analyzed considering sequential and ping-pong mechanism to develop the corresponding rate equations. Mechanisms can be conveniently represented according to the nomenclature proposed by Cleland (1963).

3.4.1.1 Ordered-Sequential

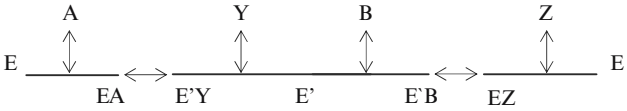
In this case, the enzyme has no preformed site for binding substrate B, which forms only when substrate A has been previously bound to the enzyme. In other words, there is no formation of secondary E-B complex.

3.4.1.2 Ordered-Random



In this case, the enzyme can bind either substrate first so that both secondary enzyme complexes, EA and EB, can be formed to then bind the other substrate generating the reactive ternary complex EAB.

3.4.1.3 Ping-Pong



In this case, E and E′ are alternative enzyme species. Species E recognizes substrate A, binds it and converts it into product Y, while suffering a structural change to species E′. That species no longer recognizes A but instead recognizes substrate B, binds it and converts into product Z, while again suffering a structural change to species E so closing the catalytic cycle.

3.4.2 Development of Kinetic Models

Kinetic models can be derived from the above mechanisms according to the rapid equilibrium hypothesis.

3.4.2.1 Ordered-Sequential

$$E + A \longleftrightarrow EA \qquad K_A = \frac{(e - c - f) \cdot a}{c} \tag{3.48}$$

$$EA + B \longleftrightarrow EAB \qquad K'_B = \frac{c \cdot b}{f} \tag{3.49}$$

$$EAB \longrightarrow E + Y + Z \qquad v = k_{cat} \cdot f \tag{3.50}$$

From Eqs. 3.48 and 3.49:

$$f = \frac{e}{1 + \frac{K'_B}{b} + \frac{K_A \cdot K'_B}{a \cdot b}} \tag{3.51}$$

and replacing in Eq. 3.50:

$$v = \frac{k_{cat} \cdot e \cdot a \cdot b}{a \cdot b + K'_B \cdot a + K_A \cdot K'_B} \tag{3.52}$$

3.4.2.2 Random-Sequential

$$E + A \longleftrightarrow EA \qquad K_A = \frac{(e - c - d - f) \cdot a}{c} \tag{3.53}$$

$$E + B \longleftrightarrow EB \qquad K_B = \frac{(e - c - d - f) \cdot b}{d} \tag{3.54}$$

$$EA + B \longleftrightarrow EAB \qquad K'_B = \frac{c \cdot b}{f} \tag{3.55}$$

$$EB + A \longleftrightarrow EAB \qquad K'_A = \frac{d \cdot a}{f} \tag{3.56}$$

$$EAB \longrightarrow E + Y + Z \qquad v = k_{cat} \cdot f \tag{3.57}$$

From Eqs. 3.53 to 3.56:

$$f = \frac{e}{1 + \frac{K'_B}{b} + \frac{K'_A}{a} + \frac{K_A \cdot K'_B}{a \cdot b}} \tag{3.58}$$

and replacing in Eq. 3.57:

$$v = \frac{V \cdot a \cdot b}{a \cdot b + K'_B \cdot a + K'_A \cdot b + K_A \cdot K'_B} \tag{3.59}$$

Eqs. 3.52 and 3.59 can be conveniently expressed in parametric form as:

$$v = \frac{V_{AP} \cdot a}{K_{AP} + a} = \frac{V'_{AP} \cdot b}{K'_{AP} + b} \tag{3.60}$$

where V_{AP} and K_{AP} are functions of b and $V_{AP}{}'$ and $K_{AP}{}'$ are in principle functions of a, as shown in Table 3.5.

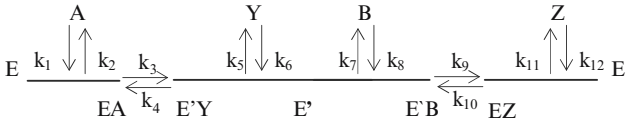
Table 3.5 Values of Kinetic Parameters for the Reaction $A+B \xrightarrow{E} Y+Z$, According to Sequential Mechanisms

	Parameter			
Mechanism	V_{AP}	K_{AP}	$V_{AP}{}'$	$K_{AP}{}'$
Ordered	$\frac{V \cdot b}{b+K_B{}'}$	$\frac{K_A \cdot K'_B}{b+K_B{}'}$	V	$\frac{a \cdot K'_B + K_A K'_B}{a}$
Random	$\frac{V \cdot b}{b+K_B{}'}$	$\frac{b \cdot K'_A + K_A K'_B}{b+K'_B}$	$\frac{V \cdot a}{a+K'_A}$	$\frac{a \cdot K'_B + K_A K'_B}{a+K'_A}$

3.4.2.3 Ping-Pong

Ping-pong mechanisms are more complex and require of certain assumptions to arrive to compact kinetic expressions amenable for evaluation by conventional experimental methods. There are several reactions of technological relevance that correspond to this type of mechanism, like the production of biodiesel with lipases (see section 6.3) and the synthesis of pharmaceuticals and fine-chemicals with oxidoreductases (see section 6.4).

The following general scheme represents the reaction of conversion of substrates A and B into products Y and Z according to ping-pong mechanisms using the nomenclature proposed by Cleland:



Certain assumptions are required to develop a sound kinetic model from that mechanism. It will be assumed that the EA to E′Y and E′B to EZ transitions are instantaneous. Steady-state balances for all enzyme species are:

$$\text{E} \qquad \frac{de}{dt} = k_2 \cdot c + k_{11} \cdot d - k_1 \cdot a \cdot (e - c - e' - d) - k_{12} \cdot z \cdot (e - c - e' - d) = 0 \tag{3.61}$$

$$\text{E}'\text{Y} \qquad \frac{dc}{dt} = k_1 \cdot a \cdot \left(e - c - e' - d\right) + k_6 \cdot y \cdot e' - (k_2 + k_5) \cdot c = 0 \tag{3.62}$$

$$\text{E}' \qquad \frac{de'}{dt} = k_5 \cdot c + k_8 \cdot d - k_6 \cdot e' \cdot y - k_7 \cdot e' \cdot b = 0 \tag{3.63}$$

$$\text{EZ} \qquad \frac{dd}{dt} = k_7 \cdot b \cdot e' + k_{12} \cdot z \cdot (e - c - e' - d) - d \cdot (k_{11} + k_8) = 0 \tag{3.64}$$

and the expression for reaction rate is:

$$v = k_{11} \cdot d \tag{3.65}$$

From Eqs. 3.61 to 3.65:

$$d = \frac{e}{\left(\frac{k_5}{(k_6 \cdot y + k_7 \cdot b)} + \frac{k_2}{(k_1 \cdot a + k_{12} \cdot z)} + 1\right) \cdot \alpha + \frac{k_8}{(k_6 \cdot y + k_7 \cdot b)} + \frac{k_{11}}{(k_1 \cdot a + k_{12} \cdot z)} + 1} \tag{3.66}$$

where:

$$\alpha = \frac{\frac{k_1 \cdot a}{k_2 + k_5} \cdot \frac{k_{11}}{k_1 \cdot a + k_{12} \cdot z} + \frac{k_6 \cdot y}{k_2 + k_5} \cdot \frac{k_8}{k_6 \cdot y + k_7 \cdot b}}{1 - \frac{k_6 \cdot y}{k_2 + k_5} \cdot \frac{k_5}{k_6 \cdot y + k_7 \cdot b} - \frac{k_1 \cdot a}{k_2 + k_5} \cdot \frac{k_2}{k_1 \cdot a + k_{12} \cdot z}} \tag{3.67}$$

Replacing in Eq. 3.65:

$$v = \frac{k_{11} \cdot e}{\left(\frac{k_5}{(k_6 \cdot y + k_7 \cdot b)} + \frac{k_2}{(k_1 \cdot a + k_{12} \cdot z)} + 1\right) \cdot \alpha + \frac{k_8}{(k_6 \cdot y + k_7 \cdot b)} + \frac{k_{11}}{(k_1 \cdot a + k_{12} \cdot z)} + 1} \tag{3.68}$$

where α is better expressed as:

$$\alpha = \frac{\frac{a}{\frac{k_2 + k_5}{k_{11}} \cdot a + k_{12} \cdot \frac{k_2 + k_5}{k_1 \cdot k_{11}} \cdot z} + \frac{y}{\frac{k_2 + k_5}{k_8} \cdot y + k_7 \cdot \frac{k_2 + k_5}{k_6 \cdot k_8} \cdot b}}{1 - \frac{y}{\frac{k_2 + k_5}{k_5} \cdot y + k_7 \cdot \frac{k_2 + k_5}{k_6 \cdot k_5} \cdot b} - \frac{a}{\frac{k_2 + k_5}{k_2} \cdot a + k_{12} \cdot \frac{k_2 + k_5}{k_1 \cdot k_2} \cdot z}} \tag{3.69}$$

Considering:

$$\frac{k_2 + k_5}{k_{11}} = K_A \qquad \frac{k_2 + k_5}{k_8} = K_Y$$

$$\frac{k_2 + k_5}{k_2} = K_{AA} \qquad \frac{k_2 + k_5}{k_5} = K_{YY}$$

$$k_7 \cdot \frac{k_2 + k_5}{k_6 \cdot k_8} = K_B \qquad k_{12} \cdot \frac{k_2 + k_5}{k_1 \cdot k_{11}} = K_Z$$

$$k_7 \cdot \frac{k_2 + k_5}{k_6 \cdot k_5} = K_{BB} \qquad k_{12} \cdot \frac{k_2 + k_5}{k_1 \cdot k_2} = K_{ZZ}$$

then:

$$K_A = \frac{K_Z}{K_{ZZ}} \cdot K_{AA}$$

$$K_B = \frac{K_Y}{K_{YY}} \cdot K_{BB}$$

and replacing in Eq. 3.69:

$$\alpha = \frac{\frac{a}{K_A \cdot a + K_Z \cdot z} + \frac{y}{K_Y \cdot y + K_B \cdot b}}{1 - \frac{y}{K_{YY} \cdot y + K_{BB} \cdot b} - \frac{a}{K_{AA} \cdot a + K_{ZZ} \cdot z}} \tag{3.70}$$

From Eq. 3.68:

$$v = \frac{V}{\left(\frac{1}{\frac{k_6 y}{k_5}+\frac{k_7 b}{k_5}} + \frac{1}{\frac{k_1 a}{k_2}+\frac{k_{12} z}{k_2}} + 1\right)\cdot\frac{\frac{a}{K_A a+K_Z z}+\frac{y}{K_Y y+K_B b}}{1-\frac{y}{K_{YY} y+K_{BB} b}-\frac{a}{K_{AA} a+K_{ZZ} z}} + \frac{1}{\frac{k_6 y}{k_8}+\frac{k_7 b}{k_8}} + \frac{1}{\frac{k_1 a}{k_{11}}+\frac{k_{12} z}{k_{11}}} + 1} \tag{3.71}$$

where $V = k_{11} \cdot e$.

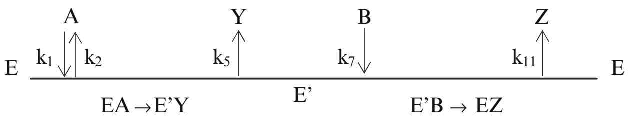
As seen, the expression for reaction rate is quite complex and contain a large number of kinetic parameters, being advisable to analyze it on a case basis. Two cases will be presented to illustrate this.

Case 1: Oxidation of Glucose with Glucose Oxidase

This is one of the few oxidoreductases which is conventionally used in the food industry and also in chemical analysis (see section 1.5). The enzyme catalyzes the oxidation of glucose to gluconolactone (that can spontaneously yield gluconic acid) by molecular oxygen which in the presence of water is reduced to hydrogen peroxide. The enzyme (E) requires the coenzyme flavin adenine dinucleotide (FAD) which acts as the electron transporter, according to:

Glucose +	E … FAD	→ Gluconalactone	+ E … FADH
(A)	(E)	(Y)	(E')
$^1/_2 O_2$	+ E … FADH	→ $^1/_2 H_2O_2$	+ E … FAD
(B)		(Z)	

The ping-pong mechanism for such reaction is, according to the given nomenclature:



A model has been derived from that mechanism considering that the formation of the EA complex is the only reversible step ($k_6 = k_8 = k_{12} = 0$), and the transition of EA into E′Y and E′B into EZ are instantaneous, which greatly simplifies the formulation of the model, which according to Eq. 3.68 is:

$$v = \frac{k_{11}\cdot e}{\left[\left(\frac{k_5}{k_7\cdot b}+\frac{k}{k_1\cdot a}+1\right)\cdot\frac{\left(\frac{k_1}{(k_2+k_5)}\cdot a\cdot\frac{k_{11}}{k_1\cdot a}\right)}{\left(1-\frac{k_1}{(k_2+k_5)}\cdot a\cdot\frac{k_2}{k_1\cdot a}\right)}+\frac{k_{11}}{k_1\cdot a}+1\right]} \tag{3.72}$$

which can be simplified to:

$$v = \frac{V}{\frac{k_{11}}{k_5}+1+\frac{k_{11}}{k_7\cdot b}+\frac{k_{11}}{k_1\cdot a}\cdot\left(1+\frac{k_2}{k_5}\right)} \tag{3.73}$$

that can be expressed as (Marangoni 2003):

$$v = \frac{V \cdot a \cdot b}{(K_{ZY} + 1) \cdot a \cdot b + K_{ZB} \cdot a + K_{ZY} \cdot K_{YY} \cdot b} \tag{3.74}$$

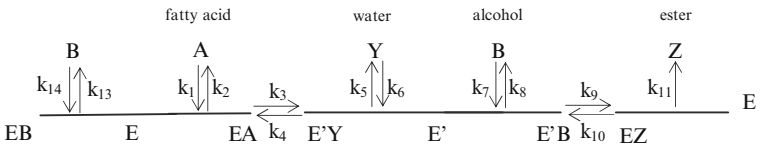
where: $K_{ZY} = \frac{k_{11}}{k_5}$, $K_{ZB} = \frac{k_{11}}{k_7}$.

For the particular case when $k_5 >>> k_{11}$:

$$v = \frac{V \cdot a \cdot b}{a \cdot b + K_{ZB} \cdot a + K_{ZY} \cdot K_{YY} \cdot b} \tag{3.75}$$

Case 2: Production of Biodiesel by Transesterification with Lipase

The enzymatic transesterification of fats and oils with short chain alcohols is a promising technology for the production of biodiesel as highlighted in section 6.3. Several mechanisms of reaction have been proposed to develop the corresponding kinetic models, considering lipase inhibition by the alcohol or the water present in the reaction medium (van Tol et al. 1995). The scheme below represents the ping-pong mechanism of reaction considering inhibition by alcohol (Janssen et al. 1999):



Steady-state balances for all enzyme species are:

$$E \qquad \frac{de}{dt} = k_2 c + k_{11} d + k_{13} i - k_1 a(e - c - e' - d - i) - k_{14} b(e - c - e' - i) = 0 \tag{3.76}$$

$$E'Y \qquad \frac{dc}{dt} = k_1 \cdot a(e - c - e' - d - i) + k_6 \cdot y \cdot e' - (k_2 i + k_5) = 0 \tag{3.77}$$

$$E' \qquad \frac{de'}{dt} = k_5 \cdot c + k_8 \cdot d - k_6 \cdot e' \cdot y - k_7 \cdot e' \cdot b = 0 \tag{3.78}$$

$$EZ \qquad \frac{dd}{dt} = k_7 \cdot b \cdot e' - d \cdot (k_{11} + k_8) = 0 \tag{3.79}$$

$$EB \qquad \frac{di}{dt} = k_{14} \cdot b \cdot (e - c - e' - d - i) - k_{13} \cdot i = 0 \tag{3.80}$$

The expression for the reaction rate is more complex and more kinetic parameters have to be determined than in the previous case (Bousquet-Dubouch et al. 2001).

3.4.3 Determination of Kinetic Parameters

3.4.3.1 Sequential Mechanisms

Kinetic parameters for sequential mechanisms can be conveniently determined from the parametric Eq. 3.60. Experimental design consists in a matrix in which initial rate data are gathered at different concentrations of both substrates (a and b) as depicted in Table 3.6.

If initial rate data are linearized using the double reciprocal plot of Lineweaver–Burke, from Eq. 3.60:

$$\frac{1}{v} = \frac{K_{AP}}{V_{AP}} \cdot \frac{1}{a} + \frac{1}{V_{AP}} = \frac{K'_{AP}}{V'_{AP}} \cdot \frac{1}{b} + \frac{1}{V'_{AP}} \tag{3.81}$$

By plotting 1/v versus 1/a at constant b (columns in Table 3.6) values of apparent kinetic parameters V_{AP} and K_{AP} are obtained from the intercepts in the Y and X-axis respectively, as shown in Fig. 3.8 for ordered (I) and random (II) mechanisms. By plotting 1/v versus 1/b at constant a (rows in Table 3.6) values of apparent kinetic parameters $V_{AP}{}'$ and $K_{AP}{}'$ are obtained from the intercepts in the Y and X-axis respectively, as shown in Fig. 3.8 for ordered (I) and random (II) mechanisms. Ordered sequential mechanism can be easily distinguished from random sequential in Lineweaver–Burke plots: in the case of ordered mechanism, intercept in the Y-axis $(1/V_{AP}{}')$ is a constant (1/V) independent of a (the substrate who binds to the enzyme first), while in the case or random mechanism it depends on a (see Table 3.5).

Once the mechanism has been identified, the corresponding kinetic constants can be obtained from the expressions in Table 3.5.

In the case of ordered mechanism, V is directly obtained from the 1/v versus 1/b plot. Straight lines in the 1/v versus 1/a plot will intersect at a point that is easily demonstrated to correspond to $1/K_A$. However, all kinetic parameters can be obtained from secondary plots as shown in Fig. 3.9I. In the case of random mechanism, as in ordered mechanism, straight lines in the 1/v versus 1/a plot will

Table 3.6 Experimental Design for the Determination of Kinetic Parameters for the Two-Substrate Reaction: $A + B \xrightarrow{E} Y + Z$

	b_1	b_2	b_3	$\cdots$	b_n		
a_1	v_{12}	v_{12}	v_{13}	$\cdots$	v_{1n}	$V_{AP1}{}'$	$K_{AP1}{}'$
a_2	v_{21}	v_{22}	v_{23}	$\cdots$	v_{2n}	$V_{AP2}{}'$	$K_{AP2}{}'$
a_3	v_{31}	v_{32}	v_{33}	$\cdots$	v_{3n}	$V_{AP3}{}'$	$K_{AP3}{}'$
$\vdots$	$\vdots$	$\vdots$	$\vdots$	$\vdots$	$\vdots$	$\vdots$	$\vdots$
a_n	v_{n1}	v_{n2}	v_{n3}	$\cdots$	v_{nn}	$V_{APn}{}'$	$K_{APn}{}'$
	V_{AP1}	V_{AP2}	V_{AP3}	$\cdots$	V_{APn}		
	K_{AP1}	K_{AP1}	K_{AP3}	$\cdot$	K_{APn}		

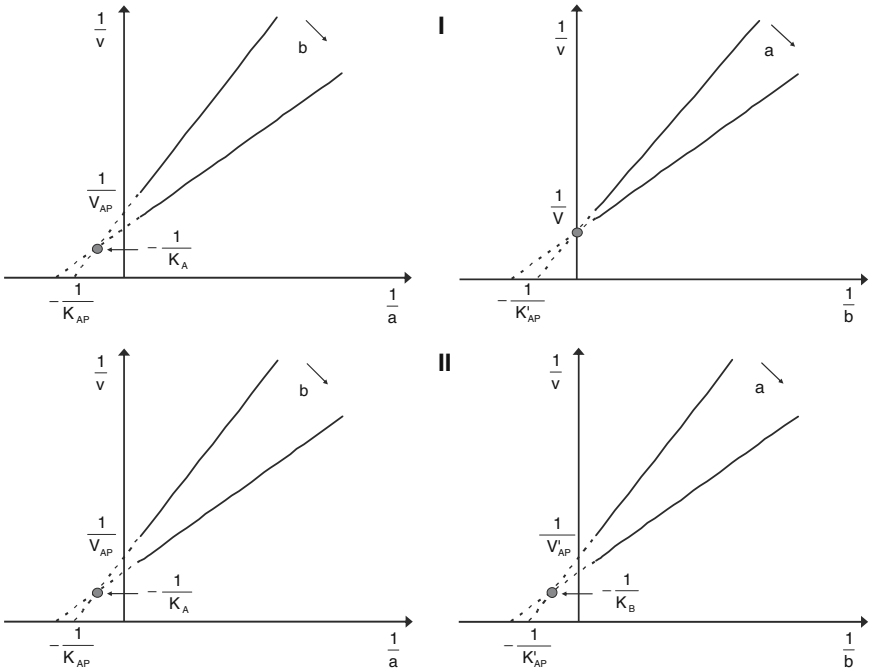


Fig. 3.8 Graphical representation in Lineweaver–Burk double reciprocal plots of kinetic models for the two-substrate reaction: $A + B \xrightarrow{E} Y + Z$, according to sequential mechanisms. I: ordered; II: random

intersect at a point that is easily demonstrated to correspond to $1/K_A$. However, all kinetic parameters can be obtained from secondary plots as shown in Fig. 3.9II.

3.4.3.2 Ping-Pong Mechanisms

Considering the case represented by Eq. 3.75, for the purpose of determining kinetic parameters, parameters K_{ZY} and K_{YY} can be lumped into one (K_L) equivalent to their product so that the equation can be rewritten as:

$$v = \frac{V \cdot a \cdot b}{a \cdot b + K_{ZB} \cdot a + K_L \cdot b} \tag{3.82}$$

Eq. 3.82 can be expresses in the parametric form of Eq. 3.45, where in this case:

$$V_{AP} = \frac{V \cdot b}{b + K_{ZB}} \qquad K_{AP} = \frac{K_L \cdot b}{b + K_{ZB}}$$

Experimental design for the determination of kinetic parameters in ping-pong mechanism is analogous to the previously described for sequential mechanisms, so that V_{AP} and K_{AP} at different values of b are determined as the Y and X-axis intercepts

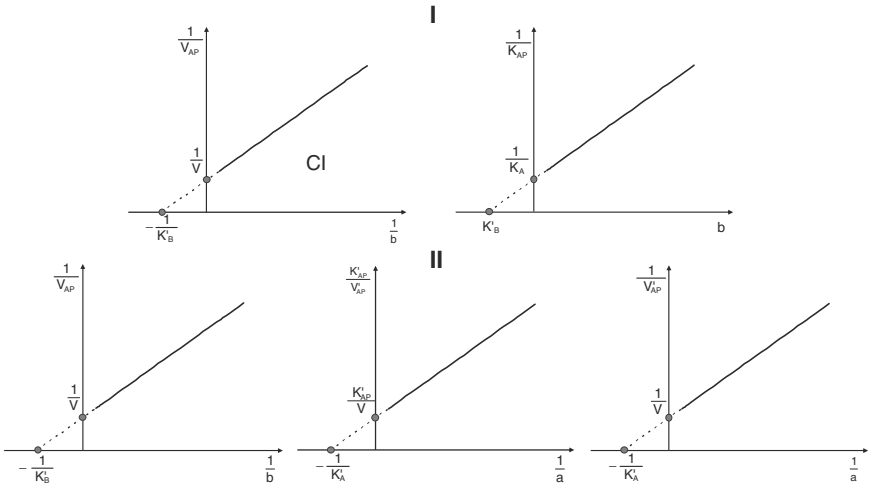


Fig. 3.9 Secondary plots for the determination of kinetic parameters in sequential mechanisms. I: ordered; II: random

($1/V_{AP}$ and $-1/K_{AP}$ respectively) in a primary double reciprocal plot of 1/v versus 1/a and then in a secondary double reciprocal plots of $1/V_{AP}$ versus 1/b, the values of parameters V and K_{ZB} are determined as the Y and X-axis intercepts (1/V and $-1/K_{ZB}$) respectively and the lumped parameter K_L is determined as the Y-axis intercept in a secondary double reciprocal plots of $1/K_{AP}$ versus 1/b.

Same guidelines as those given in section 3.2.2 are applicable for the experimental determination of kinetic parameters for two-substrate reactions. Desirably no less than eight concentrations for each substrate should be considered, data points should be evenly distributed and the range of substrates concentrations should contain the values of the respective Michaelis–Menten constants. As before, other methods of linearization (i.e. Eaddie–Hofstee; Hanes) can be used, as well as integrated methods. Even though linearization methods are valuable tools for determining the mechanism of reaction, once determined, kinetic parameters can better be evaluated by non-linear regression to the corresponding rate equations, as indicated in section 3.2.2.

3.5 Environmental Variables in Enzyme Kinetics

Besides the effect of enzyme, substrates and modulator concentrations, the catalytic potential of enzymes is affected by several environmental factors among which, pH and temperature are outstanding. These variables not only affect enzyme activity but enzyme stability as well. Enzyme stability is regarded as the capacity of the enzyme to retain its activity. Protein denaturation is an event or sequence of events leading to structural changes (not compromising its primary structure) and in most cases

such changes produce unfolding to the extent of altering the biological functionality of the protein, which in the case of enzymes means a loss in activity (Misset 1993; O'Fágáin 2003; Bommarius and Broering 2005). Such structural changes are the consequence of covalent and non-covalent bond disruption and changes in the ionization state of the active site which are promoted by altering the environment surrounding the enzyme.

Kinetic expressions seldom contain explicit functions of such environmental variables and enzyme kinetic studies are usually performed at controlled values usually regarded as optimal in the sense that they maximize the initial rate of reaction. Considered as such, they can be determined quite easily by simply profiling initial rates of reaction with respect to the variable, pH or temperature, under study. Controlling pH or temperature within a bioreactor is a rather simple task so, in principle, kinetic parameters evaluated at such "optimal" conditions will suffice for reactor design or evaluation of reactor performance (see Chapter 5). However, this concept of optimal has to be taken with precaution since it refers to initial rates, which is by no means the whole picture, since enzyme stability is a major determinant of enzyme performance and conditions optimizing activity do not necessarily match those at which the enzyme is more stable. In fact, with respect to temperature there is a strong compromise between activity and stability as will be analyzed ahead. The case of pH is usually different since optimum pH for activity usually lies within the pH range at which the enzyme is more stable (Shraboni et al. 1992). In the following section the effect of pH and temperature on enzyme kinetic parameters will be evaluated. For the case of temperature, the effect on enzyme stability will be analyzed also.

3.5.1 Effect of pH: Hypothesis of Michaelis and Davidsohn. Effect on Enzyme Affinity and Reactivity

Because enzymes are polyionic polymers, it is expected that pH will affect most of their properties. In fact, change in pH may change the distribution of charges in the active site and in the whole surface of the protein molecule. Enzymes may present polar amino acid residues at its active site whose charge depends on pH. With respect to enzyme activity, it is a well known fact that rates of enzyme catalyzed reactions tend to decrease at extremes of pH usually exhibiting maxima at some intermediate values as shown in Fig. 3.10 for the case of glucoamylase (Illanes 1983).

Based on this, even before the publication of the Michaelis–Menten hypothesis, Michaelis and Davidsohn (1911) proposed a theory for explaining the effect of pH on enzyme kinetics based on the following assumptions:

- The active center of the enzyme contains ionizable amino acid residues (in the simpler case it is assumed that here are only three ionic species of successive number of charges).
- Only one of those species (the intermediate in the case of three ionic species) is catalytically active so that the fraction of active enzyme depends on pH.

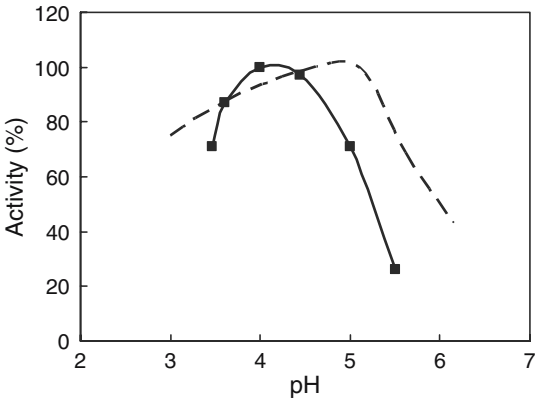


Fig. 3.10 Profile of reaction rate versus pH for the hydrolysis of maltose with glucoamylase

The first assumption is arguable as in fact more than three ionic species can exist in the active center (Dixon and Webb 1979); however, for simplicity, only three are considered which is not far from reality (the active center is usually conformed by a very low number of amino acid residues) and allows a simpler analysis of the phenomenon. The second assumption, as seen below, is supported by experimental evidence since it is consistent with the shape of the pH profiles of enzyme-catalyzed reaction rates. The following scheme represents that hypothesis:

$$
\begin{array}{ccccc}
E^{n-1} & \xleftrightarrow{K_{1E}} & E^{n} & \xleftrightarrow{K_{2E}} & E^{n+1} \\
 & & \updownarrow K & & \\
E^{n-1}S & \xleftrightarrow{K_{1ES}} & E^{n}\ S & \xleftrightarrow{K_{2ES}} & E^{n+1}S \\
 & & k_{cat}\downarrow & & \\
 & & E^{n} + P & &
\end{array}
$$

E^n represents the active species while E^{n-1} and E^{n+1} represent the inactive forms by protonation and deprotonation of the active site respectively, being n considered as the number of its negative charges (this is arbitrary and do not impose a net positive or negative charge at the active site, since n can be a positive or negative number). K_1 and K_2 are the corresponding ionic equilibrium constants, the suffix E denoting the free enzyme and suffix ES the enzyme–substrate complex, being:

$$K_{1E} = \frac{e^n \cdot h^+}{e^{n-1}} \tag{3.83}$$

$$K_{2E} = \frac{e^{n+1} \cdot h^+}{e^n} \tag{3.84}$$

$$K_{1ES} = \frac{c^n \cdot h^+}{c^{n-1}} \tag{3.85}$$

$$K_{2ES} = \frac{c^{n+1} \cdot h^+}{c^n} \tag{3.86}$$

where, as before, the lower case letters represent the molar concentration of the respective species in capital letters.

According to the scheme above, reaction rate is:

$$v = k_{cat} \cdot c^n \tag{3.87}$$

and the absolute (non pH dependent) Michaelis–Menten constant can be expressed as:

$$K = \frac{e^n \cdot s}{c^n} \tag{3.88}$$

A material balance of all enzyme species renders:

$$e = e^{n-1} + e^n + e^{n+1} \tag{3.89}$$

$$c = c^{n-1} + c^n + c^{n+1} \tag{3.90}$$

In this way, from Eqs. 3.83, 3.84 and 3.89, and Eqs. 3.85, 3.86 and 3.90, Eqs. 3.91 and 3.92 are obtained respectively:

$$e = e^n \left(\frac{h^+}{K_{1E}} + 1 + \frac{K_{2E}}{h^+} \right) \tag{3.91}$$

$$c = c^n \left(\frac{h^+}{K_{1ES}} + 1 + \frac{K_{2ES}}{h^+} \right) \tag{3.92}$$

The expressions in parenthesis are termed Michaelis functions of pH. The inverse of such functions represent the molar fraction of the total enzyme species that is in a particular ionic form. As clearly seen, that fractions (e^n and c^n) are dependent on pH ($-\log\ h^+$) and will rise and fall passing through a maximum, since the expression contains terms with h^+ in the numerator and the denominator. On the contrary, expressing them in terms of e^{n-1} and c^{n-1} or e^{n+1} and c^{n+1}, that fractions will rise or fall monotonically with pH. The second assumption of the Michaelis–Davidsohn hypothesis is then consistent with the experimental behavior, since pH profile of any enzymatic reaction resembles the dependence of c^n (and not c^{n-1} or c^{n+1}) with pH.

Based on the above equations, it is possible to derive pH-explicit expressions for the kinetic parameters. In fact, Michaelis–Menten equation can be expressed as in Eq. 3.45, where V_{AP} and K_{AP} are in this case functions of pH.

From Eq. 3.92:

$$V_{AP} = k_{cat} \cdot e^n = \frac{k_{cat} \cdot e}{\frac{h^+}{K_{1ES}} + 1 + \frac{K_{2ES}}{h^+}} = \frac{V}{\frac{h^+}{K_{1ES}} + 1 + \frac{K_{2ES}}{h^+}} \tag{3.93}$$

which is a pH explicit expression for V_{AP}.

K_{AP} can be expressed as:

$$K_{AP} = \frac{e \cdot s}{c} \tag{3.94}$$

so that, from Eqs. 3.88, 3.91 and 3.92:

$$K_{AP} = K\frac{e}{e^n} \cdot \frac{c^n}{c} = K\frac{\frac{h^+}{K_{1E}} + 1 + \frac{K_{2E}}{h^+}}{\frac{h^+}{K_{1ES}} + 1 + \frac{K_{2ES}}{h^+}} \tag{3.95}$$

which is a pH explicit expression for K_{AP}.

In this way, a pH explicit rate expression can be obtained as:

$$v = \frac{V_{AP} \cdot s}{K_{AP} + s} = \frac{\frac{V}{\frac{h^+}{K_{1ES}} + 1 + \frac{K_{2ES}}{h^+}} \cdot s}{K\frac{\frac{h^+}{K_{1E}} + 1 + \frac{K_{2E}}{h^+}}{\frac{h^+}{K_{1ES}} + 1 + \frac{K_{2ES}}{h^+}} + s} \tag{3.96}$$

The possible existence of multiple ionic forms for the substrate has not been considered, but can also be included (Mesentsev et al. 1997; Pickering et al. 1999). When this is the case, usually only one of those forms is catalytically active as it occurs, for instance, in the synthesis of β-lactam antibiotics with penicillin acylase, where only the non-ionized form of the nucleophile interacts with the enzyme (Ferreira et al. 2004; Guranda et al. 2004). If binding of the substrate does not affect the ionic equilibria among enzyme species, then pH has no effect on the affinity parameter and $K_{AP} = K$, as clearly seen from Eq. 3.96. The effect of pH on K_{AP} is usually mild or even negligible (Cornish-Bowden 1976) and, in any case, less important than the effect on V_{AP}. Optimum pH (pH^*), regarded as the one that maximizes V_{AP}, can be determined from Eq. 3.93:

$$\frac{dV_{AP}}{dh^+} = V\left(1 + \frac{h^+}{K_{1es}} + \frac{K_{2ES}}{h^+}\right)^{-2}\left(\frac{1}{K_{1es}} - \frac{K_{2ES}}{h^{+2}}\right) \tag{3.97}$$

$$\begin{aligned} h^{+*} &= \sqrt{K_{1ES} \cdot K_{2ES}} \\ pH^* &= \frac{K_{1ES} + K_{2ES}}{2} \end{aligned} \tag{3.98}$$

Ionic equilibrium constants for the free enzyme (E) and the enzyme–substrate complex (ES) have to be determined to quantify the effect of pH on reaction rate (Eq. 3.96). Experimental design is simple since it consists on a matrix in which initial rate data are collected at varying s and pH as shown in Table 3.7.

Ionic equilibrium constants for the ES complex will be determined from the V_{AP} values, while those for E will be determined from both V_{AP} and K_{AP} values.

From Eq. 3.93:

$$\log V_{AP} = \log V - \log\left(\frac{h^+}{K_{1ES}} + 1 + \frac{K_{2ES}}{h^+}\right) \tag{3.99}$$

Table 3.7 Experimental Design for the Determination of pH-Explicit Functions of Kinetic Parameters

	pH_1	pH_2	pH_3	$\cdots$	pH_m
s_1	v_{12}	v_{12}	v_{13}	$\cdots$	v_{1m}
s_2	v_{21}	v_{22}	v_{23}	$\cdots$	v_{2m}
s_3	v_{31}	v_{32}	v_{33}	$\cdots$	v_{3m}
$\vdots$	$\vdots$	$\vdots$	$\vdots$	$\vdots$	$\vdots$
s_n	v_{n1}	v_{n2}	v_{n3}	$\cdots$	v_{nm}
	V_{AP1}	V_{AP2}	V_{AP3}	$\cdots$	V_{APm}
	K_{AP1}	K_{AP1}	K_{AP3}	$\cdots$	K_{APm}
	Δ_{AP1}	Δ_{AP2}	Δ_{AP3}	$\cdots$	Δ_{APm}

If: $h^+ \rangle\rangle\rangle K_{1ES} \rangle K_{2ES}$, from Eq. 3.99:

$$\log V_{AP} = \log V + pH - pK_{1ES} \tag{3.100}$$

If: $K_{1ES} \rangle K_{2ES} \rangle\rangle\rangle h^+$, from Eq. 3.99:

$$\log V_{AP} = \log V - pH + pK_{2ES} \tag{3.101}$$

If: $K_{1ES} \rangle h^+ \rangle\rangle\rangle K_{2ES}$, from Eq. 3.99:

$$\log V_{AP} = \log V \tag{3.102}$$

When plotting log V_{AP} versus pH, from the data in Table 3.7, a curve like the one in Fig. 3.11 is obtained (as represented by Eq. 3.99). Straight lines (1), (2) and (3) represent Eqs. 3.100, 3.101 and 3.102 respectively and, as easily determined, the intersection of lines 1 and 3 (Eq. 3.100 = Eq. 3.102) yields the value of pK_{1ES} and the intersection of lines 2 and 3 (Eq. 3.101 = Eq. 3.102) yields the value of pK_{2ES}. K_{1ES} and K_{2ES} are then easily calculated by tracing the slopes of the straight lines at the extreme values of pH and the tangent line to the maximum V_{AP}, as shown in Fig. 3.11.

Ionic equilibrium constants for the free enzyme (E) can be determined from the slopes ($\Delta_{AP} = K_{AP}/V_{AP}$) in Table 3.7.

From Eqs. 3.93 and 3.95:

$$\Delta_{AP} = \frac{K_{AP}}{V_{AP}} = \frac{K}{V}\left(\frac{h^+}{K_{1E}} + 1 + \frac{K_{2E}}{h^+}\right) = \Delta\left(\frac{h^+}{K_{1E}} + 1 + \frac{K_{2E}}{h^+}\right) \tag{3.103}$$

$$\log \Delta_{AP} = \log \Delta + \left(\frac{h^+}{K_{1E}} + 1 + \frac{K_{2E}}{h^+}\right) \tag{3.104}$$

If: $h^+ \rangle\rangle\rangle K_{1E} \rangle K_{2E}$, from Eq. 3.104:

$$\log \Delta_{AP} = \log \Delta - pH + pK_{1E} \tag{3.105}$$

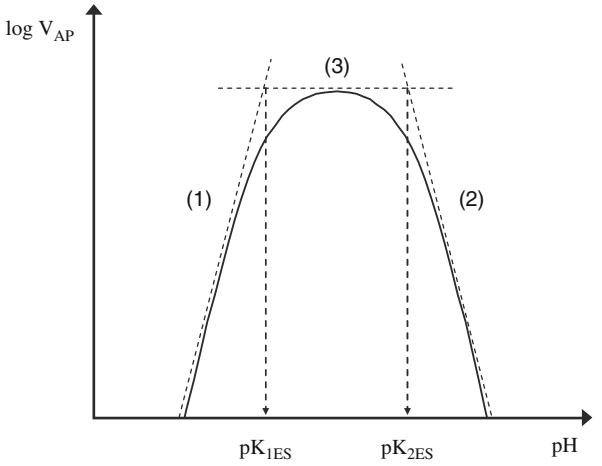


Fig. 3.11 Experimental determination of ionic equilibrium constants for the ES complex, according to the hypothesis of Michaelis and Davidsohn

If: $K_{1E}\rangle K_{2E}\rangle\rangle h^+$, from Eq. 3.99:

$$\log \Delta_{AP} = \log\Delta + pH - pK_{2E} \tag{3.106}$$

If: $K_{1E}\rangle h^+\rangle\rangle K_{2E}$, from Eq. 3.99:

$$\log \Delta_{AP} = \log\Delta \tag{3.107}$$

When plotting log Δ_{AP} versus pH, from the data in Table 3.7, a curve like the one in Fig. 3.12 is obtained (as represented by Eq. 3.104). Straight lines 1, 2 and 3 represent Eqs. 3.105, 3.106 and 3.107 respectively and, as easily determined, the intersection of lines 1 and 3 (Eq. 3.105 = Eq. 3.107) yields the value of pK_{1E} and the intersection of lines 2 and 3 (Eq. 3.106 = Eq. 3.107) yields the value of pK_{2E}. K_{1E} and K_{2E} are then easily calculated by tracing the slopes of the straight lines at the extreme values of pH and the tangent line to the minimum Δ_{AP}, as shown in Fig. 3.12.

Experimental error will be reduced if several data points are collected at high and low pH values, which can in occasions be troublesome because of enzyme instability. Values of pH two to three units apart from the isoelectric point can produce reversible inactivation so that, sometimes, optimum pH with respect to activity may be outside of the zone of maximum stability (Ladero et al. 2005); however, as said before, the opposite trend seems to be more frequent. Determination of optimal pH is not always so straightforward, since substrate and product solubility and susceptibility to contamination are also pH dependent variables that in some cases might be of relevance.

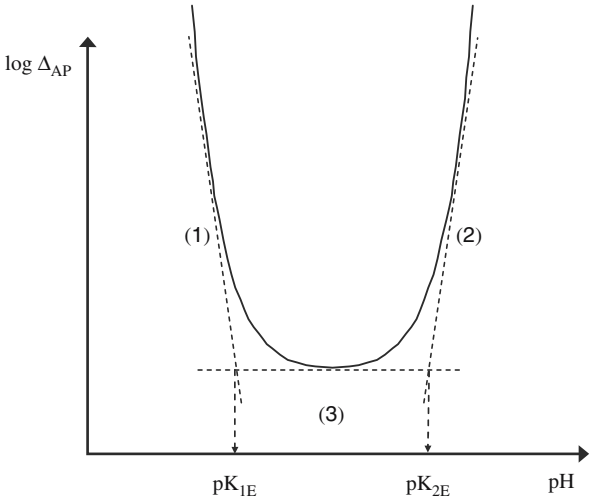


Fig. 3.12 Experimental determination of ionic equilibrium constants for the free enzyme, according to the hypothesis of Michaelis and Davidsohn

3.5.2 Effect of Temperature: Effect on Enzyme Affinity, Reactivity and Stability

Temperature is the most relevant variable in any biological system. This is particularly so for the case of enzymatic processes. Enzymes are complex labile proteins and biocatalysis refers to its use under non-natural conditions, where its native properties can be significantly altered. It is to be expected then that temperature will have a profound impact not only in enzyme activity but in enzyme stability as well. Distinctly from pH, temperature exerts opposite effects on enzyme activity and stability. An increase in temperature increases the rate of the chemical reaction being catalyzed (rates of chemical reactions are highly dependent on temperature, though not altering the order of reaction; this has been very well established and can be analyzed in depth in any textbook of thermodynamics or chemical kinetics) while it also increases the rate at which the enzyme is inactivated, creating a compromise which is schematically represented in Fig. 3.13. Under moderate temperatures (usually up to 30 °C) and for short periods of time, inactivation rate is insignificant and initial rates increase with temperature as in any chemical reaction. At higher temperatures the concentration of active enzyme decreases during the course of reaction and inactivation rates become preponderant. At sufficiently high temperatures, higher than 60 to 70 °C for mesophilic enzymes (Beadle et al. 1999) inactivation is so fast that initial rates of reaction stand only for a very short period of time making it very hard to quantify them; at the extreme, the initial rate of reaction does not last for long enough time to measure it and is considered to be zero. A very relevant consequence of that compromise is that temperature optimum for any enzyme reaction, being of the utmost relevance, is not easy to determine, as will be shown in

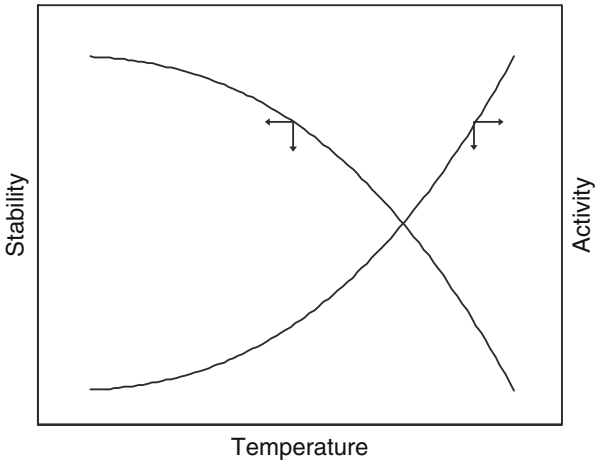


Fig. 3.13 Schematic representation of the effect of temperature on enzyme activity and stability

Chapter 5. As suggested by Fig. 3.13, optimum temperature is time-dependent since enzyme activity is essentially independent of time (it is formally an initial rate of reaction), while stability is clearly time-depending. Therefore, temperature optimum will decrease with increasing contacting time between the enzyme and the reaction medium.

3.5.2.1 Effect of Temperature on Enzyme Affinity Parameters

If in the simple kinetic scheme:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P_i$$

the first reaction, according to the hypothesis of Michaelis–Menten, is considered in equilibrium, the corresponding equilibrium thermodynamics correlations are applicable. According to the Gibss–Helmholtz correlation:

$$\Delta G^0 = \Delta H^0 - T \cdot \Delta S^0 = R \cdot T \cdot \ln K \tag{3.108}$$

Note that, according to the accepted convention for enzyme kinetics, K is the dissociation of the ES complex back into E and S, so that the minus sign in the right-hand side of the equation disappears since K is the inverse of the equilibrium constant considered in the thermodynamic correlation. From Eq. 3.108:

$$\ln K = \frac{\Delta H^0}{R \cdot T} - \frac{\Delta S^0}{R} \tag{3.109}$$

$$K = K_0 \cdot \exp\left(\frac{\Delta H^0}{R \cdot T}\right) \tag{3.110}$$

where: $K_0 = \exp\left(-\Delta S^0/R\right)$.

If the specific heat capacity of reactants and products is considered equal (which is acceptable for most enzyme-catalyzed reactions), ΔH^0 and ΔS^0 will be independent on temperature so that derivating Eq. 3.109:

$$\frac{d(\ln K)}{d(T^{-1})} = \frac{\Delta H^0}{R} \tag{3.111}$$

ΔH^0 is positive in the case of endothermic reactions and negative in the case of exothermic reactions. Considering that most enzymatic reactions occur in liquid media, ΔH^0 can be considered equal to the standard internal energy change (ΔE^0).

A similar analysis can be made for any other equilibrium reaction within a catalytic scheme, so that the effect of temperature on any inhibition constant or dissociation constants for multiple substrate reactions (see sections 3.3 and 3.4) can be determined accordingly. For any inhibition constant K_I:

$$\ln K_I = \frac{\Delta H_I^0}{R \cdot T} - \frac{\Delta S_I^0}{R} \tag{3.112}$$

$$K_I = K_{0,I} \cdot \exp\left(\frac{\Delta H_I^0}{R \cdot T}\right) \tag{3.113}$$

where: $K_{0,I} = \exp\left(-\Delta S_I^0/R\right)$.

3.5.2.2 Effect of Temperature on Enzyme Reactivity Parameters

The effect of temperature on enzyme reactivity (expressed by the rate constant k_{cat} or the parameter V) can be analyzed from the theory of the activated complex (or transition state theory, TST) or else by using the semi-empirical correlation of Arrhenius. According to TST (Rooney 1995), the equation of Eyring describes the effect of temperature on any rate constant:

$$k_{cat} = \kappa \frac{k_B \cdot T}{k_P} \exp\left(\frac{-\Delta G^{\ddagger}}{R \cdot T}\right) \tag{3.114}$$

Eyring's equation assumes that a thermodynamic equilibrium exists between the transition state and the state of the reactants and that the reaction rate is proportional to the concentration of particles at the high-energy transition state. κ accounts for the fraction of molecules going into product state and $\Delta G^{\ddagger}$ represents the difference between Gibbs energy of transition state and reactants. If $\Delta G^{\ddagger}$ is expressed in terms of enthalpy $(\Delta H^{\ddagger})$ and entropy $(\Delta S^{\ddagger})$ of activation, from Eq. 3.114:

$$k_{cat} = \kappa \frac{k_B \cdot T}{k_P} \exp\left(\frac{-\Delta H^{\ddagger}}{R \cdot T}\right) \cdot \exp\left(\frac{\Delta S^{\ddagger}}{R}\right) \tag{3.115}$$

Eq. 3.115 can be compared with the semi-empirical equation of Arrhenius, which is a powerful correlation to describe the effect of temperature on any chemical reaction

rate constant (Connors 1990):

$$k_{cat} = A \cdot \exp\left(\frac{-E_a}{R \cdot T}\right) \tag{3.116}$$

where A is a term usually designated as *frequency factor* or *pre-exponential factor* that, for most cases, can be considered properly as a constant since it is a very weak function of temperature. E_a is termed *energy of activation* and it refers to the minimum magnitude of energy required for the reaction to proceed. For most enzyme-catalyzed reaction, its magnitude ranges from 2 to 40 Kcal mol^{-1}.

The analogy between Eqs. 3.115 and 3.116 is beyond formal, since in liquid media, activation enthalpy is similar to the energy of activation, so that A would be equivalent to:

$$A = \kappa \frac{k_B \cdot T}{k_P} \cdot \exp\left(\frac{\Delta S^{\ddagger}}{R}\right) \tag{3.117}$$

The catalytic constant for the enzymatic reaction:

$$ES \xrightarrow{k_{cat}} E + P_i$$

can be expressed according to Eq. 3.116 as:

$$k_{cat} = k_{cat,0} \cdot \exp\left(\frac{-E_a}{R \cdot T}\right) \tag{3.118}$$

Derivating Eq. 3.118:

$$\frac{d(\ln k_{cat})}{d(T^{-1})} = \frac{-E_a}{R} \tag{3.119}$$

where E_a represents the energy of activation of the reaction of product formation from the enzyme–substrate complex and $k_{cat,0}$ is the pre-exponential term.

Based on the above equations, it is possible to derive temperature-explicit expressions for the kinetic parameters. In fact, Michaelis–Menten equation can be expressed as in Eq. 3.45, where V_{AP} and K_{AP} are in this case functions of temperature.

Experimental design is simple since it consists of a matrix in which initial rate data are collected at varying s and temperatures as shown in Table 3.8.

The effect of temperature on K_{AP} can be evaluated from the data in Table 3.8 and using Eq. 3.109, rewritten as:

$$\ln K_{AP} = \ln K_{AP,0} + \frac{\Delta H^0}{R \cdot T} \tag{3.120}$$

$$K_{AP} = K_{AP,0} \cdot \exp\left(\frac{\Delta H^0}{R \cdot T}\right) \tag{3.121}$$

as shown in Fig. 3.14 for an endothermic reaction.

Table 3.8 Experimental Design for the Determination of Temperature-Explicit Functions of Kinetic Parameters

	T_1	T_2	T_3	$\cdots$	T_m
s_1	v_{12}	v_{12}	v_{13}	$\cdots$	v_{1m}
s_2	v_{21}	v_{22}	v_{23}	$\cdots$	v_{2m}
s_3	v_{31}	v_{32}	v_{33}	$\cdots$	v_{3m}
$\vdots$	$\vdots$	$\vdots$	$\vdots$	$\vdots$	$\vdots$
s_n	v_{n1}	v_{n2}	v_{n3}	$\cdots$	v_{nm}
	V_{AP1}	V_{AP2}	V_{AP3}	$\cdots$	V_{APm}
	K_{AP1}	K_{AP1}	K_{AP3}	$\cdots$	K_{APm}
	Δ_{AP1}	Δ_{AP2}	Δ_{AP3}	$\cdots$	Δ_{APm}

The effect of temperature on k_{cat} can be evaluated from Eq. 3.118 which can be rewritten as:

$$V_{AP} = V_{AP,0} \cdot \exp\left(\frac{-E_a}{R \cdot T}\right) \tag{3.122}$$

since $V_{AP} = k_{cat} \cdot e$ and e can be considered a constant as long as no inactivation occurs, which is so because initial rate values are being used. Taking the logarithm of Eq. 3.122:

$$\ln V_{AP} = \ln V_{AP,0} - \frac{E_a}{R \cdot T} \tag{3.123}$$

The effect of temperature on V_{AP} can be determined as shown in Fig. 3.15.

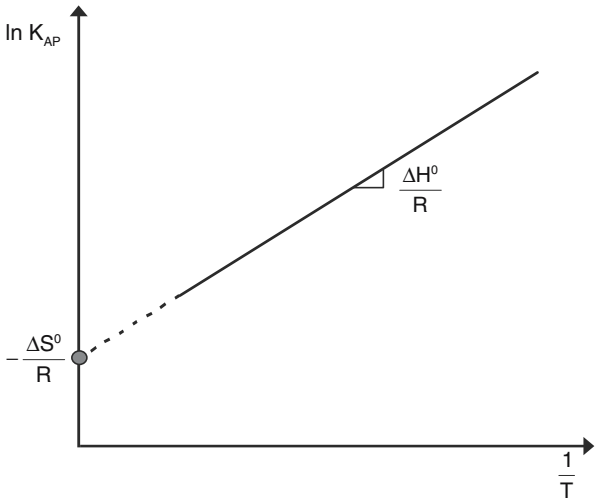


Fig. 3.14 Temperature dependence on Michaelis–Menten constant

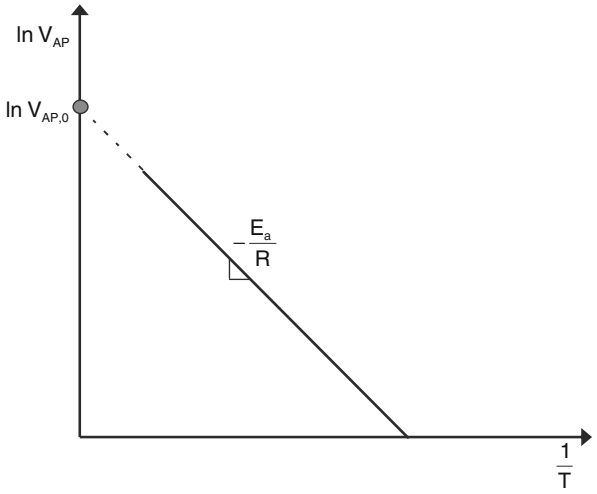


Fig. 3.15 Temperature dependence of maximum reaction rate

In this way, a temperature-explicit rate expression can be obtained as:

$$v = \frac{V_{AP} \cdot s}{K_{AP} + s} = \frac{V_{AP,0} \cdot \exp\left(\frac{-E_a}{R \cdot T}\right) \cdot s}{K_{AP,0} \cdot \exp\left(\frac{\Delta H^0}{R \cdot T}\right) + s} \tag{3.124}$$

We have obtained excellent correlations to describe temperature dependence of immobilized β-galactosidase by using Eqs. 3.121 and 3.122 (Illanes et al. 2000).

3.5.2.3 Effect of Temperature on Enzyme Inactivation Parameters

As said above, temperature exerts opposite effects on enzyme activity and enzyme stability (see Fig. 3.13) so temperature-explicit functions for inactivation parameters are required to determine the best temperature for a given process.

Enzyme thermal inactivation of enzymes is the consequence of the weakening of the intermolecular forces responsible of the preservation of its three-dimensional structure leading to a reduction in its catalytic capacity (Misset 1993). Inactivation may involve covalent or non-covalent bond disruption with subsequent molecular aggregation or improper folding (Bommarius and Broering 2005). Knowledge on enzyme inactivation kinetics under process conditions is an absolute requirement to properly evaluate enzyme performance. Enzyme inactivation can be assessed by measuring the variation of enzyme activity through time and then mathematically modeled, hopefully based on sound mechanisms of inactivation. It is wise to use the simplest model that adequately fits the experimental data so as to deal with a reasonable number of inactivation parameters.

Enzyme inactivation has been frequently described by a very simple one-stage mechanism in which it is assumed that the enzyme suffers a highly cooperative conformational transition from a native active structure to an unfolded completely inactive form (Henley and Sadana 1985). This transition is represented by a hypothetical chemical reaction:

$$E_{native} \xrightarrow{k_D} E_{unfolded}$$

whose rate constant is k_D, usually denoted as first-order inactivation rate constant. This mechanism leads to a first-order kinetic model that can be represented as:

$$-\frac{de}{dt} = k_D \cdot e \tag{3.125}$$

which by integration gives an exponential decay profile for enzyme activity with time:

$$\ln \frac{e}{e_0} = -k_D \cdot t \tag{3.126}$$

$$\frac{e}{e_0} = \exp(-k_D \cdot t) \tag{3.127}$$

Eq. 3.126 predicts a linear correlation between residual enzyme activity and time in a semilog plot. In this way, experimental data can be correlated using only one inactivation parameter, namely the first rate inactivation constant k_D. In several occasions, this simple model has been validated both for soluble and immobilized enzymes (Laidler and Bunting 1973; Ertan et al. 1997; Ortega et al. 1998; Burdette et al. 2000; O'Fágáin 2003). However, this mechanism is clearly an oversimplification and it is quite frequent to observe inactivation patterns that markedly depart from one-step first-order mechanism (Illanes et al. 1996; Schokker and van Boekel 1999; Yang et al. 1999). The modeling of enzyme inactivation kinetics was not studied systematically until the mid-eighties, where a series of works end up with the formulation of a so-called *deactivation theory* (Henley and Sadana 1985, 1986), based on a matrix of series and parallel inactivation reactions. The subject will be thoroughly reviewed in section 5.4. If simple first-order kinetics is assumed, enzyme inactivation can be entirely described by the parameter k_D. Being considered a chemical rate constant, its dependence on temperature can be adequately described by an Arrhenius-type equation:

$$k_D = k_{D,0} \cdot \exp\left(\frac{-E_{ia}}{R \cdot T}\right) \tag{3.128}$$

$$\ln k_D = \ln k_{D,0} - \frac{E_{ia}}{R \cdot T} \tag{3.129}$$

Ei_a is the energy of activation of the process of enzyme inactivation and its magnitude reflects the minimum energy required for enzyme inactivation to proceed. For most enzyme-catalyzed reactions, its magnitude ranges from 20 to 200 Kcal mol^{-1}. This means that more energy is required for promoting enzyme inactivation than for

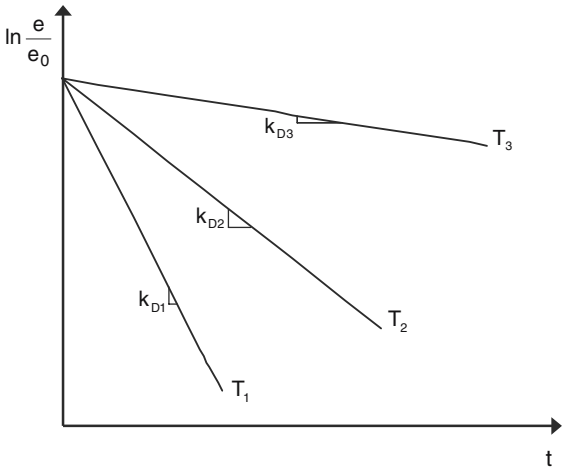


Fig. 3.16 Enzyme inactivation profiles at different temperatures according to simple first-order kinetics (T1 > T2 > T3)

product formation from the enzyme–substrate complex, so both opposing phenomena respond differently to temperature change.

Experimental design for the determination of inactivation parameters is in principle quite straightforward since it consists in sampling at different times at the selected conditions and collecting data of residual activity versus time. Figure 3.16 represents enzyme inactivation profiles at different temperatures assuming simple first-order kinetics (Eq. 3.126). The inactivation parameter k_D is then simply determined from the slopes of such curves, so that a set of data of k_D versus temperature is generated. From that data and Eq. 3.129, E_{ia} can be determined as shown in Fig. 3.17. We have obtained very good correlations to describe temperature

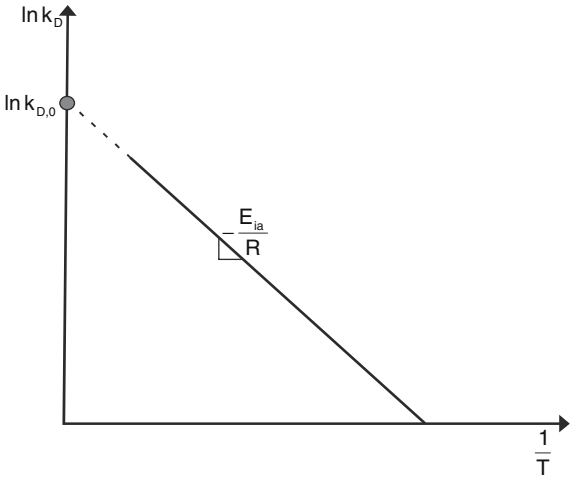


Fig. 3.17 Temperature dependence of inactivation rate constant

dependence of inactivation rate constants for immobilized β-galactosidase by using Eq. 3.128 (Illanes et al. 2000).

For the determination of inactivation parameters, several aspects deserve consideration. Experiments should be conducted at controlled values of all relevant variables (i.e. pH, temperature, ionic strength and so forth). Enzyme concentration is a potential variable, since it is frequent that enzymes exhibit different stability depending on the protein concentration, especially in the case of enzymes dissolved in the medium (Hodgson and Fridovich 1975). If this is so, caution should be taken to conduct the experiment at a concentration simulating that at which the enzyme will be used.

For the sake of simplicity, inactivation experiments are usually conducted under no reactive conditions, that is, in the absence of substrates and products of reaction (Ortega et al. 1998). This information, though useful, does not necessarily represent the actual behavior of the biocatalyst under operating conditions. In fact, it is a well-reported fact that enzyme stability is different under reactive conditions (Villaume et al. 1990; Ospina et al. 1992), suggesting that reactants and products affect enzyme stability. Substrate protection of enzyme stability was originally described by O'Neill (1972) proposing a very simple model according to which, inactivation rate constant was inversely proportional to the substrate concentration. The study on the effect of substrate protection was later on refined by Chen and Wu (1987) and then generalized in terms of modulation effects by Illanes et al. (1994), proposing that any substance that interacts with the enzyme during catalysis (i.e. substrate, product, inhibitor, analogue) is a potential modulator of enzyme stability, be it positive (protection) or negative (destabilization). This hypothesis has been sustained by experimental evidence (Illanes et al. 1996, 1998). It is indeed quite relevant to consider these modulation effects on enzyme inactivation when using inactivation parameters to design enzyme reactors or to assess enzyme reactor performance. This important aspect will be analyzed in depth in section 5.4.

3.5.3 Effect of Ionic Strength

Temperature and pH are the most relevant environmental variables affecting enzyme behavior. However, there are other variables, like ionic strength, that may also have a significant effect on enzyme kinetics. Ionic strength is defined as:

$$\mu = \frac{1}{2}\sum c_i \cdot z_i^2 \tag{3.130}$$

The effect of ionic strength in enzyme kinetics is seldom made explicit though special precautions are usually taken to obtain kinetic parameters under constant ionic strength. In fact, ionic strength has been considered as a neglected variable in enzyme kinetics, but it has been proven that it affect enzyme behavior at values over 100 mM (Dale and White 1982). Enzymes are polyionic polymers and may have charged amino acid residues at the active site; moreover, substrates may also contain

charged groups, so that the concentration of ions in the surrounding medium may certainly affect enzyme kinetics.

Starting from the Debye–Huckel theory, it is possible to formulate equations for the kinetic parameters of the enzyme considering that both the substrate and the enzyme at its active site are charged (Buchholz et al. 2005):

$$\ln K = \ln K^0 - z_S \cdot z_E \frac{\sqrt{\mu}}{1+\sqrt{\mu}} \tag{3.131}$$

$$\ln k_{cat} = \ln k_{cat}^0 + z_S \cdot z_E \frac{\sqrt{\mu}}{1+\sqrt{\mu}} \tag{3.132}$$

We have evaluated the effect of ionic strength on the kinetic parameters of lactose hydrolysis with β-galactosidase from *Aspergillus oryzae* in McIlvaine citrate-phosphate buffer at concentrations from 50 to 400 mM and found an increase in K but almost no effect on V (k_{cat}). Studies on the effect of ionic strength on enzyme kinetics are not usual, so that works with acetylcholinesterase (Nolte et al. 1980), myosin kinase (Blumenthal and Stull 1982) and cytochrome C (Hazzard et al. 1991; Harris et al. 1994) are worth mentioning. Combined effect of pH and ionic strength on enzyme kinetics has been recently analyzed (Alberty 2006).

Nomenclature

A	Pre-exponential factor in Arrhenius equation (frequency factor)	
a	molar concentration of substrate A	$[ML^{-3}]$
b	molar concentration of substrate B	$[ML^{-3}]$
c	molar concentration of enzyme complex ES alternatively: molar concentration of enzyme complex E′Y in ping-pong mechanism	$[ML^{-3}]$
c_i	ion concentration	$[ML^{-3}]$
d	molar concentration of enzyme complex SE	$[ML^{-3}]$
	alternatively: molar concentration of enzyme complex EB or enzyme complex EZ in ping-pong mechanism	$[ML^{-3}]$
E_a	energy of activation in Arrhenius equation	
e	active enzyme concentration	$[UL^{-3}]$
e_0	active enzyme initial concentration	$[UL^{-3}]$
f	molar concentration of enzyme complex SES	$[ML^{-3}]$
	alternatively: molar concentration of enzyme complex EAB	$[ML^{-3}]$
ΔG^0	standard free energy change of dissociation of ES into E and S	
$\Delta G^{\ddagger}$	activation Gibbs energy	

g	molar concentration of enzyme complex EP_1	$[ML^{-3}]$
ΔH^0	standard enthalpy change of dissociation of ES into E and S	
$\Delta H^0{}_I$	standard enthalpy change of dissociation of EI (or EIS) into E (or ES) and I	
$\Delta H^{\ddagger}$	activation enthalpy	
h	molar concentration of enzyme complex EP_2	$[ML^{-3}]$
h^+	molar concentration of protons	$[ML^{-3}]$
i	molar concentration of enzyme complex EP_1P_2	$[ML^{-3}]$
	alternatively: molar concentration of inhibitor I	$[ML^{-3}]$
j	molar concentration of enzyme complex EP_2S	$[ML^{-3}]$
K	Michaelis–Menten (dissociation) constant for the ES complex into E and S	$[ML^{-3}]$
K^0	Michaelis–Menten (dissociation) constant at infinite dilution	$[ML^{-3}]$
K_P	Michaelis–Menten (dissociation) constant for the ES complex into E and P	$[ML^{-3}]$
K_{eq}	equilibrium constant of dissociation of ES into E and S	$[ML^{-3}]$
K_{EQ}	global equilibrium constant of reversible reaction	
K_{AP}	apparent Michaelis–Menten (dissociation) constant	$[ML^{-3}]$
K_i	equilibrium constants of dissociation of enzyme complexes according to reaction scheme	$[ML^{-3}]$
K_A	dissociation constant for the EA complex into E and A	$[ML^{-3}]$
K_B	dissociation constant for the EB complex into E and B	$[ML^{-3}]$
K'_A	dissociation constant for the EAB complex into EB and A	$[ML^{-3}]$
K'_B	dissociation constant for the EAB complex into EA and B	$[ML^{-3}]$
K_P	dissociation constant of EP into E and P	$[ML^{-3}]$
k	rate constant	
k_B	Boltzmann universal constant	$[FL\Theta^{-1}]$
k_D	first-order inactivation rate constant	$[T^1]$
k_P	Planck universal constant	[FLT]
k_{cat}	catalytic rate constant	
$k_{cat}{}^0$	catalytic rate constant at infinite dilution	
k_i	reaction rate constants according to reaction scheme	
p	molar concentration of product	$[ML^{-3}]$
p_1	molar concentration of competitive inhibitor product	$[ML^{-3}]$
p_2	molar concentration of non-competitive inhibitor product	$[ML^{-3}]$
R	universal gas constant	$[FLM^{-1}\Theta^{-1}]$
ΔS^0	standard entropy of dissociation of ES into E and S	
$\Delta S^0{}_I$	standard entropy of dissociation of EI (or EIS) into E (or ES) and I	
$\Delta S^{\ddagger}$	activation entropy	

s	molar concentration of substrate S	$[ML^{-3}]$
s_i	initial molar concentration of substrate S	$[ML^{-3}]$
T	absolute temperature	$[\Theta]$
t	time	$[T]$
V	maximum reaction rate of conversion of S into P	$[ML^{-3}T^{-1}]$
V'	maximum reaction rate of conversion of EIS into EI and P	$[ML^{-3}T^{-1}]$
V_P	maximum reaction rate of conversion of P into S	$[ML^{-3}T^{-1}]$
V_{AP}	apparent maximum reaction rate	$[ML^{-3}T^{-1}]$
v	reaction rate	$[ML^{-3}T^{-1}]$
z_E	enzyme charge at its active site	
z_S	substrate charge	
z_i	ion charge	
v	reaction rate	$[ML^{-3}T^{-1}]$
Δ	K/V	$[T]$
Δ_{AP}	K_{AP}/V_{AP}	$[T]$
κ	transition constant in Eq. 3.114	
μ	ionic strength	$[ML^{-3}]$

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Chapter 4
Heterogeneous Enzyme Kinetics

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4.1 Enzyme Immobilization

Because of their excellent functional properties (activity, selectivity, specificity), enzymes have a great potential as industrial catalysts in a number of areas of chemical industry: fine chemistry, food chemistry, analysis and so on (Koeller and Wong 2001). However, the enzymes have been modified during evolution to optimize their behavior in the framework of complex catalytic chains inside the living cells under stress and subjected to regulation. Obviously, enzymes have not been optimized by evolution in order to work as catalysts in industrial reactors so that some of their properties are not well suited for that purpose: they are water soluble, unstable at conditions different from physiological, frequently inhibited by substrates and products of reaction and have rather narrow substrate specificity. In most cases, enzymes have to be greatly improved for their application as industrial catalysts. The engineering of enzymes for such purpose is one of the most exciting, complex and interdisciplinary goals of biotechnology, considering different techniques like: a) the screening, inside the biodiversity, of enzymes with improved properties; b) the improvement of enzyme properties via techniques of molecular biology; c) the improvement of enzyme properties via immobilization and post-immobilization techniques; d) the improvement of enzyme properties via reaction and reactor engineering. These techniques complement each other to succeed in improving enzyme properties for delivering catalysts for a much more sustainable chemical industry, where very complex and useful compounds are synthesized under very mild and cost-effective conditions.

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For technical and economical reasons, most chemical processes catalyzed by enzymes require the re-use or the continuous use of the biocatalyst for a very long time (Chibata et al. 1986; Bickerstaff 1997; Katchalski-Katzir and Kraemer 2000). In this context, immobilization of enzymes may be defined as any technique that allows the re-use or continuous use of the biocatalyst. Immobilization of enzymes is maybe the most relevant approach for stabilization and recovery of enzymes, as already highlighted in Chapter 1. From an industrial perspective, simplicity and cost-effectiveness are key properties of immobilization techniques, but a long term industrial re-use of immobilized enzymes also requires the preparation of very stable derivatives having the right functional properties (activity, selectivity ...) for a given reaction (Gianfreda and Scarfi 1991; Bornscheuer 2003; Cao 2005a). At first glance, the practical development of protocols for immobilization of enzymes is tightly related to simplicity, cost-effectiveness and stabilization. Enzyme immobilization has profound influence on the nature of the catalytic process which is now heterogeneous in nature, since catalysis occurs in the surface or within a solid structure where the enzyme is located while substrates and products of reaction are in the liquid reaction medium where its course is being monitored. Enzyme immobilization will be reviewed in section 4.1 as the necessary framework to treat heterogeneous enzyme catalysis in sections 4.2 to 4.4.

4.1.1 Methods of Immobilization

There is a large number of methods of immobilization that can be broadly divided into those than involve the interaction of the enzyme with a matrix (usually through a chemical bond) and those in which the enzyme is contained within a restricted space, as shown in Fig. 4.1.

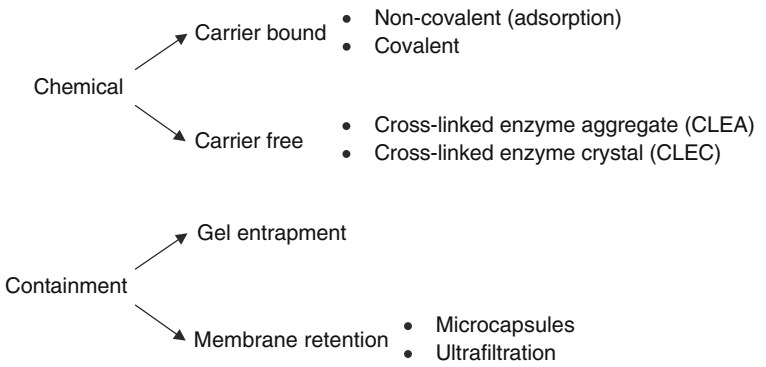


Fig. 4.1 Methods of enzyme immobilization

4.1.1.1 Chemical Bond

Consider those methods that include the chemical binding of the enzyme molecules to an inert carrier (*carrier-bound*), where linkage can be covalent or non-covalent, and those in which the enzyme protein molecules are chemically linked among themselves, usually through a bifunctional reagent, without the participation of an inert carrier (*carrier-free*).

Carrier Bound

Different types of materials have been used as carriers for immobilized enzymes, considering both organic and inorganic compounds. Desirable properties of the carrier are a high surface to volume ratio, high protein binding capacity, compatibility and insolubility in the reaction medium, high mechanical and chemical stability, recoverability after use and conformational flexibility. There is no material that fulfills all these requirements so that in practice all kind of materials have been tested to suit the particular needs of a given process. Many of the materials tested at laboratory scale are, however, not well suited to perform at productive scale, either because of their intrinsic properties or because of their high costs. At the end, availability and cost are key factors in determining the carrier to be used. It must be pointed out that most of the early developments in enzyme immobilization referred to its use in conventional aqueous medium so that a thorough analysis should be made when defining suitable carriers to perform in non-aqueous media (Adlercreutz 2006).

Covalent Immobilization to Solid Supports

A covalent bond is established between the functional groups in the activated carrier and the functional groups in the amino acid residues of the enzyme, like $-OH$, $-SH$, $-NH_2$, and $-COOH$. Covalent immobilization has been thoroughly studied and detailed information on methods and procedures can be found in several publications devoted to it (Zaborsky 1973; Cao 2005b; Guisan 2006). It is a rather complex method where the carrier is hardly recoverable after enzyme exhaustion, immobilization yield is rather low and the kinetic properties of the enzyme can be severely altered. However, operational stability is high and it is quite flexible, so that directed immobilization can be done to suit the particular characteristics of the process.

Among the many systems for covalent enzyme immobilization, multi-point covalent attachment, where the enzyme is linked to the support through several amino acid residues is particularly interesting and has been developed using different solid supports like porous glass, polyacrylamide, cellulose, magnetic particles and so on. Very likely, immobilization on glyoxyl-agarose is the most precise protocol to get very intense enzyme-support multipoint covalent attachments, the enzyme being immobilized through its surface region having the highest density of lysine residues (Pedroche et al. 2007). Multi-point covalent attachment has allowed to significantly

increase the stability of a large number of enzymes like α-chymotrypsin (Guisan et al. 1991), trypsin (Blanco and Guisán 1988; Blanco et al. 1988), carboxypeptidase A (Pedroche et al. 2002), lipases (Otero et al. 1991; Fernández-Lafuente et al. 1998), D-aminoacid oxidases (Fernández-Lafuente et al. 1999), ferredoxin NADP oxidoreductase (Bes et al. 1995), esterases (Fernández-Lafuente et al. 1995), rennin (Penzol et al. 1998) and penicillin G acylase from *Escherichia coli* and *Kluyvera citrophila* (Guisan et al. 1990; Alvaro et al. 1990, 1991). The establishment of a number of attachments between every immobilized enzyme molecule and the support exerts very strong stabilizing effects. When spacer arms (between the enzyme and the support) are very short and the support is very rigid, it can be assumed that all the relative positions among the enzyme residues involved in multipoint immobilization have to remain unmodified during any conformational change induced by any distorting agent (heat, organic cosolvents …). In this way, the intensity of these conformational changes should be strongly diminished. This hypothesis has been raised from the beginnings of enzyme technology to explain the strong stabilizations obtained with conventional immobilization protocols (Martinek et al. 1977; Mozhaev et al. 1990). Despite this, after more than 40 years of research, there are very few immobilization protocols useful to promote very intense enzyme-support multipoint covalent immobilizations. The internal morphology of agarose gels is composed by large fibers with a high geometrical congruence with protein surfaces (Mateo et al. 2006a). At first glance, these gels, when activated with glyoxyl groups (small aliphatic aldehyde groups) are unsuitable for enzyme immobilization. However, at the very end, under tailor-made conditions, they seem to be the most adequate to get dramatic immobilization–stabilization of industrial enzymes. These activated gels are very stable and easy to prepare and are commercially available (Mateo et al. 2005). A scheme is presented in Fig. 4.2. Details on immobilization protocol have been published by Guisan et al. (1997).

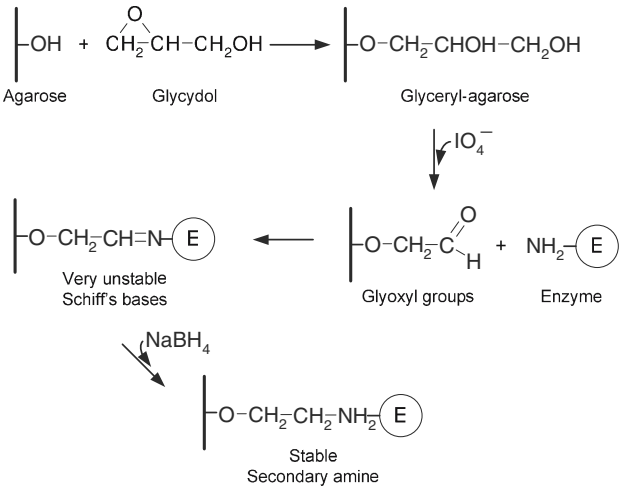


Fig. 4.2 Scheme for enzyme immobilization in glyoxyl-agarose

Table 4.1 Stabilization of Enzymes by Multi-Point Covalent Attachment to Glyoxyl-Agarose

Enzyme	Expressed Activity (%)	Stabilization Factor
Chymotrypsin	70	60,000
Trypsin	75	10,000
Penicillin G acylase (*Escherichia coli*)	70	8000
Penicillin G acylase (*Kluyvera citrophila*)	70	1000
Glutamate racemase	70	1000
Esterase (*Bacillus stearothermophilus*)	70	1000
Lipase (*Candida rugosa*)	70	150
Thermolysin (*Bacillus thermoproteolyticus*)	100	100

The strong stabilization effect promoted by immobilization in glyoxyl agarose can be appreciated in Table 4.1 for different monomeric enzymes with a very small decrease in catalytic activities. Inactivation experiments were made to compare multipoint immobilized derivatives with single point immobilized ones (prepared by using poorly activated supports). In this way, the stabilization factors reported (ratio of half-life of the multi point over the single point immobilization) really represent the 3D stiffening of enzyme structures. Moreover, stiffening of enzymes by a very intense multipoint covalent immobilization should promote stabilization against any distorting agent.

Amino-epoxy supports are other quite interesting matrices for enzyme immobilization by covalent attachment. These derivatives may be easily prepared by reaction of highly activated amino-supports with butanediol diglycidyl ether (BDDGE) and are commercially available from Resindion (Mitsubishi Chem. Co). Ideally, the amino groups on the support should have a pK around 9–10 in order to be easily modified with epoxy reagents and, after modification, to become ionized (as secondary amino groups) at pH 7.0 and 4 °C. In this way a very simple first adsorption of enzymes by anionic exchange may be developed. A three step immobilization protocol on heterofunctional epoxy supports is represented in Fig. 4.3.

Epoxy groups are very poorly reactive towards intermolecular immobilization and hence the first step (at pH 7.0) is always the ionic adsorption of the enzyme through the region having the highest density of negative charges. Then the reaction between the epoxy groups in the support and the amino groups in the enzyme takes place and in the third step (incubation under alkaline conditions) a much more intense multipoint covalent attachment is promoted contributing significantly to enzyme stabilization (Mateo et al. 2007). In addition to commercial supports (i.e. Sepabeads from Resindion), any aminated support may be activated and utilized in a similar way. An additional merit of this immobilization protocol (adsorption plus intramolecular covalent attachment) is the possibility to completely immobilize enzymes that hardly adsorb on the support. The very small percentage of adsorbed molecules becomes also covalently immobilized and hence the equilibrium of adsorption is completely shifted towards the adsorption of enzymes with poor affinity. Epoxy groups in Eupergit C and EP-Sepabeads have been thiolated and the thiol derivative used to immobilize enzymes through their thiol groups via thiol disulfide interchange. For those enzymes lacking cysteine residues, like *E. coli* penicillin

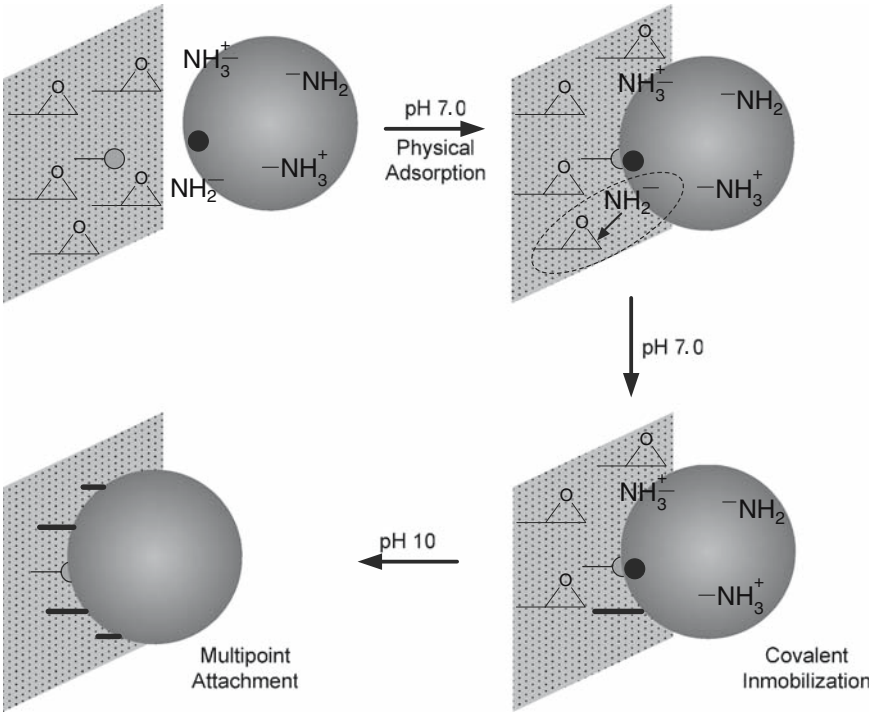


Fig. 4.3 Immobilization of enzymes on heterofunctional epoxy supports

acylase and *Rhizomucor miehei* lipase, they were introduced chemically to promote multipoint covalent attachment to the thiolated support, obtaining dramatic increases in stabilization (Grazú et al. 2005).

Glutaraldehyde is a bifunctional reagent quite useful for developing protocols for covalent immobilization to solid supports (Betancor et al. 2006; Hamerska-Dudra et al. 2006). Enzyme immobilization on amine-activated supports activated with glutaraldehyde is a simple process (Monsan et al. 1975; Alonso et al. 2005; López-Gallego et al. 2005). Amino-supports are chemically very stable and can be stored at 4 °C, for prolonged periods of time and activation is simpler, being glutaraldehyde a non toxic and GRAS reagent frequently used for enzyme immobilization. Nevertheless this method has some drawbacks: multipoint covalent attachment is not very strong nor its chemical stability and the reactivity of the lysine groups cannot be increased further by using more alkaline pH because of the instability of the glutaraldehyde groups.

Non-Covalent Immobilization to Solid Supports

It considers all kind of interactions between the enzyme and the support not involving covalent bonds, including short-range interactions like van der Waals forces, but also stronger ones like hydrophobic interactions and ionic bonds; sometimes they

are referred generically as adsorption. It is a simple method, where the carrier can be easily recovered after enzyme exhaustion by promoting protein desorption, immobilization yields are usually high and no obnoxious reagents are involved. However, its main drawback is that the enzyme can be easily desorbed from its carrier by subtle changes in the reaction medium. This is particularly so in the case of aqueous systems, but it is worthwhile to point out that there is a much lesser tendency to desorption in the case of non-aqueous medium, like organic solvents, where simple immobilization by adsorption can be a good option.

Ionic exchange is a rather simple and effective method for enzyme immobilization since the vast majority of proteins adsorb very fast on anion or cation exchange resins. However, the strength of the interaction between conventional supports (surfaces fairly covered with ionic groups) and proteins is weak: most of the proteins are desorbed at relatively low ionic strengths (200–300 mM) and pH changes may also promote desorption. The use of new solid supports fully covered with ionic polymers like polyethylenimine (PEI) and dextran sulfate (DS), mimicking "ionic flexible and deep beds", has been proposed to increase the strength of the ionic interaction between proteins and supports. These supports have a much higher concentration of ionic groups than conventional ones and their flexible structure allows a better adaptation of the ionic layer to the immobilized protein (Mateo et al. 2000; Torres et al. 2003; Fuentes et al. 2004; Pessela et al. 2005). Many kinds of supports (porous glass, agarose gels, magnetic particles, etc.) covered with such ionic polymers are good candidates to prepare active, stable and selective enzyme biocatalysts that can be used (and re-used) in several types of industrial reactors (see Chapter 5).

Carrier Free

Enzymes can be insolubilized by straight chemical cross-linking of the protein molecules with bifunctional reagents, like glutaraldehyde. Cross-linking can be performed over the soluble enzyme protein (cross-linked enzyme, CLE) (Broun 1976; Tyagi et al. 1999), over a crystallized enzyme protein (cross-linked enzyme crystal: CLEC) (Margolin 1996; Häring and Schreier 1999; Roy and Abraham 2004), or over a protein enzyme aggregate (cross-linked enzyme aggregate: CLEA) (Cao et al. 2000, 2001; López-Serrano et al. 2002; Mateo et al. 2004; Schoevaart et al. 2004; Sheldon et al. 2007). These systems only differ in the protein precursor to be cross-linked and have the obvious advantage that no inert support is involved since the enzyme is auto-immobilized in its own protein mass and, therefore, the specific activity of the biocatalyst is very high, being the enzyme concentration within the biocatalyst close to its theoretical limit of packing (Cao et al. 2003).

CLEs are produced by straight cross-linking of the soluble enzyme. A delicate balance among different factors (amount and type of cross-linking reagent, temperature, pH, ionic strength) is required to control the process. The intermolecular cross-linking of the highly solvated enzyme produces significant losses of activity (immobilization yields are usually below 50%), and results are difficult to reproduce (Cao et al. 2003). Although used for some industrial purposes some decades ago

(Carasik and Carroll 1983), CLEs are no longer used mainly because of their poor mechanical properties.

CLECs are produced by cross-linking of purified enzyme crystals and are endowed with excellent properties: high stability under harsh conditions (high temperatures, extreme pHs, organic solvents), resistance to autolysis (in the case of proteases) and exogenous proteolysis (St Clair and Navia 1992; Vaghjiani et al. 1999) and extremely high volumetric (and specific) activity (Tischer and Kasche 1999; Xu and Klibanov 1996), which is quite relevant for the rather slow reactions of synthesis. They have excellent mechanical properties (Lalonde 1997) and biocatalyst recovery is quite simple (Persichetti et al. 1995). CLECs can be freeze-dried and indefinitely stored at room temperatures (St Clair and Navia 1992; Persichetti et al. 1995). However, a main drawback is the high cost of the biocatalyst that arises from the requirement of a high degree of purity of the enzyme protein to crystallize. So, in practice, despite its excellent properties, very few processes are being conducted with this kind of biocatalysts (Lee et al. 2000). Lowering the costs of production and tailoring existing CLECs to suit particular applications will make them very attractive biocatalysts for organic synthesis (Roy and Abraham 2004).

CLEAs are produced by cross-linking of protein aggregates produced by conventional protein precipitation techniques (see Chapter 2). Stable biocatalysts can be easily prepared in a two-step protocol: firstly, aggregation of soluble enzymes is promoted by the addition of salts (salting-out), organic solvents or polymers under very mild experimental conditions to ensure good retention of enzyme activity; after aggregation strong stirring is promoted to reduce particle size of the enzyme aggregates and, finally, enzyme aggregates are stabilized by cross-linking (usually with glutaraldehyde). A scheme of the preparation of CLEAs is in Fig. 4.4a. This very simple procedure produces insoluble and stable cross-linked enzyme aggregates even after extensive washing to remove the cross-linking reagent. This method was first developed at Delft University by Prof. Sheldon's research group (Cao et al. 2000) and represents a major contribution to biocatalysis because it combines the good properties of non-supported biocatalysts with simplicity and low cost of production since, as opposite to CLECs, CLEAs do not require of a purified enzyme (actually, fractional precipitation is used for enzyme purification of rather crude preparations, as indicated in Chapter 2). This technique has had a great success in producing a number of very active derivatives of industrial enzymes (Cao et al. 2000, 2001; López-Serrano et al. 2002; Mateo et al. 2004, 2006b; Sheldon et al. 2007). Beyond that, several improvements have been introduced. Procedures have been developed for the co-immobilization of different enzymes, producing the so-called combi-CLEAs, either to perform cascade (Sheldon et al. 2007) or non-cascade reactions (Dalal et al. 2006). CLEAs are particularly well suited to develop such multiple-enzyme biocatalysts that can be quite useful for performing complex biotransformations or acting upon complex heterogeneous substrates. Co-immobilization of enzymes and their respective coenzymes is another exciting potential of CLEAs.

Enzymes and highly hydrophilic polymers (i.e. polyethyleneimine and dextran sulfate) can be co-immobilized prior to cross-linking, as shown in Fig. 4.4b creating

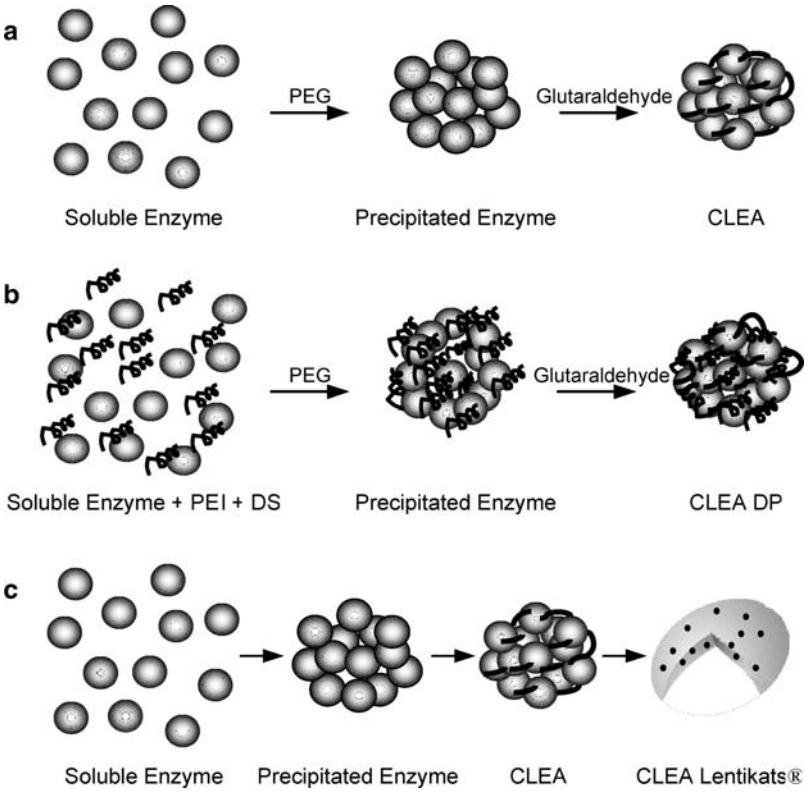


Fig. 4.4 Schematic representation of the preparation of CLEAs: a) using polyethylene glycol (PEG) as precipitating agent and glutaraldehyde as cross-linking agent; b) using highly hydrophilic microenvironment composed by polyethyleneimine (PEI) and dextran sulfate (DS), (PEG) as precipitating agent and glutaraldehyde as cross-linking agent; c) using polyethylene glycol (PEG) as precipitating agent and glutaraldehyde as cross-linking agent and encapsulation into polyvinyl alcohol lens-shaped gel particles (LentiKats)

a highly hydrophilic microenvironment that significantly improves their stability against organic solvents (Abian et al. 2001, 2002). When using high concentrations of organic cosolvents a partition effect of the cosolvent between the bulk solution and the enzyme microenvironment is produced so that the enzyme molecules are now in contact with a much lower concentration of organic cosolvent, diminishing its deleterious effect (Wilson et al. 2004a). These CLEas are particularly well suited to perform in such organic media. Polymers, like polyethyleneimine, that contain several primary amino groups contribute to enhance the intensity of chemical cross-linking in those enzymes that are relatively poor in lysine residues (Wilson et al. 2006).

CLEAs of multimeric enzymes are also very good biocatalysts since the stability is greatly improved. When multimeric enzymes are chemically cross-linked every enzyme subunit becomes also cross-linked and hence no subunit dissociation

is possible (Betancor et al. 2003; Wilson et al. 2004b). CLEAs are then much more stable than their soluble counterparts. In addition to that, inactivation of cross-linked aggregates does not depend on derivative dilution. When using non stabilized derivatives the more diluted suspension inactivates much more rapidly because dissociation of subunits is favored.

Mechanical properties and control of particle size are the main drawbacks of CLEAs. Particles are compressible and shear sensitive and size is usually small so that recovery of the biocatalyst may pose a problem for conventional reactor configurations (see Chapter 5). To solve that problem, basket-type bioreactors can be used or else the biocatalyst can be modified. An interesting approach is the encapsulation of CLEAs within polymer gels, as shown in Fig. 4.4c. Entrapment of CLEAs within polyvinyl alcohol lens-shaped gel particles (LentiKats) produced very robust biocatalysts of a convenient size to be easily recovered (Wilson et al. 2004c).

4.1.1.2 Containment

Consider those methods in which the enzyme is confined to a restricted space and includes molecular entrapment within polymeric gels and also retention within semipermeable membranes that allow free passage of substrates and products of reaction while retaining the enzyme.

Entrapment

The enzyme is confined within the inner cavities of a solid polymeric matrix, compact enough to retain the enzyme molecules within it. Immobilization occurs by polymerization of a monomer solution in which the enzyme is dissolved. Polymerization of the monomer can be induced by physical stimulation (i.e. photopolymerization) or chemically; alternatively, the dissolved polymer can be promoted to sol–gel transition by lowering the temperature. Most popular matrices for gel entrapment are alginate (Cheetham et al. 1979), polyacrylamide (Pizarro et al. 1997), polyurethane (Wang and Ruckenstein 1993); polyvinyl alcohol (Wang et al. 1995) and κ-carrageenan (Tosa et al. 1979). Entrapment in polymeric gels has been a powerful tool for cell immobilization (Klibanov 1983; Cantarella et al. 1997; Muscat and Vorlop 1997), but not as much in the case of enzymes because the tendency to leakage can be counterbalanced only by increasing gel strength which in turns magnifies mass transfer limitations. Polyvinyl alcohol (PVA) is a particularly interesting material because it is innocuous and cheap and also mechanically and chemically robust (Lozinsky and Plieva 1998; Durieux et al. 2000). Even though it has been mainly used for cell immobilization (Wang et al. 1995; Wittlich et al. 1999), it has been successfully applied to the immobilization of enzyme–polymer composites (Czichocki et al. 2001; Gröger et al. 2002) and enzyme aggregates as already mentioned (Wilson et al. 2002, 2004a). Immobilization is performed by mixing a solution of

commercial PVA with the enzyme (or composite or aggregate) and then promoting gelification by dripping the mixture over a cooled surface where regular lens-shaped particles are formed of about 5 mg, 3–4 mm in diameter and 0.2–0.4 mm in height. Biocatalysts of this kind are quite robust and easy to recover during reactor operation.

Sol–gel encapsulation of enzymes is a very attractive system of immobilization that consists in the acid or base catalyzed hydrolysis of tetraalkylosilanes, where the silane precursor undergoes hydrolysis and cross-linking condensation to form a silica matrix in which the enzyme is entrapped (Gill and Ballesteros 1996; Reetz 1997, 2006). It has been successfully applied to a number of enzymes, like esterases (Altstein et al. 1998), proteases (van Unen et al. 2001), organophosphorus hydrolase (Lei et al. 2002), alkaline phosphatase (Braun et al. 2007) and lipases (Schuleit and Luisi 2001; Reetz 2006; Meter et al. 2007). However, applications of sol–gel encapsulation have been more related to chemical analysis than bioprocesses and in that field several enzyme electrodes have been developed for the analysis of organic compounds (Przybyt and Białkowska 2002). Through careful selection of sol–gel precursors and additives, these materials are being designed for specific application, and can produce useful, robust enzyme analytical devices. A comprehensive review on the subject has been published recently (Pierre 2004).

Membrane Retention

The enzyme is retained by a semi-permeable membrane that allows free-passage of the substrates and products of reaction. More than one enzyme can be retained so that cascade reactions can be performed. Retention can be attained by microencapsulation and by containment in ultrafiltration membranes

Microencapsulation

Enzyme microcapsules are produced by promoting a polymerization reaction in the surface of drops of enzyme aqueous solution dispersed in a water-immiscible organic solvent with the aid of a surfactant. Reverse micelles and liposomes are forms of microcapsules. The former are those in which the hydrophilic head of the surfactant is oriented to the inner enzyme aqueous drop while its tail is oriented to the outer organic phase. Liposomes are micelles composed by a double layer of surfactant so that the external solvent is the aqueous enzyme phase. Enzyme reverse micelles have interesting properties since the microenvironment is adequate for the enzyme and mass transfer limitations are negligible, so that several enzymes have been microencapsulated by this technique (Fadnavis and Luisi 1989; Serralheiro et al. 1990; Vicente et al. 1994). The main drawback of enzyme reverse micelles is mechanical weakness and the tendency of the enzymes to denature at the water-organic interface. Lipases are particularly well-endowed to perform at interfaces so their immobilization in reverse micelles has been thoroughly studied (Carvalho and Cabral 2000; Zaman et al. 2005). Liposomes have also been used for enzyme

immobilization (Dufour et al. 1996; Li et al. 2007) though its main potential relies in their biomedical applications (Özden and Hasirci 1990; Esquisabel et al. 2006).

Containment by Ultrafiltration Membranes

In these systems the enzyme is confined within a space delimited by an ultrafiltration membrane that allows the free passage of substrates and products while retaining the enzyme. The enzyme may be free in the inner or outer space of the ultrafiltration device or else attached to either side of the membrane. Nowadays, ultrafiltration membranes are having much better performances being more hydrophilic and inert. One of the drawbacks of enzyme containment in ultrafiltration devices is inactivation by the interaction with interfaces (i.e. air bubbles or any other interface in stirred reactors) and by undesirable aggregation (Caussette et al. 1998; Colombie et al. 2001; Bommarius and Karau 2005). A solution for overcoming those problems is the complete cross-linking of the soluble enzymes with aldehyde-dextran polymers (plus borohydride reduction) to promote the formation of new enzyme molecules fully surrounded by hydrophilic and inert polymers, as illustrated by the dramatic stabilization of glucose oxidase (Betancor et al. 2005). Containment by ultrafiltration membranes is particularly relevant for processes involving coenzyme-requiring enzymes, where both the enzyme and the derivatized coenzyme are retained (Kragl et al. 1996; Liu and Wang 2007).

4.1.2 Evaluation of Immobilization

From a bioprocess perspective the main purpose of enzyme immobilization is to increase its stability and allow its prolonged utilization either in continuous or sequential batch processes (see Chapter 5). Immobilization may also be used for other very relevant purposes, like the modification of enzyme kinetic parameters. The controlled and directed interaction between a solid surface and an enzyme (i.e. immobilization of an enzyme through a specific residue, the stiffening of selected areas of the immobilized enzyme, the interaction of the adsorbed enzymes with polymers) are valuable tools for enhancing the activity and the selectivity when the enzyme is to be used on non-natural substrates or in non-conventional media. The case of lipases (see section 6.3) may serve as a good example to illustrate this. Lipases are mainly active when acting on hydrophobic interfaces of their natural substrates (fats). This catalytic behavior, called *interfacial activation*, involves the opening of a polypeptide chain (*lid*) that covers the active site. The closed (inactive) conformation is favored in aqueous medium, while the open conformation is favored by the presence of interfaces. Lipases interact with any hydrophobic surface much like they do on fat surfaces, so that the enzyme attaches to the surface with the active centre lid wide open (Fernández-Lafuente et al. 1998; González-Navarro et al. 2001). In addition to that, this interaction is quite specific: lipases adsorb on hydrophobic surfaces at very low ionic strength and under these conditions most of the proteins

are unable to do it so that a highly selective adsorption of lipase may occur. When using larger or highly hydrophilic substrates, lower hyperactivation is found. This is presumably due to steric hindrances: the active site of the enzyme is so close to the hydrophobic surface that large and hydrophilic substrate molecules have no access to it (Palomo et al. 2004). Immobilization may also alter enzyme enantioselectivity, which is a very relevant aspect in enzymatic organic synthesis (see section 1.6). Using different immobilization strategies for lipases, regions of the enzyme close to the active site were involved, different degrees of stiffening were promoted and also the interaction of the enzyme with positively or negatively charged polymers, producing several lipase derivatives of quite different enantioselectivity when acting on different chiral ester substrates (Palomo et al. 2002; Turner 2003; Chaubey et al. 2006; Torres et al. 2006).

Enzyme immobilization has evolved to progressively more directed protocols to suit particular process requirements. However, novel methods and materials are still needed to achieve a massive implementation of enzymes as catalysts for complex chemical processes, which explains why, after more than 40 years of work, research in this field keeps on very active. A recently published book covers extensively the most relevant methods of immobilization in the form of critical reviews by the most prominent research groups worldwide (Guisan 2006).

Any immobilization protocol will involve an activation step in which the support (and the enzyme) is activated, and a contacting step in which the activated partners interact with each other to deliver the immobilized enzyme. Optimization of the process of immobilization is a complex task, since many variables are involved in both stages and a sound objective function is required. Some of the most relevant variables to consider are the ratio of activating agent to support in the first stage, the ratio of enzyme contacted to activated support in the second, pH, temperature, time of contact and agitation rate in both stages. These variables are likely to have interactions among them so that a careful statistical design is required for optimization. An even more complex task is to define a proper objective function to optimize. To do so, relevant immobilization parameters have to be taken into account.

4.1.2.1 Parameters of Enzyme Immobilization

During the process of immobilization a fraction of the enzyme protein is immobilized, while the rest remains unbound. The immobilized enzyme protein expresses only a fraction of the expected activity and this can be due to enzyme inactivation, steric hindrance or mass transfer limitations. On the other hand the unbound enzyme protein may be partly inactive, so enzyme immobilization yield (Y_E), as defined by Eq. 4.1, has to be carefully analyzed in its meaning.

$$Y_E = \frac{E_I}{E_C} = \frac{E_I}{E_I + E_R + E_L} \tag{4.1}$$

As defined, Y_E simply represents the fraction of the contacted activity that is expressed in the biocatalyst. Although enzyme is quantified in units of activity (not

mass), Eq. 4.1 is presented as a material balance, so that the term E_L has been included to close that balance. The meaning of E_L is simply the fraction of the contacted enzyme activity which is not expressed either in the biocatalyst or in the medium and may be attributed to inactivation (of the bound, but mostly of the unbound enzyme) or to mass transfer limitations and steric hindrances of the immobilized enzyme. An insight of the reasons underlying the partial expression of the contacted activity may be obtained by defining protein immobilization yield, which is simply the ratio of bound protein to contacted protein, as expressed by Eq. 4.2:

$$Y_P = \frac{P_I}{P_C} = \frac{P_C - P_R}{P_C} \tag{4.2}$$

Comparing P_I with E_I may give an insight of the incidence of mass transfer limitations and steric hindrances. If Y_P is significantly higher than Y_E, then those effects are probably relevant, but immobilization can have degree of selectivity (positive or negative) for the enzyme with respect to the whole protein that cannot be ruled out.

Sometimes enzyme immobilization yield is defined as:

$$Y'_E = \frac{E_I}{E_I + E_L} = \frac{E_I}{E_T - E_R} \tag{4.3}$$

which means that the enzyme remaining active in solution after immobilization is not considered a loss. Although strictly true, for practical purposes it usually represents a loss because it is not economic to recover this small fraction of diluted enzyme. Obviously Y_E is lower than $Y_E{}'$, but the former should be used since it better reflects enzyme recovery.

Other very relevant parameter of enzyme immobilization is biocatalyst mass (or volumetric) specific activity, which is simply the amount of enzyme activity expressed per unit mass (or unit volume) of biocatalyst:

$$A_{sp} = \frac{E_I}{M} \tag{4.4}$$

This parameter may differ significantly from enzyme load, which can be defined as the amount of enzyme loaded to the support. Enzyme load can be calculated by subtracting the total activity in suspension to the activity in solution after immobilization. Such difference simply represents the enzyme protein loaded but gives no insight on the expression of such enzyme protein. Alternatively, protein load can be determined as:

$$P_{load} = \frac{P_C - P_R}{M} \tag{4.5}$$

but protein load may differ from enzyme load since some selectivity of immobilization (whether positive or negative) of the enzyme with respect to protein as a whole may occur. In fact, there are several cases reported where selective immobilization of enzymes has been obtained (Bastida et al. 1998; Fernández-Lafuente et al. 1998).

4.1.2.2 Optimization of Enzyme Immobilization

Immobilization yield and mass (or volumetric) specific activity are usually in compromise so that usually rather low specific activities are obtained at conditions that maximize yield and vice versa (Illanes et al. 1988). Therefore, an objective function that adequately weighs these two parameters could be a good criterion for optimization. However, this is not an easy task since the impact of each parameter on process economics is hard to evaluate and relates not only to the immobilization process itself but also to the process of biocatalyst utilization. Immobilization yield is strictly related to the immobilization process and will have a strong impact when the cost of the enzyme is high; on the other hand, mass specific activity is more related to the quality of the biocatalyst and its impact is related to its utilization in a bioreactor: the higher the specific activity, the higher the volumetric productivity of the reactor. Unfortunately, to properly weigh these impacts in a cost-objective function is not easy at an early step of process development. An objective function based on the a priori evaluated as the most significant parameter, under certain restriction of minimum acceptable values of the other, may be adequate. For instance, for not very expensive enzymes optimization can be based on maximizing specific activity for immobilization yields over a pre-established value. Besides these two parameters, operational stability of the biocatalyst should also be taken into account when optimizing an immobilization process. Half-life of the biocatalyst at simulated operation conditions can be used as a first approach to include enzyme stability as a parameter to optimize immobilization conditions. Ideally, a cost-based objective function based on those three parameters adequately weighed according to its impact on the cost of the process of enzyme utilization should be developed.

4.2 Heterogeneous Kinetics: Apparent, Inherent and Intrinsic Kinetics; Mass Transfer Effects in Heterogeneous Biocatalysis

Enzyme kinetic principles developed early in the 20th century were based on homogeneous systems, this is, when the biocatalyst and their substrates and products of reaction are in a single phase where the reaction occurs. This made sense since by that time enzymes were regarded as catalyst only effective in aqueous media where proteins are soluble. Nowadays, more and more applications of enzymes are based on heterogeneous systems. In fact, as reviewed in section 1.6, a significant portion of the applications of enzymes in organic synthesis is based on non-aqueous media where the enzyme biocatalyst is mostly insoluble (Klibanov 1989; Halling 2004), and in some cases a biphasic system occurs being the enzyme in one phase and the substrates and/or products in the other (Bordusa 2002; Krieger et al. 2004).

The most relevant case of heterogeneous catalysis from a technological perspective is represented by immobilized enzymes (see section 4.1) with reaction

taking place at the surface or inside the biocatalyst particle, where conditions are different (and sometimes hardly predictable) from the bulk reaction medium in which the process is being monitored. Enzyme immobilization may produce both conformational and micro-environmental effects that will affect the kinetics of the enzyme catalyzed reaction (Kobayashi and Laidler 1973). Conformational effects refer to the structural changes produced in the enzyme molecule as a consequence of the immobilization procedure (see section 4.1). Alteration of the native three-dimensional structure of the enzyme protein and steric effects due to its close proximity to the surface of the support are conformational changes that may produce differences in kinetic behavior with respect to the free enzyme. Micro-environmental effects refer to partition and mass transfer limitations. Partition of substrates (and products) to the enzyme phase and mass transport of substrates from the bulk reaction medium to the biocatalyst and products transport from it back into the bulk reaction medium affect the kinetics of the enzyme catalyzed reaction.

At this point it is worthwhile to introduce some definitions:

- *Intrinsic kinetics* of the immobilized enzyme represents its proper behavior and corresponds to that observed in the absence of partition and mass transfer limitations of the reacting species. This kinetic behavior and the corresponding kinetic parameters are not directly measurable for an immobilized enzyme, except in special conditions where these effects are purposely avoided. Even if the intrinsic behavior could be revealed, it may differ from that of the free enzyme counterpart because of conformational changes.
- *Inherent kinetics* is the behavior observed in the absence of mass transfer limitations. This behavior, and the corresponding kinetic parameters, may differ from the intrinsic because of partition of the reactive species between the biocatalyst phase, where the reaction occurs, and the bulk medium phase, where the reaction is monitored.
- *Effective (or apparent) kinetics* of the immobilized enzyme is that directly determined from the observed behavior. Effective and apparent seem opposite concepts, but this is not so, since it is effective from the standpoint of the enzyme user (it is what one gets), but apparent from the standpoint of the enzyme since it does not reflect its actual catalytic potential which is obscured by the heterogeneous nature of the system.

Partition and mass transfer limitation make the substrate (and product) concentration in contact with the enzyme different from that in the bulk reaction medium producing the corresponding profiles, as shown in Fig. 4.5. Partition produces a discontinuity of the profiles at the medium-biocatalyst interface while mass transfer limitations produce profiles in the immediate vicinity of that surface and on the inside of the biocatalyst support (if allowed to host the enzyme).

If kinetic rate data from an immobilized enzyme are collected directly, as presented in section 3.2.2, only effective (apparent) parameters are obtained that do not reflect the actual behavior of the enzyme. This information, though useful, is valid only at the precise conditions at which the experiment was performed. For design

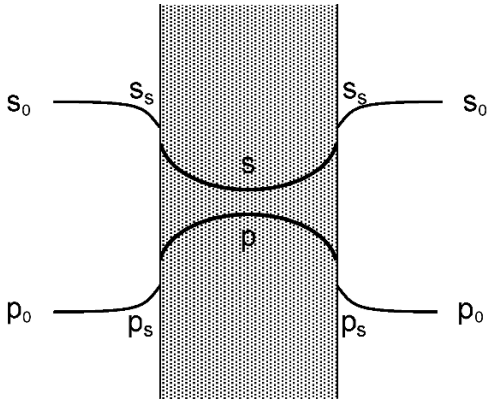


Fig. 4.5 Substrate and product profiles in an immobilized enzyme system as a consequence of partition and mass transfer limitations

and scale-up purposes, this information is absolutely insufficient so that the intrinsic (or inherent) behavior must be determined as will be exposed in the following sections.

4.3 Partition Effects

Partition effects are important in macro-heterogeneous biphasic systems (see section 1.6) where the enzyme is in one phase, while the substrates and/or products are in the other. In that case, effective substrate (and product) concentration is the one in the enzyme phase (usually the aqueous phase in the case of biphasic systems) so that partition coefficient of substrate (and product) between both phases can be a very relevant parameter that needs to be determined.

In the case of immobilized enzymes, partition at the biocatalyst–medium interface can be the consequence of the different physicochemical properties of the bulk medium and the enzyme environment within the support. Mostly relevant is partition due to electric charges. This situation occurs when the enzyme support bears some charge: in that case, partition of reactive species occurs as long as they are charged molecules at the conditions of reaction and it always occur with respect to protons, so that it has an effect on the pH dependence on the immobilized enzyme (Goldstein 1976).

For a charged support:

$$K_{ps} = \frac{s}{s_0} = \exp\left(\frac{-Z \cdot \in \cdot \varphi}{k_B \cdot T}\right) \tag{4.6}$$

If the substrate charge is of the same type as the net charge of the support ($\varphi > 0$) $s < s_0$, while if of the opposite type of charge as the net charge of the support ($\varphi < 0$) $s > s_0$, meaning that the partition effect is unfavorable in the first case, while favorable in the second. This is in agreement with the expected behavior.

For protons:

$$K_{ph^+} = \frac{h^+}{h_0^+} = \exp\left(\frac{-\in \cdot \varphi}{k_B \cdot T}\right) \tag{4.7}$$

$$pH - pH_0 = 0.43\frac{\in \cdot \varphi}{k_B \cdot T} \tag{4.8}$$

If the net charge of the support is positive $(\varphi > 0)$, then $pH > pH_0$; if negative $(\varphi < 0)$, then $pH < pH_0$. Eq. 4.8 allows predicting the displacement of the pH profile of an immobilized enzyme to a charged support. The magnitude of that displacement $(0.43 \in \cdot \varphi / k_B \cdot T)$ represents the difference with respect to the pH profile of the free enzyme, provided no other effects are occurring as a consequence of immobilization. This displacement in the activity versus pH curve should be to the left in the case of cationic supports and to the right in the case of anionic supports. This behavior has been observed in several cases (Goldstein 1976). Figure 3.10 (see section 3.5.1) shows the pH displacement of glucoamylase immobilized in a DEAE-cellulose (net positive charge within the pH range considered) which is to the left, as expected, except at very low pH values where other effects are probably intervening (Illanes 1983). Similar results have been obtained with a β-galactosidase from *Aspergillus oryzae* immobilized in cross-linked chitin (Illanes et al. 1988, 1990). This partition effect can be used advantageously when pH conditions for enzyme and substrate stability do not match as it may occur, for instance, in the hydrolysis of milk with fungal β-galactosidase. The magnitude of the pH displacement is reduced by increasing the ionic strength of the medium since in that case other ions will compete with hydrogen ions for partition. This has been verified experimentally (Goldstein 1976) being a good way to discriminate partition effects to reveal the intrinsic behavior of the enzyme.

4.4 Diffusional Restrictions

Mass transfer limitations may severely restrain the expression of the catalytic potential in the case of immobilized enzymes. In such cases it is necessary to assess the impact of mass transfer limitations to properly evaluate the biocatalyst performance. Mass transfer limitations are usually expressed as *diffusional restrictions* because substrate transport from the bulk reaction medium to the biocatalyst and product transport from there again to the bulk reaction medium is governed by molecular diffusion. Intrinsic kinetic behavior of the enzyme will be obscured by diffusional restrictions and it is the purpose of this section to quantify that effect to be able to incorporate it into the scheme leading to models for enzyme reactor design or performance evaluation. Diffusional restrictions can be external or internal to the biocatalyst particle. *External diffusional restrictions* (EDR) are the consequence of a layer of stagnant liquid surrounding the solid enzyme particle, across which no convection exists and substrate transport occurs only by molecular diffusion (Goldstein 1976). EDR could be significant for those

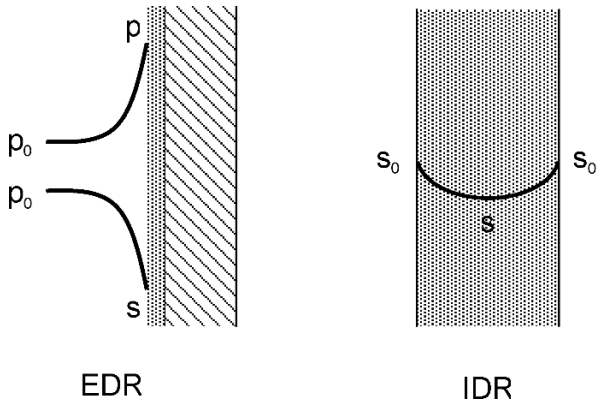


Fig. 4.6 Schematic representation of external (EDR) and internal (IDR) diffusional restrictions

enzymes immobilized onto the surface of an impervious carrier. *Internal diffusional restrictions* (IDR) occur when the enzyme is contained within a solid matrix, as it occurs in a gel or within the structure of a microporous solid support. IDR are usually more severe than EDR since substrates and products should diffuse through a medium in which mass transfer will be even slower than in a liquid medium as it occurs in EDR. This is schematically represented in Fig. 4.6.

4.4.1 External Diffusional Restrictions

4.4.1.1 Enzyme Kinetics Under External Diffusional Restrictions; Effectiveness Factor

To analyze this case, let us assume that the enzyme is homogeneously distributed over the surface of an impervious support as shown in Fig. 4.6. Substrate conversion into product occurs in three consecutive steps: substrate transportation from the bulk medium to the surface of the biocatalyst, enzymatic conversion into product at that surface and product transportation back from the surface to the bulk medium. As shown, substrate and product profiles will develop across the stagnant layer as long as substrate and or product diffusion limits the catalytic potential of the enzyme. Any of these steps can be rate-limiting.

For the simple case of the reaction $S \xrightarrow{E} P$, at steady-state:

$$r = v' = J \tag{4.9}$$

Substrate transportation through the stagnant layer occurs by molecular diffusion, so:

$$J = h(s_0 - s_S) = h' \cdot A(s_0 - s_S) \tag{4.10}$$

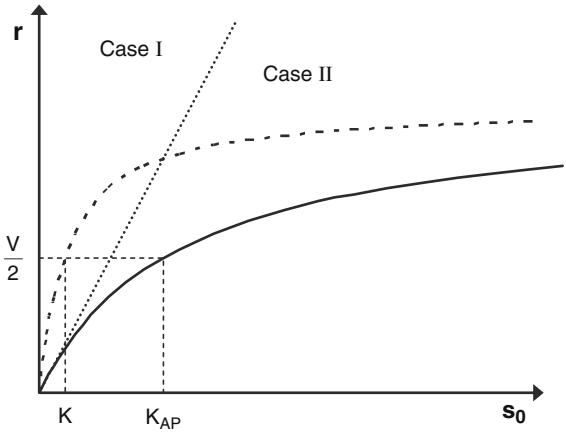


Fig. 4.7 Substrate conversion rate (r) as a function of bulk substrate concentration, showing limiting cases I and II (see text)

while, for simple Michaelis–Menten kinetics, enzyme reaction at the surface is represented by:

$$v' = \frac{V' \cdot s_S}{K + s_S} \tag{4.11}$$

being V' and K the intrinsic parameters of the enzyme (assuming no partition effects).

Two limit situations can be envisaged. One (Case I) in which r is solely determined by substrate transport rate (diffusion limited) and another (case II) in which r is solely determined by the catalytic potential of the enzyme (kinetically limited). In Case I, reaction rate is so fast with respect to substrate transport rate that substrate profile is steep, s_S being negligible with respect to s_0, while in Case II substrate transport rate across the stagnant layer is fast enough with respect to reaction rate so that no substrate profile develops and s_S is equal to s_0. Eq. 4.10 and 4.11 become Eqs. 4.12 and 4.13 respectively:

$$\text{Case I} \qquad r = J = h \cdot s_0 = h' \cdot A \cdot s_0 \tag{4.12}$$

$$\text{Case II} \qquad r = v' = \frac{V' \cdot s_0}{K + s_0} \tag{4.13}$$

that are represented in Fig. 4.7. Diffusional restrictions tend to disappear as s_0 (and therefore the substrate gradient) increases.

Then, from Eqs. 4.9 to 4.11, Eq. 4.14 is obtained that represents the enzyme behavior under EDR.

$$h(s_0 - s_S) = \frac{V' \cdot s_S}{K + s_S} \tag{4.14}$$

Even though the linear mass transfer coefficient (h') is more frequently used in heterogeneous chemical kinetics, in enzyme heterogeneous kinetics it is customary to

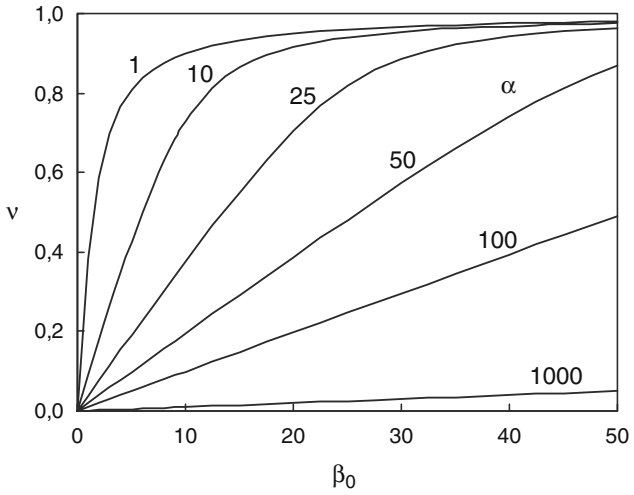


Fig. 4.8 Kinetics of immobilized enzyme under external diffusional

use the volumetric mass transfer coefficient (h) as in Eq. 4.14 (Engasser and Horvath 1976). h' is related to h, and also to the film diffusion coefficient according to:

$$h' = \frac{h}{A} = \frac{D}{\delta} \tag{4.15}$$

Eq. 4.14 is more conveniently expressed in dimensionless form as:

$$\beta_0 - \beta_S = \frac{\alpha \cdot \beta_S}{1 + \beta_S} = \alpha\nu \tag{4.16}$$

where: $\beta_0 = s_0/K$; $\beta_S = s_S/K$; $\nu = v'/V'$; $\alpha = (V'/h \cdot K)$. K and V' are intrinsic kinetic parameters and α is the dimensionless Damkoehler number (sometimes referred as substrate modulus). α represents the relative incidence of enzyme catalytic potential and substrate mass transfer rate. A high value of α implies that mass EDR are relevant since V'/K is higher than h meaning that the system is limited by substrate diffusion. The opposite holds for small values of α: if h is higher than V'/K, the system is limited by the catalytic potential of the enzyme, which is represented by the magnitude of V'/K, known as the first-order kinetic constant. Values of $\alpha < 1$ mean that the system is free of EDR (see Fig. 4.8). From Eq. 4.16, β_S can be obtained as a function of measurable β_0 and α:

$$\beta_S = \frac{-(1 + \alpha - \beta_0) \pm \sqrt{[(1 + \alpha - \beta_0)^2 + 4\beta_0]}}{2} \tag{4.17}$$

This second-order equation is unambiguous since due to the characteristics of the determinant of that equation, only the positive root will give positive values of β_S. Graphical representation of Eq. 4.16 is in Fig. 4.8. At low values of α, the behavior

is typically Michaelian, while at very high values of α (very low β_S) correlation between ν and β_0 becomes linear, as predicted from Eq. 4.16. In principle, low values of α are desirable but this has to be taken with caution since this may be the consequence of a poorly active biocatalyst, which is undesirable at least from a technological perspective. Ideally one would like to have a low value of α for a reasonably active biocatalyst, which implies a high rate of substrate transport. There are cases in which high values of α are advantageous, as in the case of enzyme electrodes (Enfors and Molin 1978; Lemke 1988) where the linear correlation between reaction rate and substrate (analyte) concentration is desirable.

The magnitude of EDR can be conveniently expressed by means of the effectiveness factor. The effectiveness factor is a general concept that represents the ratio of rates of a phenomenon under the influence of a factor and freed from that influence. For the present case, it is defined as the ratio of the reaction rate under EDR and that attainable in its absence, this is, the ratio of effective to intrinsic reaction rate. For simple Michaelis–Menten kinetics:

$$\eta = \frac{\frac{V' \cdot s_S}{K+s_S}}{\frac{V' s_0}{K+s_0}} = \frac{\beta_S(1+\beta_0)}{\beta_0(1+\beta_S)} \tag{4.18}$$

The value of η describes the impact of EDR quite neatly since it represents the fraction of the catalytic potential of the enzyme that is expressed at certain conditions under the influence of EDR. In this way, a $\eta = 0.8$ means that the enzyme is expressing 80% of its maximum catalytic potential.

From Eqs. 4.17 and 4.18:

$$\eta = \frac{(1+\beta_0)\left[1+\alpha+\beta_0-\sqrt{(1+\alpha-\beta_0)^2+4\beta_0}\right]}{2\beta_0\alpha} \tag{4.19}$$

It is clear that η is a function of β_0 and α so that, as long as α and the intrinsic kinetic parameters (V' and K) are evaluated (s_0 is measurable), Eq. 4.19 is a very useful expression that allows to quantify the behavior of an enzyme biocatalyst under EDR, as will be analyzed in section 5.3. Eq. 4.19 is usually represented in a log–log plot as shown in Fig. 4.9a. As seen, η is a very strong function of α; however the range at which such dependence is observed is a strong function of β_0. In fact, if β_0 is sufficiently high, η remains close to 1 even at high values of α, meaning that, in theory, EDR can be eliminated at sufficiently high substrate concentration. Of course, in practice this will be restrained by the solubility of the substrate in the reaction medium. Though quite didactic, Fig 4.9a is rather useless and misleading from a technological perspective. Reasonable values of η are too close to the upper axis and the area of interest is not easily seen; most importantly, α, which is a parameter that depends only on the enzyme and substrate is represented in the X-axis, while β_0 which is the actual independent variable during reactor operation is represented as a parameter. A better way of representation is in a η versus β_0 plot using α as parameter, as seen in Fig 4.9b. Each of these curves represents the evolution of η with the independent variable β_0 for a given value of α and it represents the

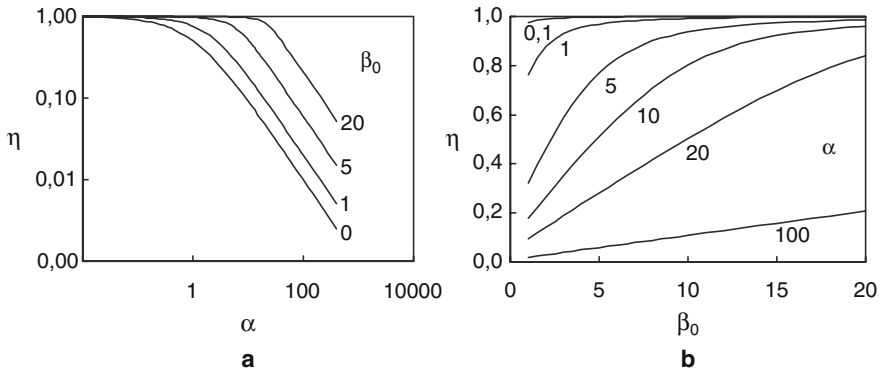


Fig. 4.9 Effectiveness factor of an enzyme biocatalyst subjected to EDR: a) η versus α log–log plot; b) η versus β_0

information required to introduce the effect of EDR in the scheme for reactor design and performance evaluation (see Fig. 3.1).

4.4.1.2 Determination of Intrinsic Kinetic and Mass-Transfer Parameters

To assess the impact of EDR on enzyme kinetics the value of α and the intrinsic kinetic parameters V and K′ have to be determined, which can be done experimentally or from empirical correlations.

Several empirical correlations have been proposed in chemical catalysis for the determination of the substrate mass transfer coefficient and few of them have been applied for enzyme catalyzed reactions (Rovito and Kittrell 1973; Buchholz 1982), which can be represented in dimensionless expressions of the type:

$$\frac{h' \cdot \rho'}{G} = C \cdot Sc^{-2/3} \cdot Re^{-m} \tag{4.20}$$

where Sc and Re are the dimensionless numbers of Schmidt and Reynolds respectively (Bennett and Myers 1982). Interestingly the exponent in Sc is always 2/3 while the exponent in Re varies considerably from one case to another (Rovito and Kittrell 1973; Traher and Kittrell 1974; Buchholz 1982). From these correlations h′ (h) is determined considering the physical characteristics of the substrate and the biocatalyst and the hydrodynamic conditions of the system. Of course, these correlations have to be validated from experimental data gathered from enzyme reactor operation.

A different approach is the direct determination of α and the intrinsic kinetic parameters from experimental rate data. This method was proposed by Chen (in Buchholz 1982) and is based on the determination of initial rates within a broad range of bulk substrate concentration. The kinetics of enzyme reaction, represented by the right-hand side of Eq. 4.14 is a very complex function of

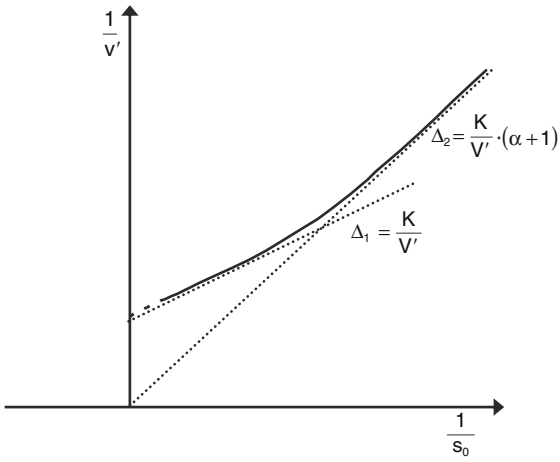


Fig. 4.10 Determination of intrinsic kinetic parameters and Damkoehler number by linearization of initial rate data in double reciprocal plot

the measurable concentration of substrate (s_0) which cannot be linearized. If the rate data is represented in a double reciprocal plot a curve is obtained as the one shown in Fig. 4.10. But if an analysis is made on the regions of very high $(\beta_0 \gg 1)$ and very low $(\beta_0 \ll 1)$ substrate concentrations, linear correlations are obtained.

In the first case, if $\beta_0 \gg 1$ $(s_0 \gg K)$, substrate gradient is very high and reaction is limited only by enzyme kinetics, so substrate profile within the stagnant film will be negligible and $s_S = s_0$. In that case the rate equation reduces to a simple Michaelis–Menten equation that can be linearized as already described in Chapter 3. Using the double reciprocal plot:

$$\frac{1}{v'} = \frac{K}{V'} \cdot \frac{1}{s_0} + \frac{1}{V'} \tag{4.21}$$

that allows the conventional determination of V' and K as shown in Fig. 4.10.

In the second case, if $\beta_0 \ll 1$ $(s_0 \ll K)$ the reaction is limited by diffusion so from these data it is possible to determine the value of. At $s_S < s_0 \ll K$:

$$v = \frac{V'}{K} \cdot s_S \tag{4.22}$$

and Eq. 4.16 becomes:

$$\beta_0 - \beta_S = \alpha \cdot \beta_S \tag{4.23}$$

$$s_0 - s_S = \alpha \cdot s_S \tag{4.24}$$

From Eqs. 4.22 and 4.24:

$$v' = \frac{V'}{K} \cdot \frac{s_0}{\alpha + 1} \tag{4.25}$$

$$\frac{1}{v'} = \frac{K}{V'} \cdot (\alpha + 1) \cdot \frac{1}{s_0} \tag{4.26}$$

Eq. 4.26 shows that, at low $s_o(\beta_0)$, a straight line extrapolating to the origin is obtained, whose slope allows the determination of α as show in Fig. 4.10. If the slopes of the regions at high and low s_0 are designated as Δ_1 and Δ_2 respectively, from Eqs. 4.21 and 4.26:

$$\Delta_1 = \frac{K}{V'} \tag{4.27}$$
$$\Delta_2 = \frac{K}{V'} \cdot (\alpha + 1)$$
$$\alpha = \frac{\Delta_2}{\Delta_1} - 1$$

so that α can be determined as shown in Fig. 4.10.

This method, although simple and straightforward, has some pitfalls. First of all, enough data points should be collected at sufficiently low and high values of s_0 so that $\beta_0 \ll 1$ and $\beta_0 \gg 1$ respectively. This might be not too simple: those regions where linear correlations are obtained are precisely those where the experimental error is higher (see section 3.3) and solubility of substrate may impose restrictions in the high concentration zone. Besides, experimental conditions have to be carefully specified, especially agitation rate, since this variable will affect the results significantly. Ideally one should try to mimic the reactor flow pattern as much as possible to generate meaningful results. Taking these precautions, the method is useful and strongly recommended to identify the presence of mass-transfer limitations. If a straight line is obtained throughout the substrate concentration range, this is indicative that no mass transfer limitations exist; if it is a monotonic curve (with no inflection point) it indicates that EDR are significant; a curve with inflection point is indicative that IDR are also significant.

4.4.1.3 Interplay Between External Diffusional Restrictions and Inhibition

For enzyme inhibition kinetics it is interesting to analyze the interplay between inhibition and EDR. To illustrate this, the case of competitive inhibition by product will be analyzed. In this case, not only substrate transport from the bulk medium to the biocatalyst surface has to be considered, but also product transport from that surface back into the bulk medium. Making a steady sate analysis, in this case:

$$h(s_0 - s_S) = \frac{V' \cdot s_S}{K\left(1 + \frac{p_S}{K_I}\right) + s_S} = h_P(p_S - p_0) \tag{4.28}$$

Dimensionless equations for both substrate and product are then derived from Eq. 4.28:

$$\beta_0 - \beta_S = \frac{\alpha \cdot \beta_S}{1 + \beta_S + \gamma_S} \tag{4.29}$$

$$\gamma_S - \gamma_0 = \frac{\alpha \cdot \beta_S \cdot a_e}{1 + \beta_S + \gamma_S} \tag{4.30}$$

where: $a_e = (h/h_P) \cdot (K/K_I)$

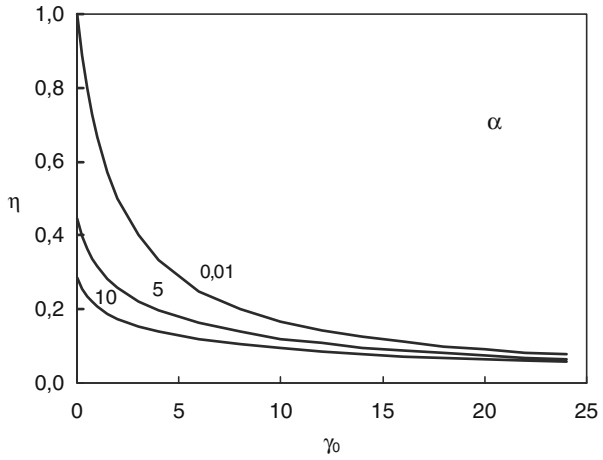


Fig. 4.11 Effectiveness factor of an enzyme biocatalyst subjected to EDR and competitive inhibition by product

Eqs. 4.29 and 4.30 are second order in β_S and γ_S respectively:

$$\beta_S^2 + \beta_S(\alpha - \beta_0 + 1 + \gamma_S) - \beta_0(1 + \gamma_S) = 0 \tag{4.31}$$

$$\gamma_S^2 + \gamma_S(1 + \beta_S - \gamma_0) - \gamma_0(1 + \beta_S) - \alpha \cdot a_e \cdot \beta_S = 0 \tag{4.32}$$

Solving the system of Eqs. 4.31 and 4.32, functions of β_S and γ_S are obtained:

$$\beta_S = f(\alpha, \beta_0, \gamma_0, a_e) \qquad \gamma_S = f(\alpha, \beta_0, \gamma_0, a_e)$$

The effectiveness factor under inhibition can be defined as:

$$\eta_I = \frac{\frac{V' \cdot s_S}{K\left(1 + \frac{p_S}{K_I}\right) + s_S}}{\frac{V' \cdot s_0}{K + s_0}} = \frac{\beta_S(1 + \beta_0)}{\beta_0(1 + \beta_S + \gamma_S)} \tag{4.33}$$

Kinetic behavior represented by Eq. 4.33 is shown in Fig. 4.11. As can be seen, there is an interplay between EDR and inhibition. The upper curve represents the situation under no EDR and reflects the pure inhibition effect. The lower curves highlight the effect of EDR. As seen, the presence of EDR reduces the impact of enzyme inhibition on enzyme performance, which is reasonable, since mass transfer limitations will move the system away from kinetic control.

A similar analysis can be made for any other type of inhibition. An interesting situation occurs in the case of uncompetitive inhibition by high substrate concentration. In this case, a steady-state analysis renders a third-order equation in β_S that for certain values of the kinetic and diffusion parameters may give three positive values of β_S for one value of β_0 and a stability analysis should be made to assess the right value. The intermediate value is always unstable but the upper or lower

value of β_S will represent the system according to the path-dependence of β_S (hysteresis), which for the case of an enzyme reactor will always be in the downward direction.

4.4.2 Internal Diffusional Restrictions

Diffusional restrictions can be quite severe when the enzyme is contained within a solid matrix. In this case, substrates and products should diffuse through a medium (a gel or the viscous fluid within micropores) in which diffusion will be even slower than in a liquid medium as it occurs in EDR. In the case of IDR, substrate and product profiles will be developed as a consequence of diffusional restrictions, as shown in Fig. 4.6. The situation in this case is much more complex than in EDR, since each enzyme molecule is subjected to different environmental conditions according to its relative position within the matrix; intrinsic reaction rates and substrate and products concentrations within the matrix are obviously not measurable and vary from on enzyme molecule to the other, according to its position within the biocatalyst. In this case, a differential analysis within the biocatalyst particle is required to properly describe the system and the corresponding equations, even for simple Michaels–Menten kinetics, should be solved numerically to yield the corresponding profiles. In the case of EDR, the behavior of the system was considered independent on the particle geometry of the impervious support, which is reasonable to assume since the reaction takes place at the surface of the biocatalyst particle and so was considered in the analysis done on section 4.4.1. In the case of IDR this is not so, and the behavior of the system is highly dependent on particle geometry.

4.4.2.1 Models for Enzyme Kinetics Under Internal Diffusional Restrictions for Different Particle Geometries; Effectiveness Factor

Two cases will be analyzed in detail: the flat slab geometry and the spherical geometry. These are cases amenable for a rigorous analysis and represent extreme cases of particles with infinite and minimum radius of curvature respectively. Notwithstanding, they represent rather usual immobilized enzyme configurations. Enzymes immobilized in membranes illustrate the first case and particles of approximate spherical geometry, represent the second case. Other geometries have been also analyzed, like the case of cylindrical particles that are also amenable for rigorous analysis (Xiu et al. 2001). We have analyzed the case of spheroid particles using oblate spheroidal coordinates as a way of approximating the behavior of lens-shaped particles (LentiKats) of immobilized penicillin acylase (Soto et al. 2002; Wilson et al. 2002). For particles of undefined geometry the problem is quite complex to analyze so that for such cases an approximate solution can be found based on a defined geometry that resembles that of the particle, by redefining

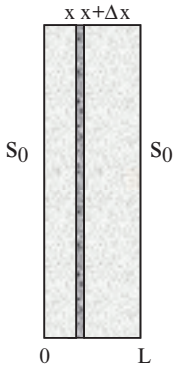
the mass transfer parameter (Thièle modulus, see below) according to the particle geometry.

The analysis will be done in three steps. In the fist step, differential equations will be developed by combining enzyme kinetics and mass transfer to obtain the substrate (and product) profile within the biocatalyst particle; in the second step, local effectiveness factor profiles will be obtained from the previous step; in the third step a global effectiveness factor will be obtained by adequately averaging that distribution. This global effectiveness factor describes the behavior of the biocatalyst particle as a whole and will be obtained in terms of measured and calculated parameters, being a useful way of incorporating IDR into enzyme reactor design and performance evaluation, as considered in section 5.3.

Case1: Slab Geometry (Enzyme Membrane)

Considering an enzyme membrane of width L, a material balance for substrate over the section of analysis of width Δx is:

$$J' \cdot A\,|_x - J' \cdot A\,|_{x+\Delta x} - v'' \cdot A \cdot \Delta x = A \cdot \Delta x \cdot \left(-\frac{ds}{dt}\right) \qquad (4.34)$$



Letting $\Delta x \to 0$ in Eq. 4.34 and considering Michaelis–Menten intrinsic kinetics and steady state:

$$-\frac{dJ}{dx} = v'' = \frac{V'' \cdot s}{K + s} \qquad (4.35)$$

Considering now the first Fick's law of diffusion:

$$J' = -D \cdot \frac{ds}{dx} \qquad (4.36)$$

From Eqs. 4.35 and 4.36:

$$D \cdot \frac{d^2 s}{dx^2} - \frac{V'' \cdot s}{K + s} = 0 \qquad (4.37)$$

Differential Eq. 4.37 has no analytical solution for Michaelis–Menten intrinsic kinetics and has to be solved numerically considering the following boundary

conditions:

$$\text{I)}\quad \begin{matrix} x = 0 \\ s = s_0 \end{matrix} \qquad \text{II)}\quad \begin{matrix} x = \dfrac{L}{2} \\ \dfrac{ds}{dx} = 0 \end{matrix}$$

Boundary condition I) assumes that EDR is negligible with respect to IDR, as may frequently occur for enzymes immobilized inside solid supports. If not, it is wrong since at the surface of the biocatalyst particle $s = s_S \neq s_0$ and boundary condition I) should be replaced by an equation of continuity at the medium-particle interface. This situation will be analyzed afterwards.

It is convenient to write Eq. 4.37 in dimensionless form by defining the corresponding dimensionless terms (see Nomenclature):

$$\frac{d^2\beta}{dz^2} - \Phi^2 \cdot \frac{\beta}{1+\beta} = 0 \tag{4.38}$$

$$\text{I)}\quad \begin{matrix} z = 0 \\ \beta = \beta_0 \end{matrix} \qquad \text{II)}\quad \begin{matrix} z = 0.5 \\ \dfrac{d\beta}{dz} = 0 \end{matrix}$$

where z is the dimensionless membrane width (x/L).

Φ is the dimensionless Thièle modulus (Engasser and Horvath 1973), that for the present case can be defined as:

$$\Phi = L\sqrt{\frac{V''}{K \cdot D}} \tag{4.39}$$

Φ (as α in the case of EDR) represents the relative incidence of enzyme catalytic potential and substrate mass transfer rate. A high value of Φ implies that either $V''/K \gg D$ or, not being so, L is big enough, meaning that the system is mass transfer limited. A small value of Φ implies that either $D \gg V'/K$ or, not being so, L is small enough, meaning that the system is limited by the catalytic potential of the enzyme.

Eq. 4.38 with its corresponding boundary conditions can be solved by numerical methods (Euler's, Runge-Kutta ...). This can be done now rather easily by using available software. Numerical solution of Eq. 4.38 is obtained as a set of data of β for different values of the independent variable z and the parameter Φ, that can be represented as:

$$\beta = f(\beta_0, z, \Phi) \tag{4.40}$$

so that the substrate profile within the biocatalytic particle can be calculated for any value of Φ, as shown in Fig. 4.12.

In the case of first order kinetics ($v'' = V''/K \cdot s$), Eq. 4.38 is simplified to:

$$\frac{d^2\beta}{dz^2} - \Phi^2 \cdot \beta = 0 \tag{4.41}$$

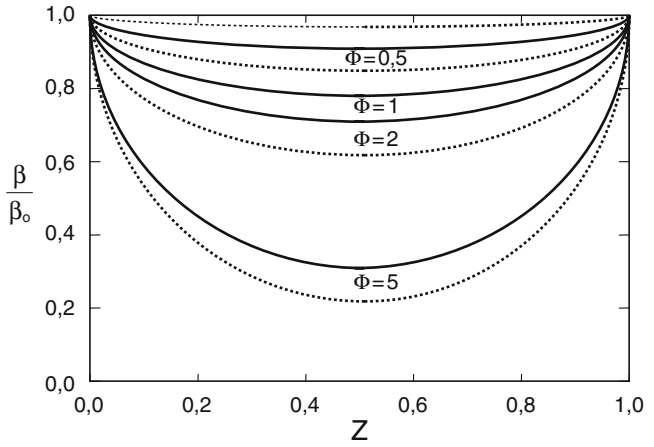


Fig. 4.12 Substrate profile of an immobilized enzyme under IDR inside the catalytic membrane. —— numerical solution for Michaelis Menten kinetics; ····· analytical solution for first-order kinetics

which is easily solved analytically with the same boundary conditions of Eq. 4.38, being its solution:

$$\beta = \beta_0 \frac{\cosh\left[\Phi(z-0.5)\right]}{\cosh\left[0.5\Phi\right]} \tag{4.42}$$

This analytical solution is of little interest from a bioreactor perspective since first-order kinetics ($s \ll K$) never applies; however, in certain applications (i.e. enzyme electrodes) it can be appropriate (Newman 1978; Lemke 1988).

The second step is the determination of the local effectiveness factor profile that by definition (see Eq. 4.18) is:

$$\eta = \frac{\frac{V''\cdot s}{K+s}}{\frac{V''s_0}{K+s_0}} = \frac{\beta(1+\beta_0)}{\beta_0(1+\beta)} \tag{4.43}$$

Then, from the numerical solution (Eq. 4.40), replacing β in Eq. 4.43

$$\eta = f(\beta_0, z, \Phi) \tag{4.44}$$

which, for the case of first-order kinetics is simply:

$$\eta = \frac{\cosh\left[\Phi(z-0.5)\right]}{\cosh\left[0.5\Phi\right]} \tag{4.45}$$

The final step is the determination of a global effectiveness factor from the profile of local effectiveness factors that adequately describe the behavior of the biocatalyst particle (membrane in this case) as a whole. Since the distribution of η values is

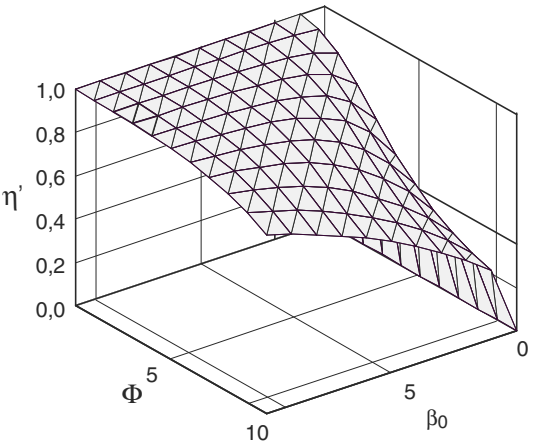


Fig. 4.13 Global effectiveness factor (mean integral value) of a membrane immobilized enzyme with Michaelis–Menten kinetics as a function of bulk substrate concentration and Thièle modulus

highly non-linear with respect to z, the mean integral value of η distribution over the volume of the biocatalyst particle is adequate. Then, for a flat membrane:

$$\eta' = \frac{\int_0^1 \eta \cdot A \cdot dz}{\int_0^1 A \cdot dz} = \int_0^1 \eta \cdot dz \tag{4.46}$$

so that from Eqs. 4.44 and 4.46:

$$\eta' = f(\beta_0, \Phi) \tag{4.47}$$

which, for the case of first-order kinetics, from Eqs. 4.45 and 4.46, is simply:

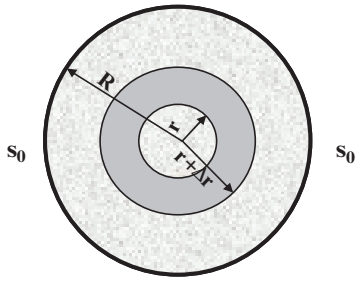
$$\eta' = \frac{\tanh[0.5 \cdot \Phi]}{0.5 \cdot \Phi} \tag{4.48}$$

Eq. 4.47 is represented as a surface of response plot in Fig. 4.13. Analog to the case of EDR, in IDR η' is a very strong function of Φ; however, the range at which such dependence is observed is a strong function of β_0. The evolution of η' with the independent variable β_0 for a given value of Φ represents the information required to introduce the effect of IDR in the scheme for reactor design and performance evaluation (see Fig. 3.1).

Case 2: Spherical Geometry

Considering now a spherical enzyme particle of radius R, a material balance for substrate over the section of analysis of width Δr is:

$$J' \cdot 4\pi r^2\big|_{r+\Delta r} - J' \cdot 4\pi r^2\big|_{r} = v'' \cdot \frac{4}{3}\pi\left[(r+\Delta r)^3 - r^3\right] \quad (4.49)$$



Letting $\Delta r \to 0$ in Eq. 4.49 and considering Michaelis–Menten intrinsic kinetics and steady state:

$$\frac{d(J' \cdot r^2)}{dr} = r^2 \cdot v'' = r^2 \cdot \frac{V'' \cdot s}{K+s} \quad (4.50)$$

and considering now the first Fick's law of diffusion:

$$J' = D \cdot \frac{ds}{dr} \quad (4.51)$$

from Eqs. 4.50 and 4.51:

$$D\frac{d^2s}{dr^2} + \frac{2D}{r}\frac{ds}{dr} - \frac{V'' \cdot s}{K+s} = 0 \quad (4.52)$$

As in the case of the enzyme membrane (Eq. 4.37), Eq. 4.52 has no analytical solution for Michaelis–Menten intrinsic kinetics and has to be solved numerically considering the following boundary conditions:

$$\text{I)} \quad \begin{matrix} r = R \\ s = s_0 \end{matrix} \qquad \text{II)} \quad \begin{matrix} r = 0 \\ \dfrac{ds}{dr} = 0 \end{matrix}$$

Again, boundary condition I) assumes that EDR is negligible with respect to IDR so that $s_S = s_0$. Writing Eq. 4.52 in dimensionless form, by defining the corresponding terms (see Nomenclature):

$$\frac{d^2\beta}{d\rho^2} + \frac{2}{\rho}\frac{d\beta}{d\rho} - 9\Phi_{sp}^2\frac{\beta}{1+\beta} = 0 \quad (4.53)$$

$$\text{I)} \quad \begin{matrix} \rho = 1 \\ \beta = \beta_0 \end{matrix} \qquad \text{II)} \quad \begin{matrix} \rho = 0 \\ \dfrac{d\beta}{d\rho} = 0 \end{matrix}$$

where ρ is the dimensionless radius (r/R) and:

$$\Phi_{sp} = \frac{R}{3}\sqrt{\frac{V''}{K \cdot D}} \quad (4.54)$$

Numerical solution of Eq. 4.53, obtained by using available software, is given as:

$$\beta = f(\beta_0, \rho, \Phi_{sp}) \quad (4.55)$$

so that the substrate profile within the biocatalytic spherical particle can be calculated for any value of Φ_{sp}, as in the case of the enzyme membrane (Fig. 4.12).

In the case of first order kinetics Eq. 4.53 is simplified to:

$$\frac{d^2\beta}{d\rho^2} + \frac{2}{\rho}\frac{d\beta}{d\rho} - 9\Phi_{sp}^2 \cdot \beta = 0 \tag{4.56}$$

whose analytical solution is:

$$\beta = \beta_0 \frac{\sinh\left[3\Phi_{sp} \cdot \rho\right]}{\rho \cdot \sinh\left[3\Phi_{sp}\right]} \tag{4.57}$$

Then, from the numerical solution (Eq. 4.55), replacing β in Eq. 4.43:

$$\eta_{sp} = f(\beta_0, \rho, \Phi_{sp}) \tag{4.58}$$

which, for the case of first-order kinetics, is simply:

$$\eta_{sp} = \frac{\sinh\left[3\Phi_{sp} \cdot \rho\right]}{\rho \cdot \sinh\left[3\Phi_{sp}\right]} \tag{4.59}$$

From Eq. 4.58, the mean integral value of the spherical particle can be determined as:

$$\eta'_{sp} = \frac{\int_0^1 \eta_{sp} \cdot \rho^2 d\rho}{\int_0^1 \rho^2 d\rho} = 3\int_0^1 \eta_{sp} \cdot \rho^2 d\rho \tag{4.60}$$

so that from Eqs. 4.58 and 4.60:

$$\eta'_{sp} = f(\beta_0, \Phi_{sp}) \tag{4.61}$$

For the case of first-order kinetics, from Eqs. 4.59 and 4.60:

$$\eta'_{sp} = \frac{1}{\Phi_{sp}} \cdot \left[\frac{1}{\tanh(3\Phi_{sp})} - \frac{1}{3\Phi_{sp}}\right] \tag{4.62}$$

Eq. 4.61 is represented in a three-dimensional plot in Fig. 4.14.

As in the case of the enzyme membrane, the evolution of η' with the independent variable β_0 for a given value of Φ_{sp} represents the information required to introduce the effect of IDR in the scheme for reactor design and performance evaluation (see Fig. 3.1).

Case 3: Other Geometries

As mentioned before, the effect of IDR has also been studied for biocatalysts of other defined geometries, like cylinders and spheroids. However, for more complex or undefined geometries the problem can be extremely difficult to solve. In those

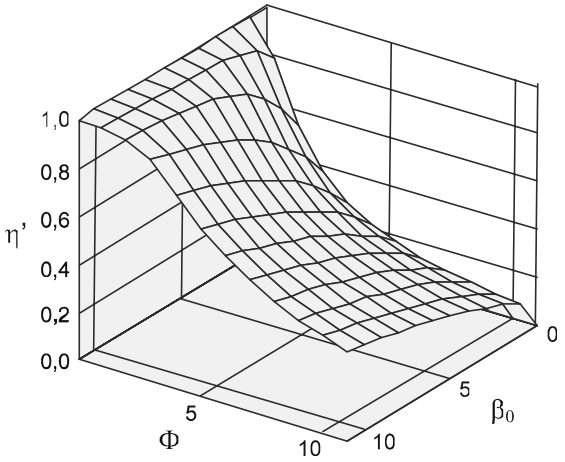


Fig. 4.14 Global effectiveness factor (mean integral value) of an immobilized enzyme with Michaelis–Menten kinetics in a spherical particle as a function of bulk substrate concentration and Thièle modulus

cases an approximate solution can be obtained by redefining the Thièle modulus in terms of the particle equivalent length as:

$$\Phi = L_{eq} \cdot \sqrt{\frac{V''}{K \cdot D}} \qquad (4.63)$$

$$L_{eq} = \frac{\text{volume of particle}}{\text{surface area of particle}}$$

Defined in this way, the solution can be approximated by using the equations derived for the particle of defined geometry (i.e. a flat membrane or a sphere) that better resembles it. However, solution can be quite approximate. We compared the solution for oblate spheroidal particles with the approximate solution of the sphere with the redefined Thièle modulus according to Eq. 4.63 and found differences as high as 30% (Soto et al. 2002).

4.4.2.2 Determination of Intrinsic Kinetic and Mass-Transfer Parameters

To assess the impact of IDR on enzyme kinetics, the value of intrinsic kinetics (V'' and K) and mass transfer (D_{eff}) parameters must be evaluated. Several strategies have been proposed to approximate the value of the intrinsic kinetic parameters. A reliable experimental procedure is the one proposed by Benaiges et al. (1986) which is basically based on comminuting the support to obtain particles so small than IDR becomes negligible (very low Φ_{sp}; see Eq. 4.54). Kinetic parameters can be determined then with that comminuted biocatalyst to have an estimate on the intrinsic values. Effectiveness factor can be approached then to the ratio of initial rates for the intact and comminuted biocatalyst (Kobayashi and Laidler 1973). An obvious drawback of this approach is that not always a biocatalyst particle small enough can be obtained to be free of IDR (effectiveness factor = 1). If a smooth correlation exists between effectiveness factor and particle size, extrapolation to size zero could give an approximate value and intrinsic kinetic parameters can be

estimated. Another objection to this approach is to what extent the comminuted biocatalyst has suffered significant structural changes so as to poorly represent the structure of the intact biocatalyst. Another approach is to freed the immobilized enzyme from its support and determine the kinetic parameters of the freed enzyme. The effectiveness factor will be in this case the ratio between the specific activity of the immobilized enzyme, and the specific activity of the freed enzyme from a similar amount of support (Müller and Zwing 1982). This procedure can be more objectionable than the former, since conditions for removing the enzyme from its support may severely affect enzyme structure (this will be particularly so in the case of covalently bound enzymes) and, as a consequence, inactivation may occur and their kinetic behavior change significantly. However, it has been argued that it is a good approach for reversibly immobilized enzymes. Another strategy to approach the values of the intrinsic parameter is to reduce the protein load in the support to a point in which the mass specific activity of the biocatalyst (units of activity per unit mass of support) is low enough to move the system away from mass transfer control, but again it is arguable to what extent that biocatalyst resembles in its kinetic behavior the one with the higher protein load. Experiments to determine intrinsic parameters of immobilized enzymes under IDR are conveniently designed to avoid EDR. For that purpose, the effect of agitation rate is evaluated to the point when the rate of reaction becomes independent on it; at higher agitation rates the system can be considered free of EDR and the net effect of IDR be revealed.

Besides intrinsic kinetic parameters, mass transfer parameter, this is, the effective diffusion coefficient for substrate within the biocatalyst particle should be determined. Values of diffusion coefficients for a large number of substances can be found in chemical or biochemical handbooks of properties. However, these values correspond to diffusion in water, usually at a reference temperature. Some empirical correlations, like Eq. 4.64, have been proposed to determine D_{eff} within porous matrices from the corresponding values in water:

$$D_{eff} = D_0 \frac{\varepsilon}{\zeta} \tag{4.64}$$

Depending on the pore structure of the biocatalyst, ε can vary from 0.4 and 0.8, while ζ may vary from 1 to 2 (Engasser 1978); therefore, D_{eff} can vary somewhat between 20% and 80% of D_0. Values of D_{eff} may be determined experimentally for the biocatalyst particle by working with tracer substances that can be the chemically or radioactively labeled substrate. A very interesting option is the procedure proposed by Grünwald (1989), according to which the biocatalyst particles devoid of enzyme (better if containing the previously inactivated enzyme) are equilibrated in a saturated solution of the corresponding solute (i.e. the substrate) and then placed in a buffer solution where the variation of concentration of solute with time (effusion) is recorded. For a spherical particle the following expression ensues:

$$\frac{s_t - s_\infty}{s_i - s_\infty} = \frac{6}{\pi^2} \sum_{n=1}^{\infty} n^{-2} \exp\left(-\frac{n^2 \pi^2 D_{eff}}{R^2} \cdot t\right) \tag{4.65}$$

where the subscripts i, t and ∞ mean zero time, any time and infinite time. If time is long enough the above equation reduces to:

$$\frac{s_t - s_\infty}{s_i - s_\infty} \cong \frac{6}{\pi^2} \exp\left(-\frac{\pi^2 D_{eff}}{R^2} \cdot t\right) \tag{4.66}$$

which, considering $s_i = 0$, can be linearized to:

$$\ln\left(\frac{s_\infty - s_t}{s_\infty}\right) = -\frac{\pi^2 \cdot D_{eff}}{R^2} \cdot t + \text{constant} \tag{4.67}$$

from which D_{eff} can be determined. The corresponding Thièle modulus can be determined then from Eq. 4.54.

The simultaneous determination of intrinsic and mass transfer parameters for biocatalysts under IDR was proposed by Engasser and Horvath (1973) using Lineweaver-Burk (and Hanes) plots. Correlations are non-linear if affected by diffusional restrictions: in the case of EDR a smooth monotonic curve is obtained, while in the case of IDR an inflection point is observed, being this a simple way of qualitatively assessing diffusional restrictions. By measuring initial rate data at different substrate concentrations, two extreme zones are revealed: zone I corresponding to $s \ggg K$ and zone II corresponding to $s \lll K$, where correlations are linear in Lineweaver-Burk plot. If enough data is gathered in zone I, intrinsic parameters V'' and K can be obtained conventionally from the Y and X-axis intercepts from the extrapolated portion of that linear region. If enough data is gathered in zone II, the effectiveness factor ca be directly obtained from the intercept in the Y-axis $(= 1/V'' \cdot \eta')$ from the extrapolated portion of that linear region. The method has the obvious drawback of working in those zones were the experimental error is at its highest level. Other methods, similar to the one above, have been proposed (Kobayashi and Laidler 1973; Lee et al. 1981; Ishikawa et al. 1987); however, in those methods the values of D_0 and D_{eff} need to be determined separately, either experimentally or using empirical correlations. Other methods are based on optimization algorithms (Luus and Jaakola 1973) that have been used for the determination of intrinsic kinetic parameters (Khorasheh et al. 2002) and applied to penicillin acylase immobilized in silica particles (Kheirolomoom et al. 2002). Nowadays, there are very robust optimization methods to evaluate kinetic parameters in non-linear systems; therefore, it is no longer strictly necessary to use linear correlations like the ones by Lineweaver-Burk or Hanes. However, they are still irreplaceable to visualize the interplay between enzyme kinetics and mass transfer limitations qualitatively.

4.4.2.3 Interplay Between Internal Diffusional Restrictions and Inhibition

A similar analysis than the one previously presented for simple Michaelis–Menten kinetics can be made for more complex kinetics involving reversible Michaelis–Menten reactions or product and substrate inhibition kinetics. Equations for each particular case and the corresponding boundary conditions for the case of spherical biocatalysts are (Jeison et al. 2003):

- For reversible Michaelis–Menten kinetics (Eq. 3.14):

$$\frac{d^2\beta}{d\rho^2}+\frac{2}{\rho}\frac{d\beta}{d\rho}-9\Phi_{sp}^2\frac{\beta-\beta_{eq}}{1+\beta+\beta_{eq}}=0 \tag{4.68}$$

$$\text{I)}\quad \rho=1;\ \beta=\beta_0 \qquad \text{II)}\quad \rho=0;\ \frac{d\beta}{d\rho}=0$$

- For uncompetitive inhibition by substrate (Eq. 3.42):

$$\frac{d^2\beta}{d\rho^2}+\frac{2}{\rho}\frac{d\beta}{d\rho}-9\Phi_{sp}^2\frac{\beta}{\beta(1+\kappa\cdot\beta)+1}=0 \tag{4.69}$$

$$\text{I)}\quad \rho=1;\ \beta=\beta_0 \qquad \text{II)}\quad \rho=0;\ \frac{d\beta}{d\rho}=0$$

- For competitive product inhibition (Eq. 3.40):

$$\frac{d^2\beta}{d\rho^2}+\frac{2}{\rho}\frac{d\beta}{d\rho}-9\Phi_{sp}^2\frac{\beta}{1+\beta+\gamma}=0 \tag{4.70}$$

$$\frac{d^2\gamma}{d\rho^2}+\frac{2}{\rho}\frac{d\gamma}{d\rho}+9\Phi_{sp,P}^2\frac{\beta}{1+\beta+\gamma}=0 \tag{4.71}$$

$$\text{I)}\quad \rho=1;\ \beta=\beta_0;\ \gamma=\gamma_0 \qquad \text{II)}\quad \rho=0;\ \frac{d\beta}{d\rho}=\frac{d\gamma}{d\rho}=0$$

where:

$$\Phi_{sp,P}=\frac{R}{3}\sqrt{\frac{V''}{K_P\cdot D_P}}$$

- For non-competitive product inhibition:

$$\frac{d^2\beta}{d\rho^2}+\frac{2}{\rho}\frac{d\beta}{d\rho}-9\Phi_{sp}^2\frac{\beta}{(1+\beta)\cdot(1+\gamma)}=0 \tag{4.72}$$

$$\frac{d^2\gamma}{d\rho^2}+\frac{2}{\rho}\frac{d\gamma}{d\rho}+9\Phi_{sp,P}^2\frac{\beta}{(1+\beta)\cdot(1+\gamma)}=0 \tag{4.73}$$

$$\text{I)}\quad \rho=1;\ \beta=\beta_0;\ \gamma=\gamma_0 \qquad \text{II)}\quad \rho=0;\ \frac{d\beta}{d\rho}=\frac{d\gamma}{d\rho}=0$$

Substrate (and product) profiles are obtained from the numerical resolution of the above differential equations (system of differential equations in the case of product inhibition). The corresponding local effectiveness factors (ratio of effective and intrinsic reaction rates) are then calculated and the global effectiveness factor determined from their profiles, as in the case of simple Michaels–Menten kinetics. Results are represented in three-dimensional plots in Figs. 4.15 to 4.18 respectively.

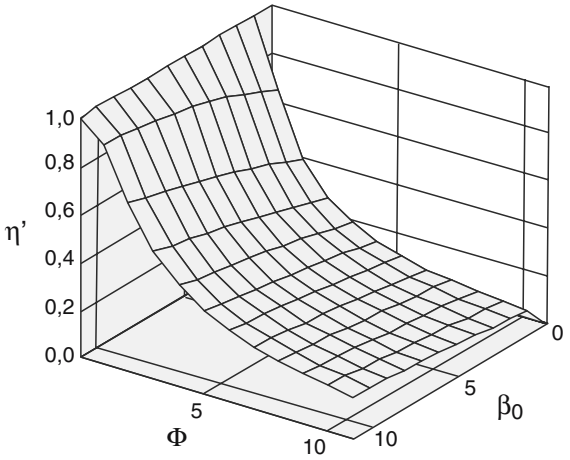


Fig. 4.15 Global effectiveness factor (mean integral value) of an immobilized enzyme with reversible Michaelis–Menten kinetics in a spherical particle as a function of bulk substrate concentration and Thièle modulus (substrate conversion 0.4; substrate conversion at equilibrium: 0.5)

4.4.3 Combined Effect of External and Internal Diffusional Restrictions

Up to now, the impact of EDR on biocatalysts subjected to IDR has been considered negligible. This can be so in many but not all occasions. The combined effect of

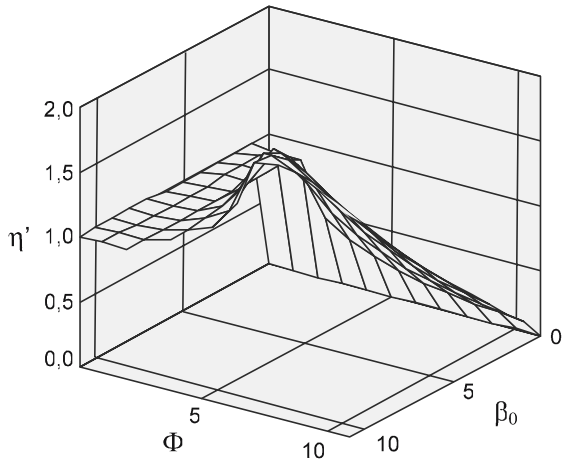


Fig. 4.16 Global effectiveness factor (mean integral value) of an immobilized enzyme with uncompetitive substrate inhibition kinetics in a spherical particle as a function of bulk substrate concentration and Thièle modulus ($\kappa = 1$)

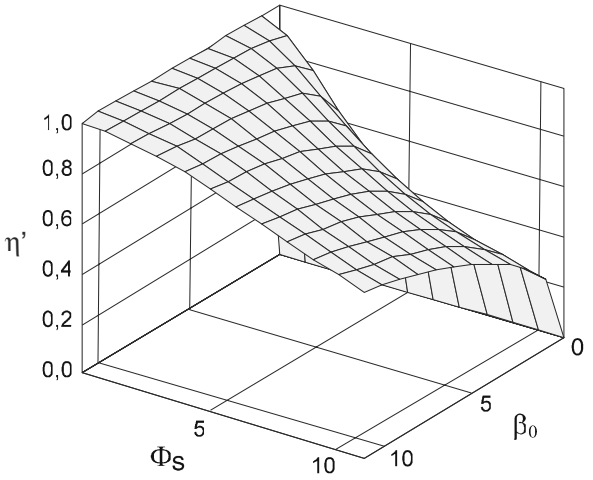


Fig. 4.17 Global effectiveness factor (mean integral value) of an immobilized enzyme with competitive product inhibition kinetics in a spherical particle as a function of bulk substrate concentration and Thièle modulus ($K/K_1 = 1$; substrate conversion = 0.9)

EDR and IDR can be conveniently analyzed by replacing the boundary conditions I) in the corresponding differential equations. For instance, in the case of the enzyme membrane (Eq. 4.38 or 4.41), and the spherical particles (Eq. 4.53 or 4.56), boundary conditions I) are replaced by equations of continuity in the biocatalyst surface

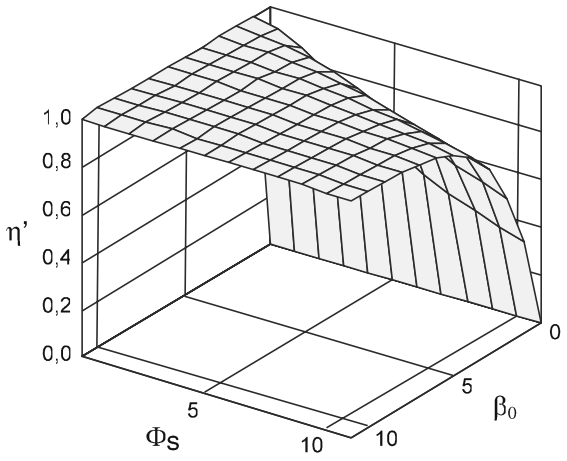


Fig. 4.18 Global effectiveness factor (mean integral value) of an immobilized enzyme with noncompetitive product inhibition kinetics in a spherical particle as a function of bulk substrate concentration and Thièle modulus ($K/K_2 = 1$; substrate conversion = 0.9)

(Eqs. 4.74 and 4.75 respectively):

$$h'(s_0 - s_b) = -D \cdot \left.\frac{ds}{dx}\right|_{x=0} \tag{4.74}$$

$$h'(s_0 - s_b) = D \cdot \left.\frac{ds}{dr}\right|_{r=R} \tag{4.75}$$

whose dimensionless forms are Eqs. 4.76 and 4.77 respectively:

$$Bi \cdot (\beta_0 - \beta_S) = -\left.\frac{d\beta}{dz}\right|_{z=0} \tag{4.76}$$

$$Bi' \cdot (\beta_0 - \beta_S) = \left.\frac{d\beta}{d\rho}\right|_{\rho=1} \tag{4.77}$$

Bi is the dimensionless Biot number defined as:

$$Bi = \frac{h' \cdot L_{eq}}{D} \tag{4.78}$$

being $L_{eq} = L$ for he membrane and $L_{eq} = R/3$ for the sphere.

The Biot number represents the relative impact of EDR with respect to IDR. A high Bi implies that external mass transfer rate is higher than internal (or not being so, the particle size is large enough), so that EDR are negligible and the former analysis is adequate. On the contrary a small Bi indicates that EDR are relevant, either because external mass transfer rate is smaller than internal or, not being so, the particle size is small enough. In this case, Eqs. 4.76 and 4.77 have to be used as boundary conditions I). The problem has been solved for the case of first-order kinetics (Eqs. 4.41 and 4.56 respectively), being the corresponding analytical solutions (named asymptotic solution) for the effectiveness factors:

$$\eta' = \frac{\tanh\frac{\Phi}{2}}{\frac{\Phi}{2}\left(1 + \frac{\Phi}{2} \cdot \tanh\frac{\frac{\Phi}{2}}{Bi}\right)} \tag{4.79}$$

$$\eta'_{sp} = \frac{Bi\left(\frac{1}{\tanh(3\Phi_{sp})} - \frac{1}{3\cdot\Phi_{sp}}\right)}{\Phi_{sp}\left(Bi - 1 + \frac{3\Phi_{sp}}{\tanh(3\cdot\Phi_{sp})}\right)} \tag{4.80}$$

It is easily seen that if $Bi \ggg 1$ (EDR irrelevant) Eqs. 4.79 and 4.80 are reduced to Eqs. 4.48 and 4.62 respectively.

Nomenclature

A	cross-section area of the biocatalyst	$[L^2]$
A_{sp}	mass (or volumetric) specific activity of enzyme biocatalyst	$[UIM^{-1}]$ $[UIL^{-3}]$
a_e	product accumulation factor at biocatalyst surface	
Bi	dimensionless number of Biot	
C	constant in Eq. 4.20	
D	substrate diffusion coefficient	$[L^2T^{-1}]$
D_P	product diffusion coefficient	$[L^2T^{-1}]$
D_0	substrate diffusion coefficient in water	$[L^2T^{-1}]$
D_{eff}	substrate diffusion coefficient in the biocatalyst particle	$[L^2T^{-1}]$
E_C	contacted enzyme activity	[UI]
E_I	immobilized enzyme activity	[UI]
E_L	activity lost by immobilization	[UI]
E_R	residual enzyme activity in solution after immobilization	[UI]
G	mass flux	$[ML^{-2}T^{-1}]$
h	film volumetric mass transfer coefficient for substrate	$[L3T^{-1}]$
h_P	film volumetric mass transfer coefficient for product	$[L3T^{-1}]$
h'	film linear mass transfer coefficient	$[LT^{-1}]$
h^+	proton molar concentration	$[ML^{-3}]$
$h^+{}_0$	proton molar concentration in the bulk	$[ML^{-3}]$
J	substrate flow rate	$[MT^{-1}]$
J'	substrate flux	$[ML^{-2}T^{-1}]$
K	intrinsic Michaelis–Menten (dissociation) constant for the ES complex into E and S	$[ML^{-3}]$
K_I	Intrinsic product competitive inhibition constant	$[ML^{-3}]$
K_P	Intrinsic product inhibition constant	$[ML^{-3}]$
K_{ph^+}	partition coefficient for protons	
K_{ps}	partition coefficient for substrate	
k_B	Boltzman universal constant	
L	catalytic membrane width [L]	[L]
L_{eq}	particle equivalent length	[L]
M	biocatalyst mass	[M]
	(or volumen)	$[L^3]$
P_C	contacted protein	[M]
P_I	immobilized protein	[M]
P_R	residual protein in solution after immobilization	[M]
P_{load}	protein load of the biocatalyst	[M]
p_0	molar concentration of product in the bulk	$[ML^{-3}]$
p_S	molar concentration of product at biocatalyst surface	$[ML^{-3}]$
R	radius of spherical particle	[L]
r	rate of substrate transformation into product	$[MT^{-1}]$
	alternatively: variable radius of spherical particle	[L]

s	molar concentration of substrate	$[ML^{-3}]$
s_0	molar concentration of substrate in the bulk	$[ML^{-3}]$
s_S	molar concentration of substrate at the biocatalyst surface	$[ML^{-3}]$
T	absolute temperature	$[\Theta]$
t	time	[T]
V'	maximum reaction rate	$[MT^{-1}]$
V''	maximum reaction rate per unit mass of biocatalyst	$[ML^{-3}T^{-1}]$
v'	reaction rate	$[MT^{-1}]$
v''	reaction rate per unit mass of biocatalyst	$[ML^{-3}T^{-1}]$
x	variable width of catalytic membrane	[L]
Y_E	yield of enzyme immobilization	
Y'_E	yield of enzyme immobilization considering recovery of the unbound enzyme	
Y_P	yield of protein immobilization	
Z	valence of the species	
z	dimensionless catalytic membrane width	
α	dimensionless number of Damkoehler	
β	dimensionless substrate concentration	
β_{eq}	dimensionless equilibrium substrate concentration	
β_0	dimensionless substrate concentration in bulk reaction medium	
β_S	dimensionless substrate concentration at the biocatalyst surface	
ε	void fraction (porosity)	
$\in$	electron charge	
γ	dimensionless product concentration	
γ_0	dimensionless product concentration in bulk reaction medium	
γ_S	dimensionless product concentration at the biocatalyst surface	
δ	hypothetical stagnant film width	[L]
κ	ratio of Michaelis constant to substrate inhibition constant	
μ	fluid viscosity	
η	local effectiveness factor	
η_I	effectiveness factor under inhibition	
η_{sp}	local effectiveness factor for sphere	
η'	global (mean integral value) of effectiveness factor	
η'_{sp}	global (mean integral value) of effectiveness factor for sphere	
φ	electrostatic potential of the support	
Φ	dimensionless Thièle modulus for substrate	
Φ_{sp}	dimensionless Thièle modulus for substrate (spherical particle)	

Φ_P	dimensionless Thièle modulus for product	
$\Phi_{sp,P}$	dimensionless Thièle modulus for product (spherical particle)	
ρ	dimensionless radius of the spherical particle	
ρ'	fluid density	$[ML^{-3}]$
ζ	tortuosity of catalyst pores	
ν	dimensionless reaction rate	

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Chapter 5
Enzyme Reactors

Andrés Illanes* **and Claudia Altamirano****

5.1 Types of Reactors, Modes of Operation

More than 80% of the commercial value of enzymes is linked to their applications as process catalysts. Hydrolytic reactions conducted mainly with the enzyme dissolved in the aqueous medium has been the traditional way of using enzymes, this technology still representing a major share of enzyme processes. However, in recent decades the use of enzymes in organic synthesis has widened its scope of application to unprecedented levels.

Enzyme reactors can operate batch-wise or continuously; fed-batch operation has also been proposed (Kumar et al. 1996). Batch processes with the enzymes (usually hydrolases) dissolved in an aqueous reaction medium, despite its wide application have several drawbacks, since enzymes are poorly stable and hard to recover in such systems, leading to low productivity; besides, such processes are characterized by a rather low added value so that process optimization is critical for being and keeping competitive. Poor stability is usually the limiting factor in any enzyme process so that enzyme stabilization during reactor operation is a major concern (Ballesteros et al. 1998; O'Fágáin 2003) and among the many strategies for enzyme stabilization (Illanes 1999) enzyme immobilization is the most relevant (Guisán 2006). Immobilized enzymes can be used in batch processes but in this case the enzyme is recovered to be used in subsequent batches until the accumulated inactivation makes necessary to replace the spent biocatalyst. As a consequence, specific productivity (mass of product/mass of biocatalyst · time of operation) is increased and bioreactor design becomes flexible to suit the particular needs of a given process.

Immobilized enzymes, despite the complexities introduced by the heterogeneous nature of the catalytic process, are usually much more stable than their soluble

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counterparts, are easily recovered from the reaction medium and reused, product contamination with biocatalyst is avoided and continuous operation becomes feasible (Katchalsky-Katzir 1993). Continuous operation of enzyme reactors is to a large extent linked to immobilized enzymes since stability should be high to justify a continuous operation. The notable exception is the case of starch liquefaction with bacterial α-amylase (see section 1.5) where the soluble enzyme is used in the continuous liquefaction of cornstarch in the manufacture of high-fructose syrup; the enzyme is continuously dosed to a tubular reactor where hydrolysis and starch gelatinization occur simultaneously (Linko et al. 1975). In this case, the low cost of the enzyme and its little significance in operation cost allows a certainly quite inefficient use of the biocatalyst. But in most cases continuous processes are conducted with immobilized enzymes. Despite the advantages of continuous processes with immobilized enzymes, industry has been reluctant to adopt it. Reluctance to modify existing conventional processes and the costs associated to process control, yield losses during immobilization and mass transfer limitations during operation have precluded a more extended use of immobilized enzymes as process biocatalysts. This situation is changing as new processes emerge based on biocatalyst stability and new strategies of enzyme immobilization are developed (D'Souza 1999; Guisán 2006).

Several reactor configurations have been proposed and used for conducting enzyme-catalyzed processes, as shown in Fig. 5.1. Batch operations with soluble enzymes are conducted mainly in stirred tank reactors (BSTR) provided with mixing

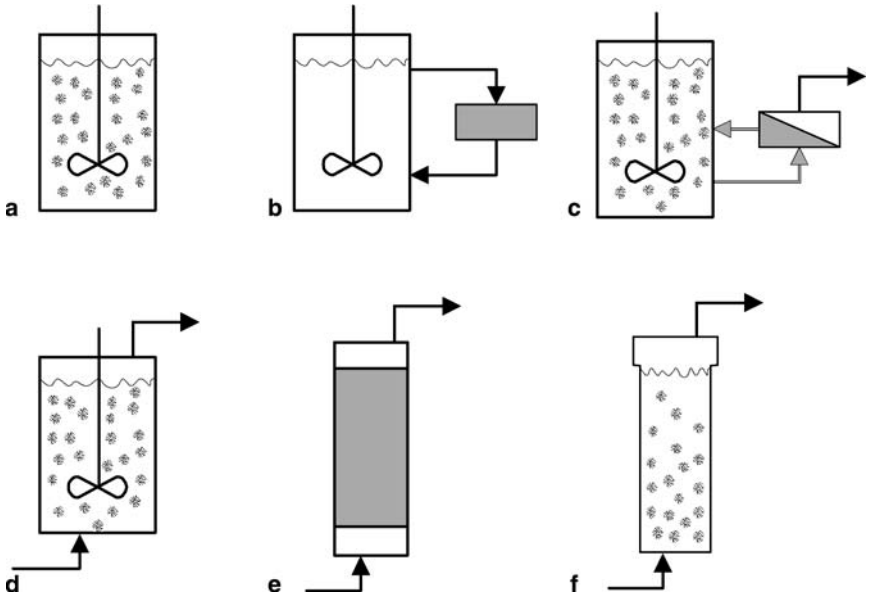


Fig. 5.1 Different configurations of reactors with immobilized enzymes: A: batch; B: recirculation batch; C: stirred tank-ultrafiltration; D: continuous stirred tank; E: continuous packed-bed; F: continuous fluidized-bed

elements and systems for temperature and pH control. Batch operations with immobilized enzymes are also conducted in stirred tank reactors, but in this case a device is included to allow biocatalyst retention after product recovery at the end of each batch. A bottom stainless steel screen is the most used system but other options exist like basket type reactors and also in-situ and ex-situ biocatalyst retention in ultrafiltration modules. Recirculation batch reactors (RBR) have been used in occasions; the enzyme is packed in the form of a narrow bed through which the reaction medium is circulated up to the point when the desired conversion is attained (Sudhakaran et al. 1992). This configuration allows a smooth operation when the reaction involves proton production or consumption and the enzyme is sensitive to pH variation. Since the conversion per pass is low (and controllable at will), so it is the pH change that is controlled in the recirculation chamber; in this way the enzyme is never in direct contact with the acid or base used for pH control. The options for continuous operation wit immobilized enzymes are many, as shown in Fig. 5.1. The most used are the packed-bed column reactors (Marrazzo et al. 1975; Munro et al. 1981; Verhoff and Schlager 1981) where the immobilized enzyme is fixed within the reactor while the substrate stream passes through, and the stirred tank reactor where the enzyme is retained in the reactor by an appropriate screen or recovered by ex-situ filtration or centrifugation and recycled back into the reactor (Vásquez-Bahena et al. 2004). An alternative is the expanded or fluidized bed reactor (Allen et al. 1979; Ching and Chu 1988) where the enzyme particles are retained by a hydrodynamic balance between gravity and drag forces promoted by the upflow substrate stream.

5.2 Basic Design of Enzyme Reactors

5.2.1 Design Fundamentals

The decision on what type of reactor to use is the first step in design and sometimes can be taken by a merely qualitative analysis, attending to the different characteristics of each type and its suitability to the particular process. Continuous processes are usually selected for rather large scale production. Continuous packed bed reactors (CPBR), if considered in a plug-flow regime (see next section), will be a better choice than continuous stirred tank reactors (CSTR) in most cases by strictly kinetic considerations (see next section); however they have the drawback that reaction conditions (i.e. pH, temperature) are difficult to control especially when scaling-up to high-level production. The opposite holds for CSTR; continuous fluidized bed reactors (CFBR) can be considered somewhat in between. Properties of the biocatalyst will also be determinants: if sensitive to compression CPBR can be inadequate because bed compaction and channeling may occur; if sensitive to shear forces, CSTR can be inadequate because of biocatalyst attrition. The properties of the feed stream are also determinants: if a limpid substrate solution is used, CPBR is adequate, but if it has a considerable amount of suspended material

bed clogging will hamper operation and make necessary frequent washing cycles that will significantly reduce productivity.

Selection of the most adequate reactor configuration for the hydrolysis of penicillin G with penicillin acylase can be used as an illustrative example (see section 6.2). The enzyme catalyzes the hydrolysis of penicillin G to 6 aminopenicillanic acid (non-competitive inhibitor) and phenylacetic acid (competitive inhibitor). The former is a weak acid poorly dissociated but the later is rather strong so that protons are produced as reaction proceeds. The enzyme is quite sensitive to pH (Ospina et al. 1996) and optimal pH is around 8 (it varies between 7.5 and 8.5 according to the source). With this information one can make an educated guess of what reactor configuration is the more adequate. For kinetic considerations CSTR is inadequate since both products of reaction are inhibitors (see next section) but it offers a good option for pH control during reaction; CPBR, though sound from a kinetic perspective, is also inadequate because of the difficulty in controlling pH throughout the biocatalyst bed. BSTR, despite its reduced productivity as a consequence of a higher proportion of unproductive time, is a better choice since it is similar to CPBR from a kinetic perspective (see section 6.2) and similar to CSTR with respect to pH control (Savidge 1984; Shewale and Sudhakaran 1997). RBR is even a better choice than CPBR since in this case pH control is exerted in a different chamber and so there is no direct contact of the enzyme with the base added to control the pH. In fact this kind of reactor has been proposed as the best option to perform the hydrolysis of penicillin G (Vandamme 1988). A similar analysis can be done for the production of 7-amino-3-desacetoxy cephalosporanic acid from cephalosporin G with immobilized penicillin acylase (Pan and Syu 2004).

Enzyme reactor design refers to the determination of the reactor size (operational volume) required to perform a given production task. For an already available reactor, design refers to the evaluation of its performance in the fulfilment of the production task. A scheme for the construction of a model for enzyme reactor design and performance evaluation was already presented in Fig. 3.1. There are basically four components on that scheme that refers to material balance, enzyme kinetics, enzyme inactivation and mass transfer limitations. The first two are fundamental components of this scheme: the material balance, that reflects the magnitude of the process and the mode of operation, and the kinetic model, that describes the rate of the reaction catalyzed by the enzyme. A basic design of enzyme reactor operation can be made considering these two components. However, other components can be taken into consideration, like non-ideal flow patterns within the bioreactor, enzyme inactivation and mass transfer limitations in the case of heterogeneous catalysis (i.e. immobilized enzymes). Non-ideal flow has been thoroughly analyzed for chemical reactors (Levenspiel 1972) and biochemical reactors (Lübbert and Jørgenssen 2001). Enzyme inactivation during bioreactor operation is a very important component of enzyme reactor design (see section 3.5) since, irrespective of their robustness, enzyme biocatalyst will always be forced to operate down to the point where a significant fraction of its initial activity has been lost (Illanes and Wilson 2003). Mass transfer limitations in heterogeneous enzyme catalysis (see Chapter 4) has been extensively studied as well (Messing 1975; Engasser and Horvath 1976; Chaplin

and Bucke 1990; Palazzi and Converti 2001) and applied to enzyme reactor design (Vieth et al. 1976; Illanes et al. 1992).

5.2.2 *Basic Design of Enzyme Reactors Under Ideal Conditions. Batch Reactor; Continuous Stirred Tank Reactor Under Complete Mixing; Continuous Packed-Bed Reactor Under Plug Flow Regime*

Basic design and performance evaluation of an enzyme reactor refers to ideal conditions of operation, this is: ideal flow regime (completely mixed if CSTR or BSTR; plug-flow if CPBR), controlled and constant operational variables (pH and temperature), full retention of enzyme activity throughout its operation and absence of mass transfer limitation in the case of heterogeneous catalysis. Departure from ideal behavior can be produced by non-ideal flow patterns (incomplete mixing in the case of stirred reactors, backmixing in the case of packed-bed reactors), non-isothermal operation because of heat transfer limitations, variable pH because of insufficient mixing and control, enzyme losses during operation because of thermal inactivation or elution from the support if immobilized, and presence of partition effects or diffusional restrictions (see sections 4.3 and 4.4) in the case of heterogeneous catalysis. Basic design of enzyme reactor operation, both batch and continuous, will be presented first as a background to include afterwards the effects of enzyme inactivation and mass transfer limitation.

A generalized kinetic expression for one-substrate reactions was presented in section 3.3.2 that considers all possible mechanisms for one-substrate reactions (or reactions in which one of the substrates is clearly limiting as it occurs in most hydrolytic reactions in aqueous medium):

$$v = \frac{k_{cat} \cdot e \cdot s \cdot \left(1 + \frac{k' \cdot s}{k \cdot K''} + \frac{k'' \cdot K \cdot p}{k \cdot K_2 \cdot K^{iv}}\right)}{s \cdot \left(1 + \frac{s}{K''} + \frac{K \cdot p}{K_2 \cdot K^{iv}}\right) + K \cdot \left(1 + \frac{s}{K'}\right) + K \cdot p \cdot \left(\frac{1}{K_1} + \frac{1}{K_2} + \frac{p}{K_1 \cdot K_2'}\right)} \tag{3.41}$$

(nomenclature for kinetic parameters is in Chapter 3).

Substrate conversion is defined as:

$$X = \frac{(s_i - s)}{s_i} = \frac{n \cdot p}{s_i} \tag{5.1}$$

which for the usual case of an equimolar reaction $(n = 1)$ is:

$$\begin{aligned} X &= \frac{(s_i - s)}{s_i} = \frac{p}{s_i} \\ s &= s_i\,(1 - X) \\ p &= s_i \cdot X \end{aligned} \tag{5.2}$$

Replacing Eq. 5.2 into Eq. 3.41 and considering inactive ternary enzyme–substrate–inhibitor complexes, the following expression is obtained considering the reaction scheme in section 3.3.2 (Illanes 1994):

$$v = \frac{k_{cat} \cdot e(1-X)}{a + bX + cX^2} \tag{5.3}$$

where:

$$a = 1 + \frac{K}{s_i} + \frac{K}{K'} + \frac{s_i}{K''}$$

$$b = K\left(\frac{1}{K_1} + \frac{1}{K'} - \frac{1}{K''}\right) + s_i\left(\frac{1}{K_2''} - \frac{2}{K''}\right) - 1$$

$$c = s_i\left(\frac{1}{K''} - \frac{1}{K_2''} + \frac{K}{K_1 K_2'}\right)$$

Reactions of synthesis with two substrates can be described according to sequential (ordered or random) or oscillatory (*ping-pong*) mechanisms, as presented in section 3.4.1 for the reaction of synthesis:

$$A + B \rightarrow Pi$$

From Eqs. 3.52, Eqs. 5.4 and 5.4′ are obtained for the sequential ordered mechanism considering A as the limiting substrate $(a = a_i(1-X);\ b = b_i - a_i \cdot X)$, and B as the limiting substrate $(b = b_i(1-X);\ a = a_i - b_i \cdot X)$ respectively:

$$v = \frac{k_{cat} \cdot e \cdot a \cdot b}{a \cdot b + K_B' \cdot a + K_A \cdot K_B'} = \frac{k_{cat} \cdot e \cdot a_i(1-X) \cdot (b_i - a_i \cdot X)}{a_i(1-X)(b_i - a_i \cdot X + K_B') + K_A \cdot K_B{}'} \tag{5.4}$$

$$v = \frac{k_{cat} \cdot e \cdot a \cdot b}{a \cdot b + K_B' \cdot a + K_A \cdot K_B'} = \frac{k_{cat} \cdot e \cdot (a_i - b_i \cdot X) \cdot b_i(1-X)}{(a_i - b_i \cdot X)(b_i(1-X) + K_B') + K_A \cdot K_B{}'} \tag{5.4'}$$

From Eq. 3.59, Eq. 5.5 is obtained for the sequential random mechanism considering A as he limiting substrate $(a = a_i(1-X);\ b = b_i - a_i \cdot X)$:

$$\begin{aligned} v &= \frac{k_{cat} \cdot e \cdot a \cdot b}{a \cdot b + K_B' \cdot a + K_A' \cdot b + K_A \cdot K_B'} \\ &= \frac{k_{cat} \cdot e \cdot a_i(1-X) \cdot (b_i - a_i \cdot X)}{a_i(1-X)(b_i - a_i X + K_B') + K_A'(b_i - a_i X) + K_A \cdot K_B'} \end{aligned} \tag{5.5}$$

A basic model to describe enzyme reactor operation can be constructed from a material balance of the process and a suitable kinetic expression, as shown in Fig. 3.1.

5.2.2.1 Batch Enzyme Reactor

Operation is discontinuous: the reactor is filled with the reaction medium containing the substrate(s) and operating conditions adjusted; then the enzyme is added

and the reaction is left to proceed until the desired conversion has been obtained. Afterwards the enzyme, if dissolved in the reaction medium, is inactivated, the reactor is emptied and the reacted medium containing the product subjected to downstream operations. If the enzyme is immobilized, the biocatalyst is retained within the reactor by a proper screen, and then washed to be ready for the following batch.

A material balance on the reactor renders:

$$v(e,X) = -\frac{ds}{dt} = s_i \frac{dX}{dt} \tag{5.6}$$

$$\int_0^X \frac{dX}{v(e,X)} = \int_0^t \frac{dt}{s_i} \tag{5.7}$$

The integral at the left hand side contains the expression for v(e,X). Therefore, if enzyme inactivation is considered $(e = f(t))$, separation of variables is not possible; however for the case of basic design since no enzyme inactivation is considered e remains constant throughout the reaction and v is only a function of X so that variables can be separated and the Eq. 5.7 can be integrated.

Considering one-substrate reaction, from Eqs. 5.3 and 5.7:

$$\int_0^X \frac{a + bX + cX^2}{1 - X} dX = \int_0^t \frac{k_{cat} \cdot e}{s_i} dt \tag{5.8}$$

$$\frac{s_i}{K}\left[-(a+b+c)\cdot\ln(1-X) - (b+c)\cdot X - 0.5\cdot cX^2\right] = \frac{k_{cat}\cdot e}{K}\cdot t = \frac{m_{cat}\cdot a_s}{K}\cdot t \tag{5.9}$$

Eq. 5.9 represents the model of batch enzyme reactor operation for one-substrate reactions. It is important to point out that this equation reduces in one the number of degrees of freedom of the system but there are still two degrees of freedom that allows flexibility of operation since from the three operational variables: m_{cat}, t and s_i, two can be established separately. A common situation is the use of Eq. 5.9 to determine the enzyme load required (m_{cat}; a_s is a property of the biocatalyst) to obtain the desired substrate conversion (X) for a certain initial substrate concentration (s_i) in a given time (t). The time of reaction can be determined at will to adapt to industrial practice (i.e. duration of one shift) and, in this case, m_{cat} will determine the X attainable for a given s_i. However, as indicated by Eq. 5.9, for a given s_i, the desired X can be obtained by any combination of $m_{cat} \cdot t$, which means that he same result can be obtained working with a high enzyme load for a short period of time or with a lower enzyme load for a longer time, provided that $m_{cat} \cdot t$ is constant. This equation can also be used for bioreactor design, since $m_{cat} = M_{cat}/V_R$. V_R is the volume of reaction and for a stirred tank it represents from 70% to 80% of the reactor volume. Graphical representation of enzyme reactor behavior is in Fig. 5.2 for Michaelis–Menten kinetics and in Fig. 5.3 for different inhibition kinetics (see section 3.3.2 for the corresponding kinetic expressions and the values of a, b and c in

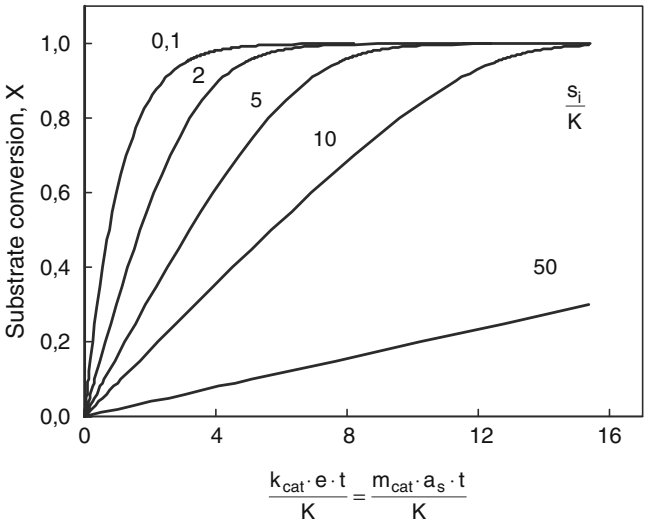


Fig. 5.2 Batch reactor performance with Michaelis–Menten kinetics

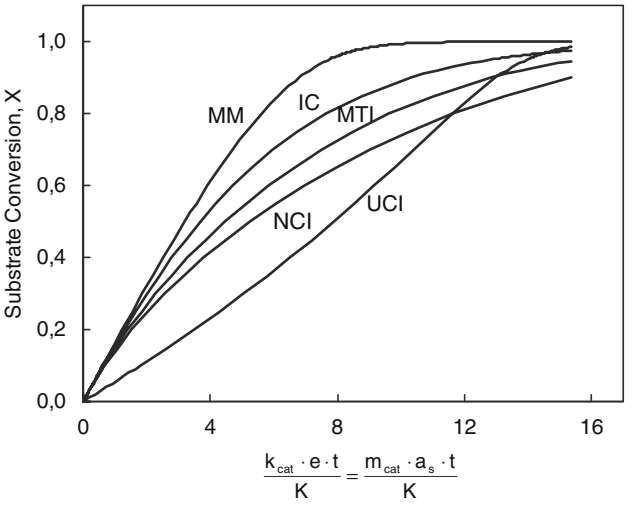


Fig. 5.3 Batch reactor performance with inhibition kinetics. $(s_i/K) = 5$. IC: competitive inhibition $(K/K_1 = 0.5)$; NCI: non-competitive inhibition $(K/K_2 = 0.5)$; MTI: mixed-type inhibition $(K/K_1 = 0.5;\ K_2/K_1 = 0.5)$; UCI: uncompetitive inhibition $(K/K'' = 0.5)$

Eq. 5.3). The expressions for the more common kinetic mechanisms are presented in Table 5.1.

For a reaction of synthesis with two substrates, a material balance over the limiting substrate, A or B, yields Eqs. 5.10 and 5.11 respectively:

$$\int_0^X \frac{dX}{v(e,X)} = \int_0^t \frac{dt}{a_i} \tag{5.10}$$

$$\int_0^X \frac{dX}{v(e,X)} = \int_0^t \frac{dt}{b_i} \tag{5.11}$$

In the case of ordered (first A then B) sequential mechanism, integrating Eqs. 5.10 or 5.11, with the corresponding kinetic expression (Eqs. 5.4 or 5.4′), Eqs. 5.12 and 5.13 are obtained with A or B as limiting substrates respectively:

$$\frac{K_A K'_B}{a_i (b_i - a_i)} \ln \left| \frac{b_i - a_i X}{b_i (1-X)} \right| - \frac{K'_B}{a_i} \ln \left(\frac{b_i - a_i X}{b_i} \right) + X = \frac{k_{cat} \cdot e}{a_i} t = \frac{m_{cat} \cdot a_s}{a_i} t \tag{5.12}$$

$$\frac{K_A K'_B}{b_i (a_i - b_i)} \ln \left| \frac{a_i - b_i X}{a_i (1-X)} \right| - \frac{K'_B}{b_i} \ln (1-X) + X = \frac{k_{cat} \cdot e}{b_i} t = \frac{m_{cat} \cdot a_s}{b_i} t \tag{5.13}$$

In the case of sequential random mechanism (in this case the designation of A or B is indistinct), Eq. 5.14 is obtained by integrating Eq. 5.10 (arbitrarily considering A as the limiting substrate) with the corresponding kinetic expression (Eq. 5.5):

$$\begin{aligned} &\frac{K_A K'_B}{a_i (b_i - a_i)} \ln \left| \frac{b_i - a_i X}{b_i (1-X)} \right| - \frac{K'_B}{a_i} \ln \left| \frac{b_i - a_i X}{b_i} \right| \\ &\quad - \frac{K'_A}{a_i} \ln (1-X) + X = \frac{k_{cat} \cdot e}{a_i} t = \frac{m_{cat} \cdot a_s}{a_i} t \end{aligned} \tag{5.14}$$

Graphical representation of enzyme reactor behavior is presented in Fig. 5.4 for sequential ordered kinetics and in Fig. 5.5 for sequential random kinetics.

5.2.2.2 Continuous Packed-Bed Reactor (CPBR)

Operation is continuous with a constant flow-rate of reaction medium fed to the reactor where the biocatalyst is packed forming a submerged bed. The reactor can be fed from the bottom or the top. At laboratory scale it is often preferred to use bottom feeding because it is easier to maintain the level of liquid above the biocatalyst bed; it also precludes from bed compaction. At large scale top feeding is frequently used

Table 5.1 Models for Enzyme Reactor Performance for One-Substrate Reactions

	$\frac{k_{cat}\cdot e}{K}\cdot t =$ or $\frac{k_{cat}\cdot E}{F\cdot K} =$	
Model	CPBR or BSTR	CSTR
General	$\frac{s_i}{K}\left[-(a+b+c)\cdot\ln(1-X)-(b+c)\cdot X-0.5\cdot cX^2\right]$	$\frac{s_i}{K}\cdot\frac{\left[aX+bX^2+cX^2\right]}{1-X}$
M–M kinetics	$\frac{s_i}{K}\cdot X-\ln(1-X)$	$\frac{s_i}{K}\cdot X+\frac{X}{(1-X)}$
CI by P	$s_i\cdot X\left[\frac{1}{K}-\frac{1}{K_1}\right]-\left[1+\frac{s_i}{K_1}\right]\cdot\ln(1-X)$	$\frac{s_i}{K}\cdot X+\frac{X}{1-X}+\frac{s_i}{K_1}\cdot\frac{X^2}{1-X}$
Total NCI by P	$s_i\cdot X\left[\frac{1}{K}-\frac{1}{K_2}\right]-\left[1+\frac{s_i}{K_2}\right]\cdot\ln(1-X)+\frac{s_i^2}{2\cdot K\cdot K_2}\cdot X^2$	$\frac{s_i}{K}\cdot X+\frac{X}{1-X}+\left[\frac{s_i}{K_2\cdot(1-X)}+\frac{s_i^2}{K\cdot K_2}\right]\cdot X^2$
Total UCI by S	$\frac{s_i}{K}\cdot X-\ln(1-X)+\frac{s_i^2\cdot X}{K\cdot K''}(1-0.5\cdot X)$	$\frac{s_i}{K}\cdot X+\frac{X}{1-X}+\frac{s_i^2\cdot X}{K\cdot K''}(1-X)$

M–M: Michaelis–Menten; CI: competitive inhibition; NCI: non-competitive inhibition; UCI: uncompetitive inhibition; CPBR: continuous packed-bed reactor; BSTR: batch stirred-tank reactor; CSTR: continuous stirred-tank reactor

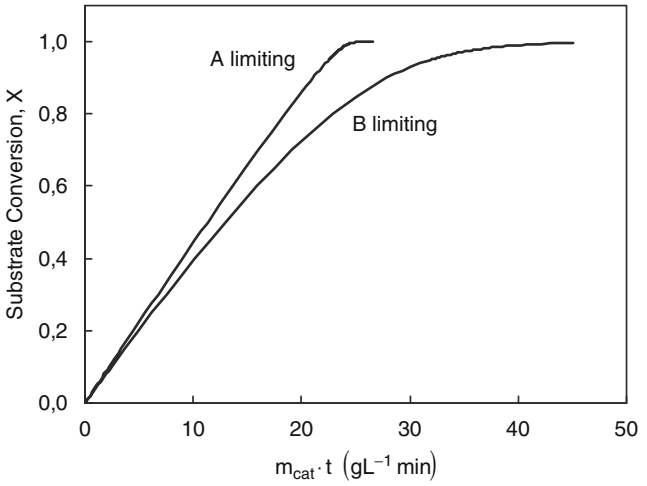


Fig. 5.4 Batch reactor performance for sequential ordered kinetics ($a_s = 500\,IU/g$; $K_A = 1\,mM$; $K_B = 2\,mM$; $K'_A = 2\,mM$; $K'_B = 4\,mM$) with A as limiting substrate ($a_i = 10\,mM$; $b_i = 20\,mM$) or B as limiting substrate ($a_i = 20\,mM$; $b_i = 10\,mM$)

to reduce the energy requirements for pumping. Bed compaction can be controlled in this case by periodic reversion of flow which also serves to the purpose of cleaning the biocatalyst. Under no enzyme inactivation, the reactor will reach steady-state after three to five residence times so for the most part of the time the operation can be considered in steady-state.

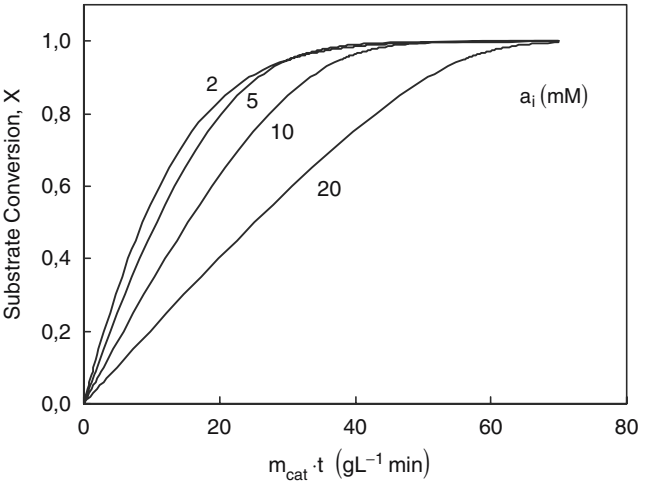


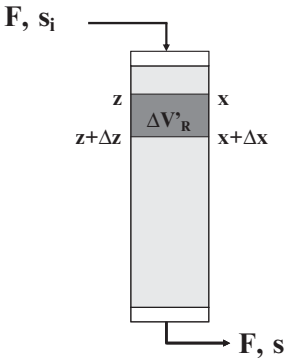
Fig. 5.5 Batch reactor performance for random ordered kinetics ($a_s = 500\,IU/g$; $K_A = 1\,mM$; $K_B = 2\,mM$; $K'_A = 2\,mM$; $K'_B = 4\,mM$) with A as limiting substrate: $b_i = 2\,a_i(mM)$

Assuming plug-flow regime through the catalyst bed, a steady-state material balance for a one-substrate reaction renders:

$$F \cdot s_i|_z - F \cdot s_i|_{z+\Delta z} = v \cdot \Delta V_R \cdot \varepsilon$$

$$F \cdot s_i(1-X)) - F \cdot s_i[1-(X+\Delta X)] = v \cdot \Delta V_R \cdot \varepsilon$$

$$\int \frac{dX}{v(e,X)} = \frac{\tau}{s_i} \qquad (5.15)$$



where: $\tau = (V_R \cdot \varepsilon)/F$.

In this case the void fraction ε, this is the fraction of the packed bed occupied by the reaction medium, can be as low as 40–50%.

For one-substrate reactions, from Eqs. 5.3 and 5.15:

$$\frac{s_i}{K}[-(a+b+c)\cdot\ln(1-X) - (b+c)\cdot X - 0.5\cdot cX^2]$$

$$= \frac{k_{cat}\cdot e}{K}\tau = \frac{k_{cat}\cdot E}{F\cdot K} = \frac{M_{cat}\cdot a_s}{F\cdot K} \qquad (5.16)$$

Eq. 5.16 represents the model of steady-state operation of CPBR. It allows the determination of the steady-state X for any given combination of M_{cat}/F. This equation reduces in one the number of degrees of freedom of the system but there are still two degrees of freedom that allows flexibility of operation, since from the three operational variables: M_{cat}, F and s_i, two can be established separately. A common situation is the use of Eq. 5.16 to determine the enzyme load required (M_{cat} of the biocatalyst) to obtain the desired substrate conversion (X) for a certain mass flow of substrate $(F \cdot s_i)$. This equation can also be used for reactor design, since its dimensions are determined by the biocatalyst bed volume, which directly depends on biocatalyst mass, according to:

$$V_B = \frac{M_{cat}}{\rho_{ap}} \qquad (5.17)$$

As seen, Eqs. 5.9 and 5.16 are formally equal, if it is considered that the residence time (τ) in a continuous reactor corresponds to the operation time (t) in a batch reactor. Both type of reactors exhibit striking similarities and the substrate profiles that develop through time in the BSTR are analogous to the substrate profiles that develop through the biocatalyst bed in the CPBR. Actually a CPBR can be considered as an infinite number of BSTR connected in series.

The corresponding kinetic expressions and the values of a, b and c for the mechanism of one-substrate reactions are in section 3.3.2. The expressions for CPBR performance for the more common kinetic mechanism are presented in Table 5.1.

For a reaction of synthesis with two substrates, a material balance over the limiting substrate, A or B, yields Eqs. 5.18 and 5.19 respectively:

$$\int \frac{dX}{v(e,X)} = \frac{\tau}{a_i} \tag{5.18}$$

$$\int \frac{dX}{v(e,X)} = \frac{\tau}{b_i} \tag{5.19}$$

In the case of ordered (first A then B) sequential mechanism, Eqs. 5.20 and 5.21 are obtained with A or B as limiting substrates respectively by integrating Eqs. 5.18 or 5.19 with the corresponding kinetic expression (Eq. 5.4 or 5.4′):

$$\frac{K_A K'_B}{a_i(b_i - a_i)} \ln\left|\frac{b_i - a_i X}{b_i(1-X)}\right| - \frac{K'_B}{a_i} \ln(1-X) + X = \frac{k_{cat} \cdot e}{a_i}\tau = \frac{k_{cat} \cdot E}{F \cdot a_i} = \frac{M_{cat} \cdot a_s}{F \cdot a_i} \tag{5.20}$$

$$\frac{K_A K'_B}{b_i(a_i - b_i)} \ln\left|\frac{a_i - b_i X}{a_i(1-X)}\right| - \frac{K'_B}{b_i} \ln(1-X) + X = \frac{k_{cat} \cdot e}{b_i}\tau = \frac{k_{cat} \cdot E}{F \cdot b_i} = \frac{M_{cat} \cdot a_s}{F \cdot b_i} \tag{5.21}$$

In the case of sequential random mechanism, Eq. 5.22 is obtained by integrating Eq. 5.18 (considering A as the limiting substrate) with the corresponding kinetic expression (Eq. 5.5):

$$\frac{K_A K'_B}{a_i(b_i - a_i)} \ln\left|\frac{b_i - a_i X}{b_i(1-X)}\right| - \frac{K'_B}{a_i} \ln\left|\frac{b_i - a_i X}{b_i}\right| - \frac{K'_A}{a_i} \ln(1-x) + X = \frac{k_{cat} \cdot e}{a_i}\tau = \frac{k_{cat} \cdot E}{F \cdot a_i} = \frac{M_{cat} \cdot a_s}{F \cdot a_i} \tag{5.22}$$

5.2.2.3 Continuous Stirred Tank Reactor (CSTR)

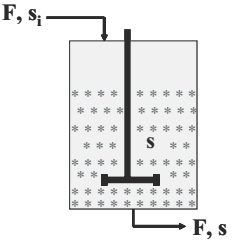
Operation is continuous with a constant flow-rate of reaction medium fed to the reactor where the biocatalyst is suspended in an agitated vessel. If well mixed, any element of fluid within the reactor has the same composition, corresponding to reactor outlet. So, in this case all enzyme particles are in contact with the same s, corresponding to its outlet value, which is quite different than in CPBR.

A steady-state material balance for a one-substrate reaction renders:

$$F \cdot s|_{inlet} - F \cdot s|_{outlet} = v \cdot V_R \cdot \varepsilon$$

$$F \cdot s_i - F \cdot s_i \cdot (1-X) = v \cdot V_R \cdot \varepsilon$$

$$\frac{X}{v(e,X)} = \frac{\tau}{s_i} \tag{5.23}$$



where: $\tau = (V_R \cdot \varepsilon)/F$. In this case the void fraction ε can be as high as 90–95%, the fraction of the volume occupied by the biocatalyst usually not exceeding 10%.

From Eqs. 5.3 and 5.23:

$$\frac{s_i}{K} \cdot \frac{aX + bX^2 + cX^3}{1 - X} = \frac{k_{cat} \cdot e}{K}\tau = \frac{k_{cat} \cdot E}{F \cdot K} = \frac{M_{cat} \cdot a_s}{F \cdot K} \tag{5.24}$$

Eq. 5.24 represents the model of steady-state operation of CSTR. As in the case of CPBR, it allows the determination of the steady-state X for any given combination of M_{cat}/F and can also be used for bioreactor design, since bioreactor dimensions will be determined from the concentration of biocatalyst that can be adequately handled in the bioreactor:

$$V_R = \frac{M_{cat}}{c_{cat}} \tag{5.25}$$

The corresponding kinetic expressions and the values of a, b and c for the mechanism of one-substrate reactions are in section 3.3.2. The expressions for CSTR performance for the more common kinetic mechanisms are presented in Table 5.1.

For a reaction of synthesis with two substrates, a material balance over the limiting substrate, A or B, yields Eqs. 5.26 and 5.27 respectively:

$$\frac{X}{v(e, X)} = \frac{\tau}{a_i} \tag{5.26}$$

$$\frac{X}{v(e, X)} = \frac{\tau}{b_i} \tag{5.27}$$

In the case of ordered (first A then B) sequential mechanism, Eqs. 5.28 and 5.29 are obtained with A or B as limiting substrates respectively by solving Eqs. 5.26 or 5.27 with the corresponding kinetic expression (Eqs. 5.4 or 5.4′):

$$X\left[\frac{K_A K'_B}{a_i(1-X)(b_i - a_i X)} + \frac{K'_B}{(b_i - a_i X)} + 1\right] = \frac{k_{cat} \cdot e}{a_i}\tau = \frac{k_{cat} \cdot E}{F \cdot a_i} = \frac{M_{cat} \cdot a_s}{F \cdot a_i} \tag{5.28}$$

$$X\left[\frac{K_A K'_B}{b_i(1-X)(a_i - b_i X)} + \frac{K'_B}{b_i(1-X)} + 1\right] = \frac{k_{cat} \cdot e}{b_i}\tau = \frac{k_{cat} \cdot E}{F \cdot b_i} = \frac{M_{cat} \cdot a_s}{F \cdot b_i} \tag{5.29}$$

In the case of sequential random mechanism, Eq. 5.30 is obtained by solving Eq. 5.18 (considering A as the limiting substrate) with the corresponding kinetic expression (Eq. 5.5):

$$X\left[\frac{K_A K'_B}{a_i(1-X)(b_i - a_i X)} + \frac{K'_B}{(b_i - a_i X)} + \frac{K'_A}{a_i(1-X)} + 1\right] = \frac{k \cdot e}{a_i}\tau = \frac{k \cdot E}{F \cdot a_i} = \frac{M_{cat} \cdot a_s}{F \cdot a_i} \tag{5.30}$$

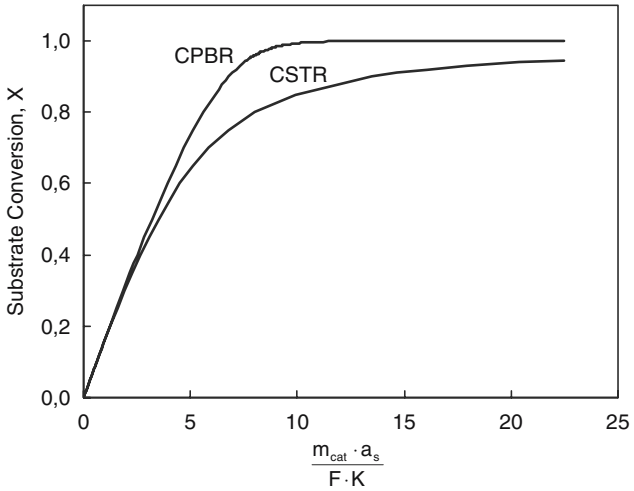


Fig. 5.6 CPBR and CSTR performance with Michaelis–Menten kinetics

5.2.2.4 Comparison Between CPBR and CSTR

Graphical representation of CPBR and CSTR performance is in Fig. 5.6 for one-substrate Michaelis–Menten kinetics and in Figs. 5.7–5.10 for different inhibition kinetics. Graphical representation of CPBR and CSTR performance is presented in Figs. 5.11 and 5.12 for the case of ordered sequential mechanism considering A

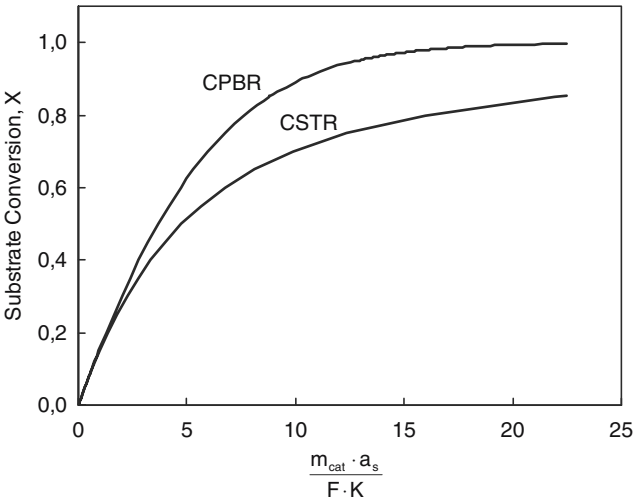


Fig. 5.7 CPBR and CSTR performance with competitive inhibition by product. $s_i/K = 5$; $K/K_1 = 0.5$

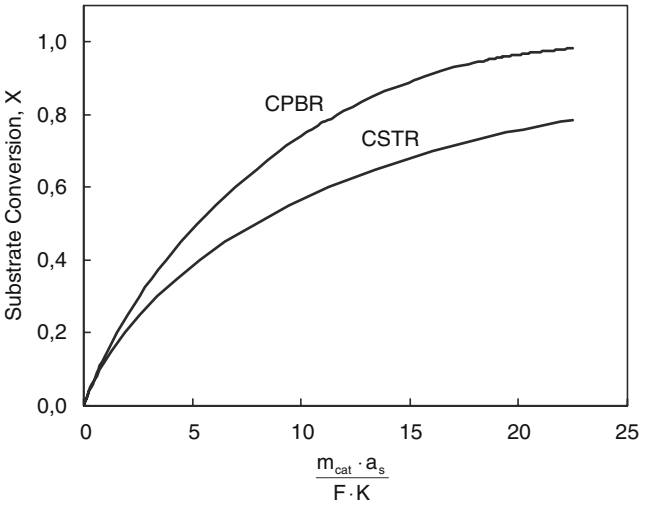


Fig. 5.8 CPBR and CSTR performance with non-competitive inhibition by product. $s_i/K = 5$; $K/K_2 = 0.5$

(Eqs. 5.20 and 5.28) or B (Eqs. 5.21 and 5.29) as limiting substrate respectively, and in Fig. 5.13 for the case of random sequential mechanism (Eqs. 5.22 and 5.30).

As expected, CPBR performance is better than CSTR for Michaelis–Menten kinetics (see Fig 5.6) and even more so in the case of product inhibition (see Figs. 5.7, 5.8 and 5.10). In fact CSTR operates in the worst condition (minimum s and maximum p, corresponding to the outlet conditions) while CPBR operates in

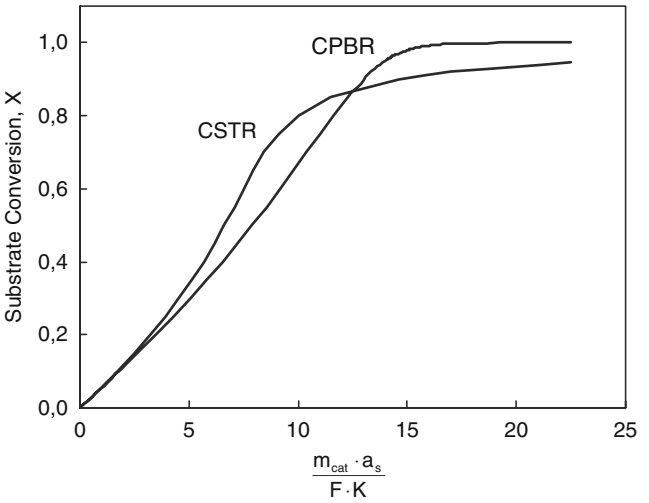


Fig. 5.9 CPBR and CSTR performance with uncompetitive inhibition by substrate. $s_i/K = 5$; $K/K'' = 0.5$

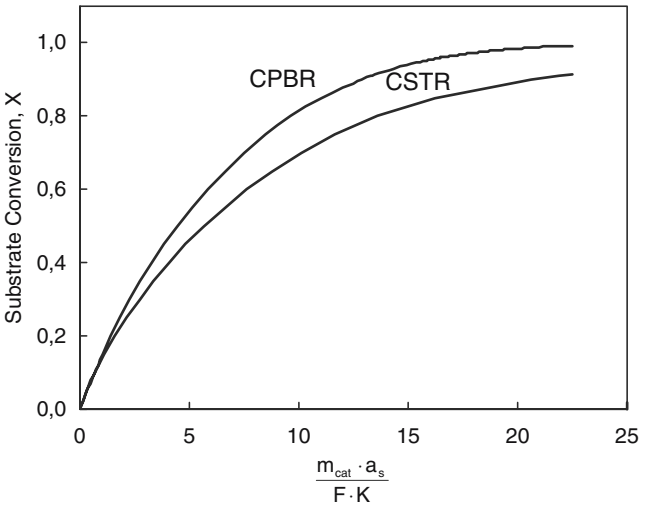


Fig. 5.10 CPBR and CSTR performance with mixed-type inhibition by product. $s_i/K = 5$; $K/K_1 = 0.5; K_1/K_2 = 0.5$

a range that goes from the best condition at the inlet (maximum s and zero p) to the worst condition at the outlet (minimum s and maximum p). This means that for a given X, a higher M_{cat} is required or a low F can be processed in the case of CSTR than in CPBR; looking in the other way, a lower X is obtained in the case of CSTR at equal M_{cat} and F. In the case of uncompetitive inhibition by high substrate concentration an inflection point is observed in Fig. 5.9, which means that CSTR performs

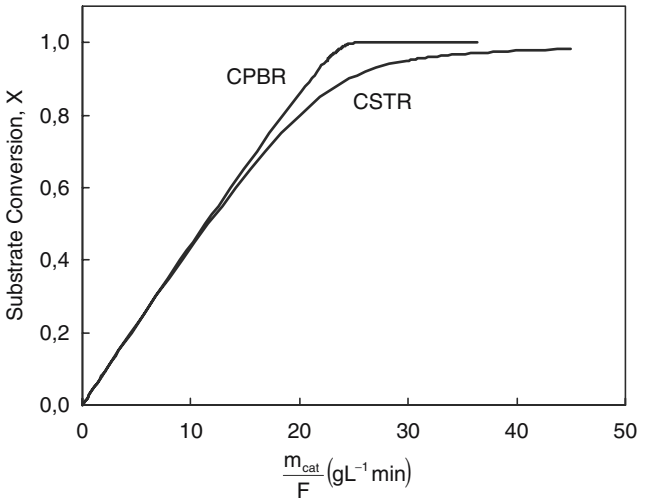


Fig. 5.11 CPBR and CSTR performance for sequential ordered kinetic mechanism (A limiting). $a_i = 10\,mM$; $b_i = 20\,mM$; $a_s = 500\,IU/L$; $K_A = 1\,mM$, $K_A' = 2\,mM$; $K_B = 2mM$; $K_B' = 4\,mM$

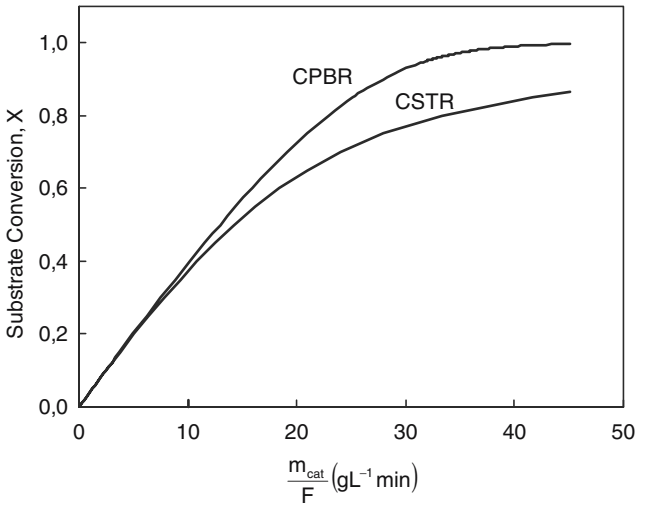


Fig. 5.12 CPBR and CSTR performance for sequential ordered kinetic mechanism (B limiting). $a_i = 20\,mM$; $b_i = 10\,mM$; $a_s = 500\,IU/L$; $K_A = 1\,mM$, $K_A' = 2\,mM$; $K_B = 2\,mM$; $K_B' = 4\,mM$

better than CPBR under certain conditions. This depends on the inlet and outlet substrate concentrations with respect to their positions in the v versus s curve and on the relative values of K and K″. However, at high X CPBR still performs better than CSTR. CPBR is also superior to CSTR for sequential ordered and random kinetics in the case of two-substrate reactions of synthesis, as shown in Figs. 5.11–5.13.

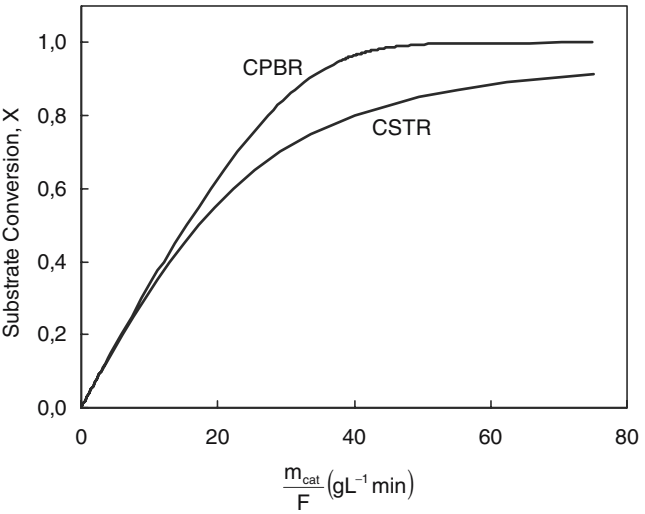


Fig. 5.13 CPBR and CSTR performance for sequential random kinetic mechanism (A limiting). $a_i = 10\,mM$; $b_i = 20\,mM$; $a_s = 500\,IU/L$; $K_A = 1\,mM$, $K_A' = 2\,mM$; $K_B = 2\,mM$; $K_B' = 4\,mM$

5.3 Effect of Diffusional Restrictions on Enzyme Reactor Design and Performance in Heterogeneous Systems. Determination of Effectiveness Factors. Batch Reactor; Continuous Stirred Tank Reactor Under Complete Mixing; Continuous Packed-Bed Reactor Under Plug Flow Regime

Mass transfer limitations can be relevant in heterogeneous biocatalysis. If the enzyme is immobilized in the surface or inside a solid matrix, external (EDR) or internal (IDR) diffusional restrictions may be significant and have to be considered for proper bioreactor design. As shown in Fig. 3.1, this effect can be conveniently incorporated into the model that describes enzyme reactor operation in terms of the effectiveness factor, defined as the ratio between the effective (or observed) and inherent (in the absence of diffusional restrictions) reaction rates. Expressions for the effectiveness factor (η), in the case of EDR, and the global effectiveness factor (η') for different particle geometries, in the case of IDR, were developed in sections 4.4.1 and 4.4.2 (see Eqs. 4.39–4.42, 4.53, 4.54, 4.71 and 4.72). Such functions can be generically written as:

$$\begin{aligned} &\text{EDR} \qquad \eta = f(\beta_0, \alpha) \\ &\text{IDR} \qquad \eta' = f(\beta_0, \Phi) \end{aligned}$$

where: $\beta_0 = \beta_{oi} \cdot (1 - X)$, so that:

$$\begin{aligned} &\text{EDR} \qquad \eta = f(X, \alpha) \\ &\text{IDR} \qquad \eta' = f(X, \Phi) \end{aligned}$$

In the case of product inhibition:

$$\begin{aligned} &\text{EDR} \qquad \eta = f(\beta_0, \gamma_0, \alpha) \\ &\text{IDR} \qquad \eta' = f(\beta_0, \gamma_0, \Phi) \end{aligned}$$

and since $\gamma_0 = \beta_{0,i} X$, then $\eta = f(X, \alpha)$ or $\eta' = f(X, \Phi)$.

These functions can now be incorporated into the models for enzyme reactor operation. Eqs. 5.7, 5.15 and 5.23 are now rewritten for a BSTR, a CPBR and a CSTR as Eqs. 5.31, 5.32 and 5.33 respectively:

$$\text{BSTR} \qquad \int \frac{dX}{v_{effective}} = \int \frac{dX}{v(e,X) \cdot \eta(X)} = \frac{t}{s_i} \tag{5.31}$$

$$\text{CPBR} \qquad \int \frac{dX}{v_{effective}} = \int \frac{dX}{v(e,X) \cdot \eta(X)} = \frac{\tau}{s_i} \tag{5.32}$$

$$\text{CSTR} \qquad \frac{X}{v_{effective}} = \frac{X}{v(e,X) \cdot \eta(X)} = \frac{\tau}{s_i} \tag{5.33}$$

(in the case of IDR, η should be replaced by η')

Eqs. 5.31, 5.32 and 5.33 can then be solved to describe enzyme reactor performance for any kinetic mechanism (v(e,X)) under mass-transfer limitations, whether external $(\eta(X))$ or internal $(\eta'(X))$.

A similar analysis can be done for kinetic mechanisms of reactions involving two substrates.

Considering the case of EDR and Michaelis–Menten kinetics as an example, from the definition of effectiveness factor, the following expression was developed (see section 4.4.1):

$$\eta = \frac{(1+\beta_0)\left[1+\alpha+\beta_0-\sqrt{(1+\alpha-\beta_0)^2+4\beta_0}\right]}{2\,\alpha\beta_0} \tag{4.11}$$

so that replacing $\beta_0 = \beta_{oi}\cdot(1-X)$ in Eq. 4.11:

$$\eta(X) = \frac{[1+\beta_{0,i}(1-X)]\cdot[1+\alpha+\beta_{0,i}(1-X)-\sqrt{Q}]}{2\cdot\alpha\cdot\beta_{0,i}(1-X)} \tag{5.34}$$

$$Q = \beta_{0,i}\cdot X^2 + 2\cdot\beta_{0,i}\cdot X(\alpha-1-\beta_{0,i}) + \beta_{0,i}(\beta_{0,i}-2\cdot\alpha+2) + \alpha(\alpha+2)+1$$

Determination of α, either experimentally or using empirical correlations, was already presented in section 4.4.1, so that $\eta = f(X)$ can be determined that reflects how the impact of diffusional restrictions changes throughout the course of the reaction.

Intrinsic kinetic expression (v(e,X)) is in this case:

$$\nu(X) = \frac{v(X)}{k_{cat}\cdot e} = \frac{\beta_{0,i}(1-X)}{1+\beta_{0,i}(1-X)} \tag{5.35}$$

so that Eqs. 5.31, 5.32 and 5.33, are in this particular case:

$$\text{BSTR} \quad \int \frac{2\cdot\alpha\cdot\beta_{0,i}}{1+\alpha+\beta_{0,i}(1-X)-\sqrt{Q}} = \frac{k_{cat}\cdot e}{K}\cdot t = \frac{m_{cat}\cdot a_s}{K}\cdot t \tag{5.36}$$

$$\text{PBCR} \quad \int \frac{2\cdot\alpha\cdot\beta_{0,i}}{1+\alpha+\beta_{0,i}(1-X)-\sqrt{Q}} = \frac{k_{cat}\cdot e}{K}\cdot\tau = \frac{k_{cat}\cdot E}{F\cdot K} = \frac{M_{cat}\cdot a_s}{F\cdot K} \tag{5.37}$$

$$\text{CSTR} \quad \frac{2\cdot\alpha\cdot\beta_{0,i}}{1+\alpha+\beta_{0,i}(1-X)-\sqrt{Q}} = \frac{k_{cat}\cdot e}{K}\cdot\tau = \frac{k_{cat}\cdot E}{F\cdot K} = \frac{M_{cat}\cdot a_s}{F\cdot K} \tag{5.38}$$

5.4 Effect of Thermal Inactivation on Enzyme Reactor Design and Performance

Loss of catalytic capacity during reactor operation is a major concern for enzyme catalyzed reactions. Such loss can be produced by different reasons like shear stress (Joshi et al. 2001), surface tension (Sarkar et al. 1987), matrix attrition and desorption in the case of supported enzymes (Regan et al. 1974), but thermal inactivation is by all means the most relevant (Peterson et al. 1989; Turner and Vulfson

2000). In fact, enzyme inactivation by exposure to the reaction temperature is unavoidable, since temperature exerts opposite effects o enzyme activity and stability so that operation temperature is always a compromise between the two. Enzyme inactivation during reaction operation occurs no matter how stable the enzyme is, since in any case operation is prolonged to the point in which a significant fraction of the initial activity is lost. Actually, the residual activity at which the biocatalyst should be disposed off is a quite relevant criterion for the optimization of reactor operation.

Inactivation of enzymes during reaction is a major concern because of their intrinsic instability. It is then of the utmost importance to develop a sound model that accounts for enzyme inactivation under process conditions. As previously presented in section 3.5, enzyme inactivation has been traditionally described according to a first-order one-step mechanism, where the first order rate constant (k_D) is the only parameter to determine, being a strong function of temperature. Such mechanism considers the one step transition of the native fully active enzyme to a completely inactive form. The model derived from it predicts an exponential decay of enzyme activity with time and customary k_D is considered as an Arrhenius type function of temperature. This model is clearly an oversimplification of the complex phenomenon of enzyme inactivation. In the first place, it is hardly conceivable that a complex protein molecule looses its biological functionality as the consequence of a single step conformational change; additionally, inactivation rate may vary not only according to temperature but other variables may affect it as well. Substrates and products of reaction may indeed affect enzyme stability, so that the values of the inactivation parameter(s) determined under non-reactive conditions may poorly predict the actual behavior of the enzyme during reaction. These two aspects will be dealt with in the following two sections.

5.4.1 Complex Mechanisms of Enzyme Inactivation

If enzyme inactivation is described by a one-step first-order mechanism, exponential decay of enzyme activity ensues, so that a straight line should be obtained in a semilog plot of residual activity versus time, as suggested by Eq. 3.126. Despite its limitation, this model has been used (and sometimes abused by forcing the data) to describe enzyme inactivation. It is quite frequent to observe behaviors that clearly depart from that simple model as revealed by semilog residual activity versus time profiles of the following type:

- Enzyme decay profile is concave to the X-axis (Henley and Sadana 1986; Ulbrich and Schellenberger 1986; Greco et al. 1993). Such curve can be approximated as a series of straight lines (two or more) with different decreasing slopes. This type of profile can be explained in terms of a *parallel mechanism* of inactivation that assumes the existence of different molecular variants of the enzyme (isoenzymes) exhibiting the same catalytic behavior but differing in their stability (Henley and

Sadana 1986; Ulbrich and Schellenberger 1986; Abraham et al. 1992). The inactivation profile results from the superposition of the (exponential) decays of each enzyme species (Dagys et al. 1984).

- Enzyme decay profile is convex to the X-axis (Henley and Sadana 1985; Illanes et al. 1996). Such curve can be described as a series of straight lines (two or more) with different increasing slopes. This situation can be explained in terms of a *series mechanism* of inactivation that assumes that the enzyme evolves from a native fully active form to a final form (partly or completely inactive) through a series of structural transitions to progressively less active forms. This mechanism can also account for the so-called *grace period*, in which the enzyme decays after a period of time in which its specific activity remains unchanged and also for *initial activation*, in which the enzyme initially evolves to a more active species prior to decay (Henley and Sadana 1985, 1986; Barclay et al. 1990). This mechanism is quite flexible since, depending on the values of the rate constants, can also accounts for concave profiles (Henley and Sadana 1985).

Based on a great amount of enzyme inactivation data gathered for different enzyme systems, a generalized theory of enzyme deactivation was proposed in the mid-1980s (Henley and Sadana 1986). This theory can be represented as a matrix in which m isoenzymes transit from their initial $(E_{i,1})$ to their final stages $(E_{i,n})$ through a series of n-2 intermediate stages of progressively lower specific activity, as shown in the following scheme:

$$
\begin{array}{ccccccccccccc}
E_{1,1} & \overset{k_{11}}{\rightarrow} & E_{1,2} & \overset{k_{12}}{\rightarrow} & E_{1,3} & \overset{k_{13}}{\rightarrow} & E_{1,4} & \overset{k_{14}}{\rightarrow} & \cdots E_{1,j} & \overset{k_{1j}}{\rightarrow} & \cdots E_{1,n-1} & \overset{k_{1(n-1)}}{\rightarrow} & E_{1,n} \\
E_{2,1} & \overset{k_{21}}{\rightarrow} & E_{2,2} & \overset{k_{22}}{\rightarrow} & E_{2,3} & \overset{k_{23}}{\rightarrow} & E_{2,4} & \overset{k_{24}}{\rightarrow} & \cdots E_{2,j} & \overset{k_{2j}}{\rightarrow} & \cdots E_{2,n-1} & \overset{k_{2(n-1)}}{\rightarrow} & E_{2,n} \\
\vdots & & \vdots & & \vdots & & \vdots & & \vdots & & \vdots & & \vdots \\
E_{i,1} & \overset{k_{i1}}{\rightarrow} & E_{i,2} & \overset{k_{i2}}{\rightarrow} & E_{i,3} & \overset{k_{i3}}{\rightarrow} & E_{i,4} & \overset{k_{i4}}{\rightarrow} & \cdots E_{i,j} & \overset{k_{ij}}{\rightarrow} & \cdots E_{i,n-1} & \overset{k_{i(n-1)}}{\rightarrow} & E_{i,n} \\
\vdots & & \vdots & & \vdots & & \vdots & & \vdots & & \vdots & & \vdots \\
E_{m,1} & \overset{k_{m1}}{\rightarrow} & E_{m,2} & \overset{k_{m2}}{\rightarrow} & E_{m,3} & \overset{k_{m3}}{\rightarrow} & E_{m,4} & \overset{k_{m4}}{\rightarrow} & \cdots E_{m,j} & \overset{k_{mj}}{\rightarrow} & \cdots E_{m,n-1} & \overset{k_{m(n-1)}}{\rightarrow} & E_{m,n}
\end{array}
$$

where k_{ij} represent the transition rate constants from enzyme species $E_{i,j}$ to $E_{i,j+1}$, $[E_{i,j}]$ is the molar concentration of the enzyme species $E_{i,j}$ (M), $a_{i,j}$ is its specific activity (IU/mol) and $e_{i,j}$ is its volumetric activity (IU/L).

At any time:

$$e = \sum_{i=1}^{m} \sum_{j=1}^{n} a_{i,j}[E_{i,j}] \tag{5.39}$$

and initially:

$$e_0 = \sum_{i=1}^{m} a_{i,1}[E_{i,1}]_0 \tag{5.40}$$

so that:

$$\frac{e}{e_0} = \frac{\sum_{i=1}^{m}\sum_{j=1}^{n} a_{i,j}[E_{i,j}]}{\sum_{i=1}^{m} a_{i,1}[E_{i,1}]_0} \tag{5.41}$$

where:

$$\frac{\sum_{i=1}^{m} e_{i,1_0}}{e_0} = 1 \tag{5.42}$$

One-step first order, parallel (Dagys et al. 1984) and series (Henley and Sadana 1985) mechanisms of inactivation are then particular cases of this generalized model.

5.4.1.1 One-Step First-Order Mechanism of Inactivation

In this case $m = 1,\ n = 2$, and if the final stage is fully inactive $a_{1j} = 0$ y $k_{1j} = 0$ for all $i > 1$ and all $j > 1$, so that according to the scheme (see section 3.5.2):

$$E_{1,1} \xrightarrow{k_{11}} E_{1,2}$$

Eq. 5.41 reduces to:

$$\frac{e}{e_0} = \frac{a_{1,1}[E_{1,1}]}{a_{1,1}[E_{1,1}]_0} \tag{5.43}$$

and assuming first order transition rate:

$$-\frac{d[E_{1,1}]}{dt} = k_{11} \cdot [E_{1,1}] \tag{5.44}$$

$$[E_{1,1}] = [E_{1,1}]_0 \cdot \exp(-k_{11} \cdot t) \tag{5.45}$$

so that replacing in Eq. 5.43:

$$\frac{e}{e_0} = \exp(-k_{11} \cdot t) \tag{5.46}$$

which is the same as Eq. 3.127, the value of the inactivation rate constant k_{11} (formerly k_D) completely describing the model. Inactivation is frequently expressed in terms of half-life $(t_{1/2})$ which is defined as the time at which the enzyme has lost one-half of its initial activity:

$$t_{1/2} = t\Big|_{e=\frac{e_0}{2}} \tag{5.47}$$

so that in this case, from Eqs. 5.46 and 5.47:

$$t_{1/2} = \frac{\ln 2}{k_{11}} \tag{5.48}$$

The inactivation rate constant and its temperature dependence can be experimentally determined as already presented in section 3.5.2.

5.4.1.2 Parallel Mechanism of Inactivation

This mechanism is represented by the first column ($n = 1$) of the generalized scheme, so that $a_{ij} = 0$ and $k_{ij} = 0$ for all $j > 1$. According to that scheme, Eq. 5.41 reduces to:

$$\frac{e}{e_0} = \frac{\sum_{i=1}^{m} a_{i,1}[E_{i,1}]}{\sum_{i=1}^{m} a_{i,1}[E_{i,1}]_0} \tag{5.49}$$

and assuming first order transition rates from $[E_{i1}]$ to $[E_{i2}]$:

$$-\frac{d[E_{i,1}]}{dt} = k_{i1} \cdot [E_{i,1}] \tag{5.50}$$

$$[E_{i,1}] = [E_{i,1}]_0 \cdot \exp(-k_{i1}t) \tag{5.51}$$

so that replacing in Eq. 5.49:

$$\frac{e}{e_0} = \frac{\sum_{i=1}^{m} a_{i,1}[E_{i,1}]_0 \exp(-k_{i1}t)}{\sum_{i=1}^{m} a_{i,1}[E_{i,1}]_0} \tag{5.52}$$

For instance, considering three isoenzymes ($m = 3$), from Eq. 5.52:

$$\frac{e}{e_0} = \frac{a_{1,1}[E_{1,1}]_0 \exp(-k_{11}t) + a_{2,1}[E_{2,1}]_0 \exp(-k_{21}t) + a_{3,1}[E_{3,1}]_0 \exp(-k_{31}t)}{a_{1,1}[E_{1,1}]_0 + a_{2,1}[E_{2,1}]_0 + a_{3,1}[E_{3,1}]_0} \tag{5.53}$$

where $\frac{a_{i,1}[E_{i,1}]_0}{a_{1,1}[E_{1,1}]_0 + a_{2,1}[E_{2,1}]_0 + a_{3,1}[E_{3,1}]_0}$ represents the fraction of the total initial activity of each isoenzyme i, so that Eq. 5.53 can now be rewritten:

$$\frac{e}{e_0} = \frac{e_{1,1_0}}{e_0}\exp(-k_{11}t) + \frac{e_{2,1_0}}{e_0}\exp(-k_{21}t) + \frac{e_{3,1_0}}{e_0}\exp(-k_{31}t) \tag{5.54}$$

and according to Eq. 5.42 with $m = 3$:

$$\frac{e_{1,1_0}}{e_0} + \frac{e_{2,1_0}}{e_0} + \frac{e_{3,1_0}}{e_0} = 1 \tag{5.55}$$

In this particular case, the model contains five parameters: k_{11}, k_{21}, k_{31}, $e_{1,1_0}/e_0$ and $e_{2,1_0}/e_0$ that can be evaluated by non-linear regression.

5.4.1.3 Series Mechanism of Inactivation

This mechanism is represented by the first row ($m = 1$) of the generalized scheme, so that $a_{ij} = 0$ and $k_{ij} = 0$ for all $i > 1$. According to that scheme, and considering that the final enzyme stage is completely inactive ($a_{1,n} = 0$) (Eq. 5.41) reduces to:

$$\frac{e}{e_0} = \frac{\sum_{j=1}^{n} a_{1,j}[E_{1,j}]}{a_{1,1}[E_{1,1}]_0} = \frac{a_{1,1}[E_{1,1}] + a_{1,2}[E_{1,2}] + \ldots + a_{1,j}[E_{1,j}] + \ldots. + a_{1,(n-1)}[E_{1,(n-1)}]}{a_{1,1}[E_{1,1}]_0} \tag{5.56}$$

and assuming first order transition rates from $[E_{1,j}]$ to $[E_{1,(j+1)}]$, for $j > 1$:

$$-\frac{d[E_{1,j}]}{dt} = k_{1,(j+1)}[E_{1,(j+1)}] - k_{1,j}[E_{1,j}] \tag{5.57}$$

with $[E_{1,1}]_{t=0} = [E_{1,1}]_0$ and $[E_{1,j}]_{t=0} = 0$. Solving Eq. 5.57 and replacing in Eq. 5.56:

$$[E_{1,j}] = (-1)^{j+1}[E_{1,1}]_0 \prod_{z=1}^{z=j-1} k_{1,z} \sum_{z=1}^{z=j} \frac{\exp(-k_{1,z}t)}{\prod_{\substack{u=1 \\ u \neq z}}^{u=j} (k_{1,z} - k_{1,u})} \tag{5.58}$$

$$\begin{aligned}\frac{e}{e_0} &= \exp(-k_{11}t) - \alpha_{1,2}k_{1,1}\left(\frac{\exp(-k_{11}t)}{(k_{11}-k_{12})} + \frac{\exp(-k_{12}t)}{(k_{12}-k_{11})}\right) + \alpha_{1,3}k_{1,1}k_{12} \\ &\times \left(\frac{\exp(-k_{11}t)}{(k_{11}-k_{12})(k_{11}-k_{13})} + \frac{\exp(-k_{12}t)}{(k_{12}-k_{11})(k_{12}-k_{13})} + \frac{\exp(-k_{13}t)}{(k_{13}-k_{11})(k_{13}-k_{12})}\right) \\ &+ \ldots + (-1)^{j+1}\alpha_{1,j} \prod_{z=1}^{z=j-1} k_{1,z} \sum_{z=1}^{z=j} \frac{\exp(-k_{1,z}t)}{\prod_{\substack{u=1 \\ u \neq z}}^{u=j} (k_{1,z} - k_{1,u})} \\ &+ \ldots + (-1)^{n}\alpha_{1,n-1} \prod_{z=1}^{z=n-2} k_{1,z} \sum_{z=1}^{z=n-1} \frac{\exp(-k_{1,z}t)}{\prod_{\substack{u=1 \\ u \neq z}}^{u=j} (k_{1,z} - k_{1,u})}\end{aligned} \tag{5.59}$$

where $\alpha_{1,j}$ is the ratio of specific activities between the intermediate species and the initial species:

$$\alpha_{1,j} = \frac{a_{1,j}}{a_{1,1}} \tag{5.60}$$

For example, assuming two transition stages $(n = 3)$ and that at prolonged time the specific activity of the enzyme will tend to $E_{1,(n-1)}$ $(k_{1,j} = 0$ for $j > 2)$, Eq. 5.60 will be:

$$\frac{e}{e_0} = \exp(-k_{11}t) - \alpha_{1,2}k_{1,1}\left(\frac{\exp(-k_{11}t)}{(k_{11}-k_{12})} + \frac{\exp(-k_{12}t)}{(k_{12}-k_{11})}\right) + \alpha_{1,3}\left(\frac{k_{1,2}\exp(-k_{11}t)}{(k_{11}-k_{12})} + \frac{k_{1,1}\exp(-k_{12}t)}{(k_{12}-k_{11})} + 1\right) \tag{5.61}$$

that can be rearranged:

$$\frac{e}{e_0} = \alpha_{1,3} + \left(1 + \alpha_{1,2}\frac{k_{11}}{(k_{12}-k_{11})} - \alpha_{1,3}\frac{k_{12}}{(k_{12}-k_{11})}\right)\exp(-k_{11}t) - \left(\alpha_{1,2}\frac{k_{11}}{(k_{12}-k_{11})} + \alpha_{1,3}\frac{k_{12}}{(k_{12}-k_{11})}\right)\exp(-k_{12}t) \tag{5.62}$$

This model contains four parameters k_{11}, k_{12}, $\alpha_{1,2}$ and $\alpha_{1,3}$ that can be determined by non-linear regression of the experimental inactivation data.

Some particular cases of Eq. 5.62 are analyzed:

- If the final enzyme species $(E_{1,3})$ is inactive, $\alpha_{1,3} = 0$ and Eq. 5.62 reduces to:

$$\frac{e}{e_0} = \left(1 + \alpha_{1,2}\frac{k_{11}}{(k_{12}-k_{11})}\right)\exp(-k_{11}t) - \left(\alpha_{1,2}\frac{k_{11}}{(k_{12}-k_{11})}\right)\exp(-k_{12}t) \tag{5.63}$$

- If, in addition, the intermediate enzyme species $(E_{1,2})$ has the same specific activity than the initial $(E_{1,1})$, $\alpha_{1,2} = 1$ and Eq. 5.63 reduces to:

$$\frac{e}{e_0} = \frac{k_{12}}{(k_{12}-k_{11})}\exp(-k_{11}t) - \frac{k_{11}}{(k_{12}-k_{11})}\exp(-k_{12}t) \tag{5.64}$$

  which corresponds to the mechanism known as *grace period*, since the enzyme remains fully stable for certain period of time after which it inactivates according to a first-order mechanism. If $\alpha_{1,2} > 1$, the intermediate species has higher specific activity than the initial, a phenomenon termed initial activation, which has been observed in certain occasions.

Series mechanism is quite versatile and can accommodate many of the observed patterns of enzyme inactivation, so it has gained increasing use to develop sound models for describing enzyme inactivation, especially in the case of immobilized enzymes (Vrábel et al. 1997; Azevedo et al. 1999; Longo and Combes 1999; Aymard and Belarbi 2000).

This generalized theory of enzyme deactivation is quite appealing since it gives a theoretical framework for developing models to describe it. However, a relatively small portion of the matricial scheme may lead to a large number of parameters that have to be determined by non-linear regression from only one set

of experimental data. This leads to uncertainty and error so that it is advisable to fit the data to the simplest mechanism that reasonably represent the experimental behavior. Such model, though being a simplified approach to a very complex phenomenon, may represent a significant improvement with respect to the traditional model based on one-stage first-order inactivation mechanism. We have obtained very good fittings for the case of immobilized penicillin acylase (Illanes et al. 1996) and β-galactosidase using a two-stage series mechanism (Illanes et al. 1998a).

5.4.2 Effects of Modulation on Thermal Inactivation

Thermal inactivation of enzymes is usually evaluated under non-reactive conditions. However, it is a well-reported fact that enzyme stability is different under reactive conditions (Villaume et al. 1990; Ospina et al. 1992), suggesting that substrates and products of reaction affect it. Results obtained under non-reactive conditions may then poorly predict the actual stability of the enzyme within the bioreactor. It has been frequently reported that the substrate protects the enzyme from thermal inactivation. Substrate protection was first modeled by O'Neill (1972) and then redefined in terms of the so-called *protection factor* by Chen and Wu (1987). We have generalized this concept in terms of *modulation factors* proposing that any substance that interacts with the enzyme during biocatalysis, is a potential modulator (M) of its stability and that such modulation can be positive (protection) or negative (destabilization) (Illanes et al. 1994). According to that hypothesis, all enzyme species (free enzyme and enzyme complexes) will inactivate at different rates so that:

$$-\frac{d[E_iM]}{dt} = k_{i,M} \cdot [E_iM] \tag{5.65}$$

where:

$$k_{i,M} = k_{i,0}(1 - n_{i,M}) \tag{5.66}$$

The modulation factor of substance M in the inactivation stage i is then defined as:

$$n_{i,M} = 1 - \frac{k_{i,M}}{k_{i,0}} \tag{5.67}$$

If $1 > n_{i,M} > 0$, M is a positive modulator; if $n_{i,M} < 0$, M is a negative modulator; if $n_{i,M} = 0$, M has no modulation effect.

Positive modulation (protection) by substrate has been explained in terms of the stiffness of the enzyme structure that it promotes (Villaume et al. 1990). Negative modulation (destabilization) has also been reported for substrate (Illanes et al. 1998a) and other catalytic modulators (Alvaro et al. 1991). This behavior can be explained in terms of the promotion of quaternary structure dissociation (Misset 1993) and alteration of the oxidation stage in the active site of the enzyme (Bourdillon et al. 1985).

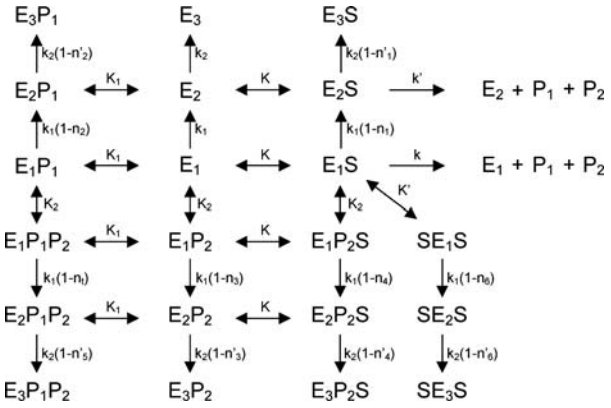


Fig. 5.14 Scheme of the kinetics of reaction $S \rightarrow P_1 + P_2$ (P_1: competitive inhibitor; P_2: non-competitive inhibitor; S: substrate, uncompetitive inhibitor), and enzyme inactivation according to a two-stage series mechanism

The generalized kinetic scheme for one-substrate reaction (see section 3.3.2) and two-stage series mechanism of enzyme inactivation (see section 5.4.1) is represented in Fig. 5.14 (if the mechanism of inactivation is one-stage first-order, the scheme simplifies accordingly). Kinetics of inactivation of each enzyme species (E, ES, EP_1, EP_2 EP_1P_2, EP_2S, SES), as represented by Eq. 5.65, is in this case described by Eq. 5.62 if the final enzyme species is active or Eq. 5.63 if it is inactive (for other mechanism of inactivation, see the corresponding equations in section 5.4.1). Transition rate constants in the presence (k_{iM}) and absence (k_{i0}) of modulators can be obtained from such equations and the corresponding modulation factors determined from Eq. 5.66.

Inactivation parameters for each enzyme species can be determined experimentally displacing the equilibrium to such species by saturating the enzyme with the corresponding modulator. If the enzyme is kept saturated with the modulator during the whole inactivation experiment, results will reflect the stability of the corresponding enzyme–modulator complex. In practical terms, a modulator concentration over ten times the value of the enzyme–modulator dissociation constant is sufficient. This condition is easily attainable, except in the case of those active complexes that include the substrate, where experimental conditions must ensure insignificant substrate conversion during the whole inactivation experiment. This can be obtained in a continuous well-mixed system maintaining a high modulator (substrate) feed flow-rate with respect to the enzyme activity within the vessel. Under such conditions, a very low substrate conversion is attained and the enzyme remains saturated with substrate (see section 5.2).

As an example, Fig. 5.15 shows the kinetics of reaction and inactivation of chitin-immobilized β-galactosidase at 27.5 °C (Illanes et al. 2000). In this case the enzyme is competitively inhibited by the product galactose but not for glucose and inactivation for all enzyme species was modeled according to a two-stage series mechanism

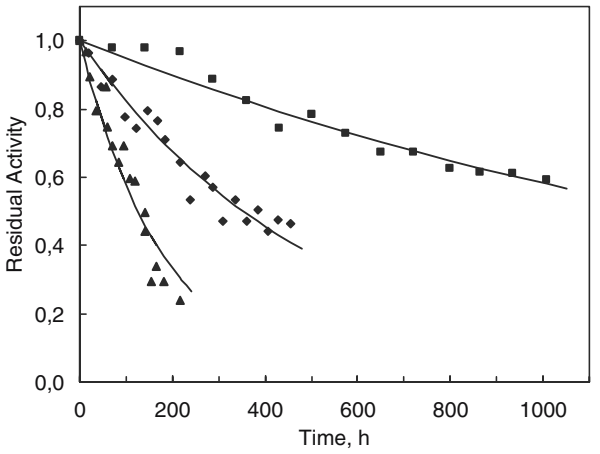


Fig. 5.15 Kinetics of thermal inactivation of chitin-immobilized β-galactosidase at pH 6.6 at 27.5 °C in phosphate buffer (♦); in lactase at a concentration of $10 \cdot K_M$(▲); in galactose at a concentration of $10 \cdot K_I$ (■)

with no residual activity (Eq. 5.63), as shown in Fig. 5.18. The substrate lactose was a negative modulator of β-galactosidase stability, while the product galactose was a positive modulator; it is interesting that glucose showed no modulation effect which supports our hypothesis about modulation (Illanes et al. 1994). Inactivation parameters were calculated by non-linear regression of experimental data in Fig. 5.15 to Eq. 5.63. The same procedure was repeated at different temperatures in the range from 20 to 40 °C (Illanes et al. 2000). Arrhenius-type functions were validated for all inactivation rate constants in the absence and presence of saturating concentrations of modulators as shown in Table 5.2. Correlations for the corresponding modulation factors are also shown in that table.

Modulation factors were mild functions of temperature in the first stage of enzyme inactivation, the effect being more pronounced in the second stage where negative modulation by lactose was always lower at higher temperature and positive modulation (protection) by galactose higher at higher temperature. From the standpoint of stability under modulation, this means that higher operating temperatures are better within the range studied. $\alpha_{1,2}$ was not a defined function of temperature, having a break-point at 30 °C and different but constant values over $(\alpha_{1,2} = 0.3)$ and below $(\alpha_{1,2} = 0.7)$ that temperature. Being A the ratio of specific activities in both stages of enzyme inactivation, this might reflect a configuration change of the enzyme at that temperature.

A similar study was conducted with immobilized penicillin acylase: in this case, both the substrate (penicillin G) and the non-competitive inhibitor product (6-aminopenicillanic acid) were positive modulators, while the competitive inhibitor product (phenylacetic acid) was a negative modulator of enzyme stability (Illanes et al. 1996); again all compounds that interacted with the enzyme during catalysis were modulators of enzyme stability.

Table 5.2 Temperature Dependence of Transition Rate Constants and Modulation Factors of β-Galactosidase Inactivation According to a Two-Stage Series Mechanism

Parameter	Temperature Dependence
$k_{1,0}$	$4.6 \cdot 10^{17} \cdot \exp\left(\frac{-13679}{T}\right)$
$k_{2,0}$	$1.59 \cdot 10^{21} \cdot \exp\left(\frac{-16746}{T}\right)$
$k_{1,S}$	$2.50 \cdot 10^{17} \cdot \exp\left(\frac{-13454}{T}\right)$
$k_{2,S}$	$1.08 \cdot 10^{4} \cdot \exp\left(\frac{-4052}{T}\right)$
$k_{1,P}$	$3.78 \cdot 10^{15} \cdot \exp\left(\frac{-12750}{T}\right)$
$k_{2,P}$	$1.14 \cdot 10^{-21} \cdot \exp\left(\frac{12377}{T}\right)$
n_{1S}	$1 - 5.43 \cdot 10^{-1} \cdot \exp\left(\frac{225}{T}\right)$
n_{2S}	$1 - 6.79 \cdot 10^{-18} \cdot \exp\left(\frac{12694}{T}\right)$
n_{1P}	$1 - 8.22 \cdot 10^{-3} \cdot \exp\left(\frac{929}{T}\right)$
n_{2P}	$1 - 7.17 \cdot 10^{-43} \cdot \exp\left(\frac{29123}{T}\right)$
$\alpha_{1,2}$	0.70 ($T \leq 30\,^{\circ}C$)
	0.30 ($T > 30\,^{\circ}C$)

S: lactose; P: galactose

5.4.3 Enzyme Reactor Design and Performance Under Non-Modulated and Modulated Enzyme Thermal Inactivation

Enzyme thermal inactivation during bioreactor operation is of paramount importance and must be considered for proper bioreactor design, as shown in Fig. 3.1. To do so, a mathematical model must be developed based on experimentally calculated and validated parameters. Mechanistic models to describe enzyme inactivation were presented in sections 5.4.1 and 5.4.2.

5.4.3.1 Reactor Design and Performance Under Non-Modulated Enzyme Inactivation

Behavior of enzyme reactors under no inactivation was presented in section 5.2. Now, the effect of enzyme inactivation will be incorporated. Conventional design of enzyme reactors considering one-stage first-order inactivation without modulation will be firstly presented in this section. In the next section, enzyme reactor design will be developed for more complex inactivation mechanisms considering modulation.

The model for one-stage first-order inactivation mechanism is represented by Eq. 5.46.

Batch Reactor

From Eq. 5.6, the model for a batch reactor performance considering one-substrate reactions (Eq. 5.3) and one-stage first-order inactivation mechanism (Eq. 5.46) is:

$$\int_0^X \frac{a + bX + cX^2}{1 - X} dX = \int_o^t \frac{k_{cat} \cdot e_0 \exp(-k_{11} \cdot t)}{s_i} dt \tag{5.68}$$

By integration:

$$\frac{s_i}{K}[-(a+b+c) \cdot \ln(1-X) - (b+c) \cdot X - 0.5 \cdot cX^2] = \frac{k_{cat} \cdot e_0 [1 - \exp(-k_{11}t)]}{K \cdot k_{11}} = \frac{m_{cat} \cdot a_s [1 - \exp(-k_{11}t)]}{K \cdot k_{11}} \tag{5.69}$$

Once k_{11} has been experimentally determined (see section 3.5.2), the curve of reactor operation (X vs t) can be obtained for a certain enzyme concentration (m_{cat}). Eq. 5.69 also allows reactor design (determination of reactor volume), since m_{cat} is simply the ratio of enzyme load to reaction volume (M_{cat}/V_R). Simulation of batch bioreactor operation under different scenarios of enzyme inactivation is presented in Fig. 5.16 for simple Michaelis–Menten kinetics $(a = 1 + K/s_i;\ b = -1;\ c = 0)$ with $s_i/K = 10$. Enzyme load in the reactor was calculated to obtain 90% conversion after 10 h of reaction under no inactivation. The strong impact of enzyme inactivation on bioreactor performance can be easily appreciated.

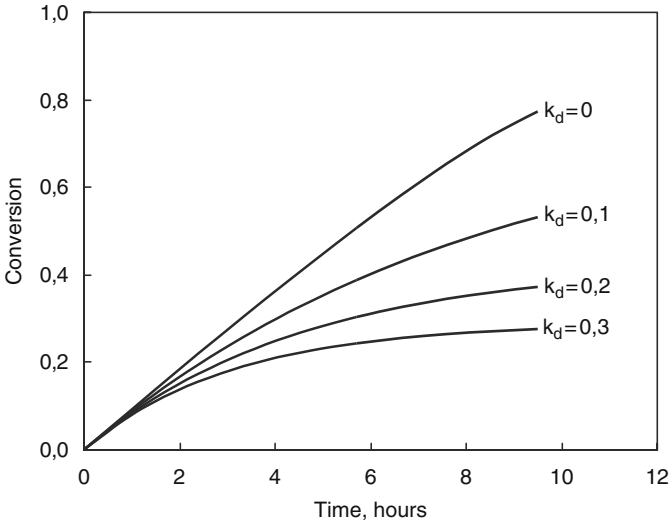


Fig. 5.16 Simulation of batch enzyme reactor operation with Michaelis–Menten kinetics $(s_i/K = 10)$ under different scenarios of enzyme inactivation. Enzyme load was calculated to obtain 90% substrate conversion at 10 h under no inactivation $(k_D = 0)$. k_D is in reciprocal hours

For reactions involving two substrates, from Eq. 5.6 and Eq. 5.46, using Eq. 5.4 or 5.4′ for sequential ordered with A and B as the limiting substrate respectively, Eqs. 5.70 and 5.71 are:

$$\frac{K_A K_B{}'}{a_i\,(b_i - a_i)} \ln\left|\frac{b_i - a_i X}{b_i\,(1-X)}\right| - \frac{K'_B}{a_i} \ln\left(\frac{b_i - a_i X}{b_i}\right) + X = \frac{k_{cat}\cdot e}{a_i} t = \frac{m_{cat}\cdot a_s\,[1-\exp(-k_{11}t)]}{a_i \cdot k_{11}} \tag{5.70}$$

$$\frac{K_A K_B{}'}{b_i\,(a_i - b_i)} \ln\left|\frac{a_i - b_i X}{a_i\,(1-X)}\right| - \frac{K_B{}'}{b_i} \ln(1-X) + X = \frac{k_{cat}\cdot e}{b_i} t = \frac{m_{cat}\cdot a_s\,[1-\exp(-k_{11}t)]}{b_i \cdot k_{11}} \tag{5.71}$$

For sequential random mechanisms (being A the limiting substrate) from Eqs. 5.6 and 5.46 and using Eq. 5.5, Eq. 5.72 is obtained after integration:

$$\frac{K_A K_B{}'}{a_i\,(b_i - a_i)} \ln\left|\frac{b_i - a_i X}{b_i\,(1-X)}\right| - \frac{K_B{}'}{a_i} \ln\left|\frac{b_i - a_i X}{b_i}\right| - \frac{K'_A}{a_i} \ln(1-X) + X = \frac{k_{cat}\cdot e}{a_i} t = \frac{m_{cat}\cdot a_s\,[1-\exp(-k_{11}t)]}{a_i \cdot k_{11}} \tag{5.72}$$

More complex inactivation models can be considered following the same procedure as above by replacing Eq. 5.46 for the corresponding to such models: for example, Eq. 5.54 for parallel inactivation with three isoenzymes; Eq. 5.62 for series with to intermediate stages; Eq. 5.63 same as before but with inactive final stage or Eq. 5.64 for grace period.

Continuous Reactors

Behavior of continuous reactors was analyzed in section 5.2 under steady-state operation. This is no longer valid if the enzyme is inactivated during reactor operation. However, if the biocatalyst is reasonably stable (as it should be for an immobilized enzyme to be used continuously), pseudo-steady-state operation can be considered (Vieth et al. 1976). In this way, the initial steady-state (X_i), obtained when the enzyme still remains fully active ($E = E_0$), can be determined from Eqs. 5.16 and 5.24 for CPBR and CSTR respectively. After certain time of reactor operation (t), the enzyme has suffered inactivation ($E = f(t)$) and pseudo-steady-state substrate conversion (X) at that time (t) can be estimated from the same Eqs. 5.16 and 5.24. Then,

if the enzyme inactivates according to Eq. 5.46:

$$\text{CPBR} \qquad \ln\frac{E}{E_0} = \ln\frac{[-(a+b+c)\ln(1-X)-(b+c)X-0.5cX^2]}{[-(a+b+c)\ln(1-X_i)-(b+c)X_i-0.5cX_i^2]} = -k_{11}\cdot t \tag{5.73}$$

$$\text{CSTR} \qquad \ln\frac{E}{E_0} = \ln\frac{(aX+bX^2+cX^3)(1-X_i)}{aX_i+bX_i^2+cX_i^3)(1-X)} = -k_{11}\cdot t \tag{5.74}$$

Once k_{11} has been determined, the curve of reactor operation (X vs t) can be obtained from Eqs. 5.73 or 5.74. Values of X_i are obtained from Eqs. 5.16 or 5.24 for a certain enzyme load and feed flow-rate in the bioreactor. Eqs. 5.73 and 5.74 also allow bioreactor design (volume determination). In the case of CPBR, the volume of the catalytic bed can be directly determined from the amount of biocatalyst required, by dividing its mass by the apparent density of the biocatalyst bed, which is easily determined. In the case of CSTR, the volume of reaction can also be determined from the amount of biocatalyst required, by dividing its mass by the biocatalyst concentration, which is usually determined by hydrodynamic considerations.

When basic design of continuous reactors was presented in section 5.2, CPBR performance was shown to be superior to CSTR for simple Michaelis–Menten kinetics, product inhibition kinetics and sequential mechanisms of two-substrate reactions. However, when considering enzyme inactivation, differences between the two reactor configurations are reduced as time elapses and the enzyme inactivates, as shown in Fig. 5.17 for simple Michaelis–Menten kinetics. The ratio of areas

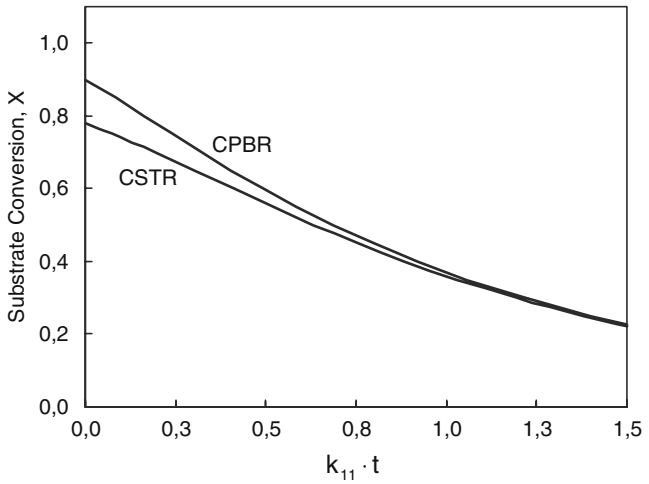


Fig. 5.17 Simulation of CPBR and CSTR with Michaelis–Menten kinetics ($s_i/K = 10$). Enzyme load was calculated to obtain a steady-state substrate conversion of 90% in CPBR

under the curves rather than the ratio of the Y-axis values represents the magnitude of superiority of CPBR over CSTR.

As in the case of the batch reactor, more complex inactivation models can be considered for continuous reactors following the same procedure as above by replacing Eq. 5.46 for the corresponding for such models: for example, for Eqs. 5.54 (parallel with three isoenzymes), 5.62 (series with to intermediate stages), 5.63 (same as before but with inactive final stage) or 5.64 (grace period) and incorporating them to Eqs. 5.73 or 5.74.

5.4.3.2 Reactor Design and Performance Under Modulated Enzyme Inactivation

Modulation of enzyme inactivation by substrates and products of reaction produces a complex effect on enzyme reactor behavior, since their concentrations are changing continuously throughout the course of reaction and, as a consequence, the enzyme inactivation rate is also changing.

CPBR with chitin-immobilized β-galactosidase (Illanes et al. 1998a) is presented to illustrate enzyme reactor design under modulated thermal inactivation. Diffusional restrictions are in this case negligible, the enzyme is inhibited by the product galactose competitively and neither glucose nor lactose at high concentrations are inhibitors (Illanes et al. 1990), so that Fig. 5.14 simplifies to the scheme in Fig. 5.18.

From Eq. 5.3 with $a = 1 + K/s_i$; $b = K/K_1 - 1$; $c = 0$, the kinetic expression for lactose hydrolysis is:

$$\nu(X) = \frac{v(X)}{k_{cat} \cdot e} = \frac{s_i(1-X)}{K\left[1 + \frac{s_i X}{K_1}\right] + s_i(1-X)} \tag{5.75}$$

Thermal inactivation of chitin-immobilized β-galactosidase has been adequately described by a two-stage series mechanism with zero residual activity. From a material balance of all enzyme species considering a two-stage series mechanism of inactivation (see Fig. 5.18), from Eq. 5.63 applied to each of the enzyme species, Eq. 5.67 and Eq. 5.75, thermal inactivation of chitin-immobilized enzyme in the bioreactor

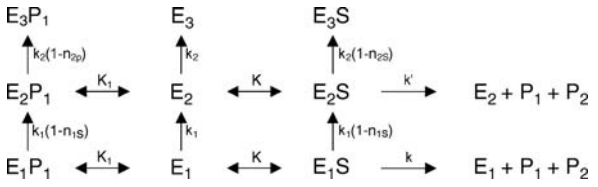


Fig. 5.18 Scheme of the kinetics of reaction catalysed by β-galactosidase: lactose (S) → galactose (P_1) + glucose (P_2) and enzyme inactivation according to a two-stage series mechanism

can be described by (Illanes et al. 1998b):

$$-\frac{de}{dt} = e \cdot k_1 \left[\frac{(1-\alpha_{12}) \cdot \exp(-k_{11} \cdot t) \cdot [1-\nu(X) \cdot N_1(X)]}{\exp(-k_{11} \cdot t) + \frac{k_{11} \cdot \alpha_{12}}{(k_{12}-k_{11})} [\exp(-k_{11} \cdot t) - \exp(-k_{12} \cdot t)]} + \frac{\alpha_{12} \cdot k_{12} \cdot [\exp(-k_{11} \cdot t) - \exp(-k_{12} \cdot t)] \cdot [1-\nu(X) \cdot N_2(X)]}{(k_{12}-k_{11}) \exp(-k_{11} \cdot t) + k_{11} \cdot \alpha_{12} \cdot [\exp(-k_{11} \cdot t) - \exp(-k_{12} \cdot t)]} \right] \tag{5.76}$$

where N_1 y N_2 are the global modulation factors corresponding to the first and second stage of enzyme inactivation:

$$N_1(X) = n_{1S} + n_{1P} \cdot \frac{K \cdot X}{K_1 \cdot (1-X)} \tag{5.77}$$

$$N_2(X) = n_{2S} + n_{2P} \cdot \frac{K \cdot X}{K_1 \cdot (1-X)} \tag{5.78}$$

From Eq. 5.15:

$$\frac{dX}{dz} = \frac{e \cdot k_{cat}(T) \cdot \nu(X,T)}{s_i} \cdot \frac{\varepsilon \cdot A_S}{F} \tag{5.79}$$

CPBR behavior under pseudo-steady-state and plug-flow regime is described by the resolution of the system of differential Eqs. 5.76 and 5.79. This model was experimentally validated in a laboratory packed-bed reactor with chitin-immobilized

Table 5.3 Design Parameters and Conditions of Operation of a Laboratory CPBER with Chitin-Immobilized β-Galactosidase

Volume of biocatalyst bed (mL)	50.3
Void fraction of biocatalyst bed	0.5
Specific activity of biocatalyst (IU/g)	147
Biocatalyst mass in reactor (g)	3
Total activity in reactor (IU)	441
Feed flow-rate (mL/min)	0.42
Lactose concentration in feed stream (mM)	526
Initial substrate conversion	0.9
Temperature (°C)	27.5
pH	6.6
K (mM)	54.4
K_1 (mM)	86.9
$k_{11} \cdot 10^3$ (/h)	7.57
$k_{12} \cdot 103$ (/h)	1.58
α_{12}	0.7
n_{1P}	0.81
n_{2P}	0.507
n_{1S}	−0.24
n_{2S}	−7.69

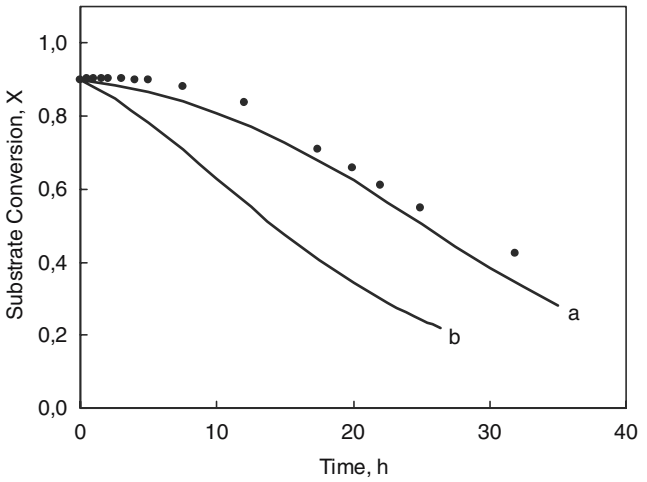


Fig. 5.19 CPBR operation with immobilized β-galactosidase at 27.5°C and pH 6.6. a) model considering modulation factors by glucose and galactose; b) model not considering modulation; (•): experimental results

β-galactosidase, whose kinetic parameters, inactivation parameters and operation conditions are in Table 5.3 (Illanes et al. 1998a; Illanes et al. 2000).

Results are presented in Fig. 5.19. A much better fit of the model was obtained considering modulation (a) than with the conventional model not considering it (b). This is just one example that illustrates the relevance of considering modulation for proper enzyme reactor design. Profile of thermal inactivation through the catalyst bed was determined and different inactivation rates were obtained, decreasing from inlet to outlet, as the modulation effects by lactose (negative) and galactose (positive) predict. Simulated profiles considering and not considering modulation have been reported (Illanes et al. 1998a). In the first case, flat profiles of enzyme decay are obtained throughout the biocatalyst bed, while in the second that profiles show a curved surface which is in agreement with the results obtained.

5.4.4 Operation of Enzyme Reactors Under Inactivation and Thermal Optimization

5.4.4.1 Operation of Enzyme Reactors Under Inactivation

The main objective of reactor operation is to deliver a product of uniform quality at the required level of production. To do so, final substrate conversion must be kept constant from batch to batch. In the case of continuous operation, outlet substrate conversion must be kept constant throughout. This means that a strategy is required to compensate for enzyme inactivation.

In the case of a batch operation with a soluble biocatalyst, the enzyme is usually unstable enough to significantly inactivate during the batch and the system is designed to obtain the desired substrate conversion by a proper combination of enzyme load and time of reaction (see Eqs. 5.69–5.72). Since the biocatalyst is not recoverable, the operation is repeated, as many times as demanded, by adding a new load of enzyme to fresh reaction medium. This can be regarded as the simplest way of conducting an enzyme catalyzed reaction and in fact has been used traditionally for several hydrolytic reactions of commercial significance (see section 1.5). However, this mode of operation is plagued with difficulties: beyond the very poor use of the enzyme catalytic potential, inactivation and removal of the biocatalyst after reaction is cumbersome and in most cases product must be completely devoid of residual biocatalyst. This is by no means trivial at large scale of operation where the usual laboratory procedures, like heat or pH denaturation, are troublesome. The first one is not easily scalable and the second may produce further product contamination.

In the case of batch operation with immobilized enzyme it is expected that the enzyme is stable enough so that after one batch most of the biocatalyst activity still remains. The biocatalyst is then recovered after each batch and inactivation from batch to batch needs to be taken into consideration to compensate for it and assure a product of uniform quality after each batch. Fresh biocatalyst can be added after each batch in an amount equivalent to the activity lost; however, the concentration or quantity of biocatalyst particles that the bioreactor can handle is limited. Other option is to compensate enzyme inactivation by progressively increasing the time of each batch (see Eqs. 5.69–5.72); however, this may be cumbersome for industrial operation, unless an on-line monitoring of reaction is settled so that reaction time can be automatically adjusted. Both strategies can be combined along the production campaign that will end when the spent biocatalyst is removed from the reactor and replaced by a new batch of fresh biocatalyst. If the biocatalyst quality changes from one campaign to the other proper adjustment should be made to deliver a product of uniform quality.

As an example of the first strategy outlined above, the simulation of sequential batch operation with immobilized penicillin acylase is presented. Penicillin acylase is inhibited by both products, being phenylacetic acid a competitive inhibitor and 6-aminopenicillanic acid a non-competitive inhibitor (Illanes et al. 1994). Simulation was done by solving differential Eq. 5.6, which represents the material balance with the corresponding kinetic expression (Eq. 3.43), and Eq. 5.76, which represents the two-phase series type enzyme inactivation kinetics. The scheme for this situation is presented in Fig. 5.20.

In this case, the global modulation factors N_1 and N_2 in Eq. 5.75 are:

$$N_1(X) = n_1 + n_2 \cdot \frac{K \cdot X}{K_1 \cdot (1-X)} + n_3 \cdot \frac{K \cdot X}{K_2(1-X)} + n_4 \frac{s_i X}{K_2} + n_5 \frac{K s_i X^2}{K_1 K_2 (1-X)} \tag{5.80}$$

$$N_2(X) = n'_1 + n'_2 \cdot \frac{K \cdot X}{K_1 \cdot (1-X)} + n'_3 \cdot \frac{K \cdot X}{K_2(1-X)} + n'_4 \frac{s_i X}{K_2} + n'_5 \frac{K s_i X^2}{K_1 K_2 (1-X)} \tag{5.81}$$

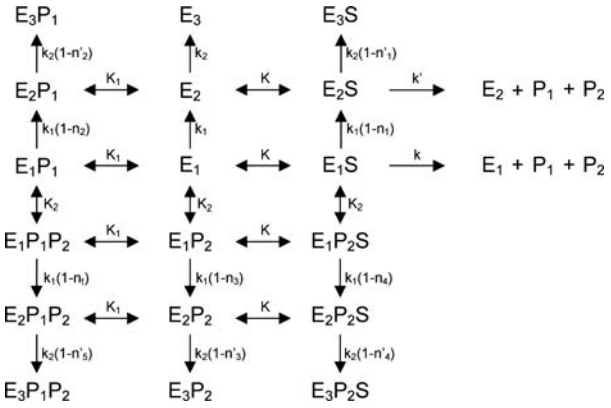


Fig. 5.20 Scheme of the kinetics of reaction of hydrolysis of penicillin G (S) to phenylacetic acid (competitive inhibitor P1) and 6-aminopenicillanic acid (non-competitive inhibitor P2) with immobilized penicillin acylase inactivation according to a two-stage series mechanism

Results of the simulation of reactor operation are presented in Fig. 5.21 (Illanes et al. 1996). The curvatures in line b (non-modulated) and line c (modulated inactivation) in Fig. 5.21 show that reaction time for each batch increases from batch to batch to compensate for enzyme inactivation. In practice, the use of very stable immobilized enzymes makes the adjustment of reaction time batch after batch unnecessary and sparse corrections are made after a number of batches when a significant reduction in substrate conversion (i.e. $> 1\%$) has occurred.

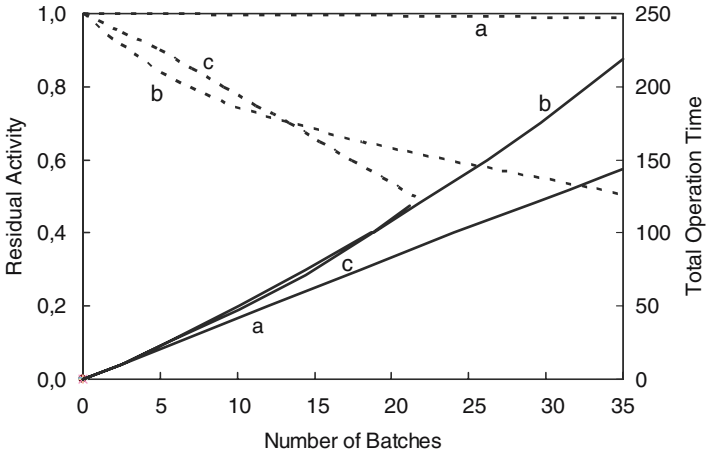


Fig. 5.21 Simulation of sequential batch reactor operation with immobilised penicillin acylase at pH 7.8 and 40 °C ($K = 6.5\,mM$; $K_1 = 56.5\,mM$; $K_2 = 59.3$; $V = 625\,\mu mol/min/g$. a) considering full protection by catalytic modulators (all $n_i = 1$; all $n_i' = 1$); b) not considering modulation (all $n_i = 0$; all $n_i' = 0$); c) with experimentally determined values for modulation factors (Illanes et al. 1996)

In the case of continuous operation of immobilized enzyme reactors, several strategies are available to obtain a product of uniform quality (Illanes et al. 1999). A rather straightforward strategy considers biocatalyst make-up, but this is cumbersome, especially for CPBR (Verhoff and Schlager 1981). Another strategy is based on a rising temperature profile to compensate enzyme inactivation by increasing reactivity (Faqir and Abu-Reesh 1998). The rational behind this strategy is that energy of activation is lower for the enzyme-catalyzed reaction than for the reaction leading to enzyme inactivation. This strategy has been used at industrial scale but it is applicable only in a rather narrow temperature range and can easily lead to operational instability. A very convenient strategy is based on profiling feed flow-rate according to the profile of enzyme inactivation. In fact, the equations that describes the continuous operation of enzyme reactors (Eqs. 5.16, 5.20–5.22, 5.24, 5.28–5.30) show that the reactor can operate at a constant outlet X, provided that E/F remains constant. However, this strategy has the drawback of producing a variable output to the downstream operations (Illanes et al. 1992). Variation in total output can be attenuated by using multiple staggered reactors. Defining a flow-rate variation tolerance (R_F) and an enzyme replacement policy based on biocatalyst life-span (H), the number of required reactors can be determined by (Pitcher 1978):

$$N_R = -H\frac{\ln 2}{\ln R_F} \tag{5.82}$$

and the time interval between each reactor start-up by:

$$t_s = \frac{d}{N_R} \tag{5.83}$$

Usually, $N_R + 1$ reactors will be required to absorb non-productive time (discharge, cleaning and filling of reactor). Solving the equation that represents enzyme inactivation under operation conditions (i.e. Eq. 5.76) and the equation that model conversion profiles within the biocatalyst bed in CPBR (Eq. 5.79), residual enzyme activity in each bioreactor after each time interval can be determined and feed flow-rate to each bioreactor during each interval calculated as:

$$F_{0i} = \frac{F_T}{\sum_{i=1}^{N_R} \frac{E_{t=t_s \cdot i}}{E_0}} \tag{5.84}$$

5.4.4.2 Thermal Optimization of Enzymatic Reactors

Temperature is a variable of paramount importance in any bioprocess. Temperature optimization of bioreactor operation is a complicated task since many variables and parameters are involved that are strongly dependent on temperature. Besides, temperature exerts opposite effects on enzyme activity and stability. Then, thermal optimization of enzyme reactor operation requires that temperature explicit functions for all parameters involved be determined and validated. Optimization will

Table 5.4 Design Parameters and Conditions of Operation in the Simulation of an Industrial CPBR for the Hydrolysis of Cheese Whey Permeate with Immobilized β-Galactosidase

Total flow-rate (m^3/h)	3
Void fraction of biocatalyst bed	0.6
Lactose concentration in feed stream (mM)	146
Specific activity of biocatalyst (IU/g)	350
Substrate conversion	$0.7 \pm 0.01\%$
Biocatalyst replacement policy (% initial activity)	5–75
Number of reactors	$8+1$
Height/diameter ratio of biocatalyst bed	5

be illustrated by considering the hydrolysis of lactose with chitin-immobilized β-galactosidase in CPBR. The following expressions for thermal dependence of its kinetic parameters were validated, based on thermodynamic and Arrhenius correlations (Illanes et al. 2000):

$$k_{cat} = 1.9 \cdot 10^7 \exp\left(\frac{-5246.6}{T}\right)$$

$$K = 5.2 \cdot 10^5 \exp\left(\frac{-2956}{T}\right)$$

$$K_1 = 2.7 \cdot 10^{11} \exp\left(\frac{-6535.7}{T}\right)$$

Temperature explicit functions for inactivation parameters considering substrate and product modulation and a two-stage series mechanism were also determined and validated as already presented in Table 5.2. Temperature optimization of CPBR was then accomplished by replacing these temperature-explicit functions in Eqs. 5.76 and 5.79. A battery of staggered reactors was considered to absorb output flow fluctuations due to enzyme inactivation (see Eqs. 5.82 and 5.83). Flow-rate was considered constant during each time interval (see Eq. 5.84) so that variation in substrate conversion was below 1%. Conditions of operation and design parameters used in the simulation of an industrial CPBR for the hydrolysis of whey permeate with immobilized β-galactosidase are summarized in Table 5.4.

Based on that data, temperature was optimized in terms of an annual cost objective function (AC), considering the costs of biocatalyst (EC), utilities (UC) and equipment (RC). A summary of results is presented in Table 5.5 for biocatalyst replacement at 25% initial activity; sensitivity with respect to biocatalyst replacement policy is presented as a surface of response in Fig. 5.22 (Illanes et al. 2001).

Optimum temperature is close to 20 °C and varies slightly with biocatalyst replacement policy. AC increases sharply over optimum temperature because of increasing cost of biocatalyst, since more frequent replacement is required as temperature increases. Below optimum temperature, AC increases smoothly due to an increase in energy consumption and in bioreactor size as temperature decreases below ambient. If no modulation effects are considered, AC is sensibly lower

Table 5.5 Temperature Optimization Based on a Cost-Objective Function of Multiple Staggered CPBER with Chitin-Immobilized β-Galactosidase Under Biocatalyst Replacement at 25% of Initial Activity. Operation Conditions Are Those in Table 5.3

T (°C)	V_R (m^3)	t_s (h)	d (h)	EC	UC	RC	AC
10	0.213	109.4	875	99.2	38.6	10.4	148.2
15	0.149	81.3	650	93.2	19.3	8.6	121.1
20	0.108	53.1	425	103.7	0.4	7.3	111.4
25	0.081	33.8	270	122.4	19.3	6.2	147.9
30	0.063	21.6	173	147.4	38.6	5.4	191.4
35	0.053	10.1	81	265.0	57.9	4.9	327.8
40	0.042	5.7	45.5	373.0	77.2	4.4	454.7

EC: enzyme cost; UC: utility cost; RC: reactor cost; AC: annual cost. All costs are in thousands of US$ per year

(Illanes et al. 2001), giving an erroneous underestimate, as a consequence of the negative modulation exerted by the substrate in this case. Considering modulation on enzyme inactivation is then quite relevant for proper bioreactor design.

5.4.5 Enzyme Reactor Design and Performance Under Thermal Inactivation and Mass Transfer Limitations

Immobilized enzymes are apparently stabilized when subjected to diffusional restrictions, since reduced activity pushes the system away from mass transfer limitations (Naik and Karanth 1978). The simultaneous effect of diffusional restrictions

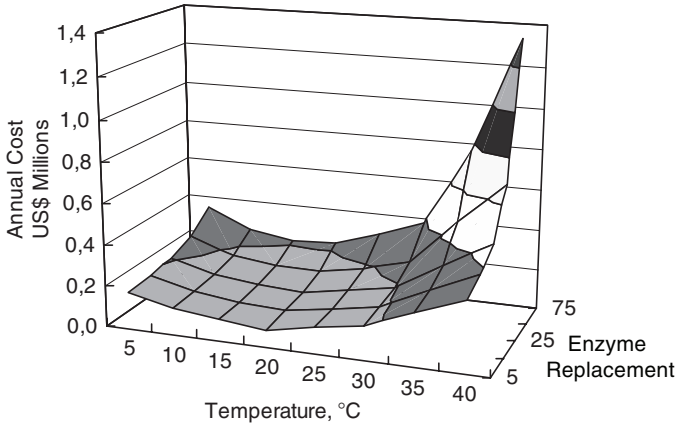


Fig. 5.22 Surface of response for temperature optimization of continuous staggered CPBR with chitin-immobilised β-galactosidase, based on data in Table 5.3, considering the annual cost of reactor operation as the objective function

and enzyme inactivation in enzyme reactor performance is then worthwhile to analyze.

As an example, the case of a batch reactor with Michaelis–Menten kinetics will be analyzed under enzyme inactivation and external diffusional restrictions (EDR).

Eq. 5.31 can be rewritten as:

$$\frac{dX}{dt} = \eta(X,t)\frac{v(X,t)}{K \cdot \beta_i} \tag{5.85}$$

where, from Eq. 5.3

$$v(X,t) = \frac{k_{cat} e(t) \cdot \beta_i (1-X)}{1+\beta_i(1-X)} \tag{5.86}$$

and from Eq. 4.11:

$$\eta(X,t) = \frac{[1+\beta_i(1-X)] \cdot \left[1+\alpha(t)+\beta_i(1-X)) - \sqrt{[1+\alpha(t)+\beta_i(1-X)]^2 + 4\beta_i(1-X)}\right]}{2\beta_i(1-X)\cdot\alpha(t)} \tag{5.87}$$

Under enzyme inactivation α cannot be considered as a constant anymore since it contains the term e which represents the concentration of active enzyme that now varies with time:

$$\alpha(t) = \frac{k \cdot e(t)}{h \cdot K} \tag{5.88}$$

Substituting Eq. 5.86, 5.87 and 5.88 in Eq. 5.85 and using a suitable model for enzyme inactivation (i.e. Eq. 5.76 for modulated two-stage sequential inactivation kinetics) batch enzyme reactor performance will be described by the resolution of differential Eqs. 5.85 and 5.76. The same procedure can be used considering other mechanisms of enzyme kinetics and enzyme inactivation. We have constructed a didactic software game in Visual Basic in which kinetic and inactivation parameters can be modified at will and analyze its impact on enzyme reactor performance.

The simultaneous effect of diffusional restrictions and enzyme inactivation in enzyme reactor performance represents a step forward to a more proper design of enzymatic reactors.

Nomenclature

A_S	cross-section area of bioreactor	$[L^2]$
a	molar concentration of substrate A (alternatively: coefficient in Eq. 5.3)	$[ML^{-3}]$
a_i	initial molar concentration of substrate A	$[ML^{-3}]$
$a_{i,j}$	specific activity of enzyme species $E_{i,j}$ (IU/mol)	$[T^{-1}]$

a_s	specific activity of biocatalyst	$[IUM^{-1}]$
b	molar concentration of substrate B (alternatively: coefficient in Eq. 5.3)	$[ML^{-3}]$
b_i	initial molar concentration of substrate B	$[ML^{-3}]$
c	coefficient in Eq. 5.3	
c_{cat}	concentration of biocatalyst	$[ML^{-3}]$
d	time of a cycle of reactor operation	[T]
E	enzyme activity	[IU]
E_0	initial enzyme activity	[IU]
$[E_{i,j}]$:	molar concentration of enzyme species $E_{i,j}$	$[ML^{-3}]$
$e_{i,j}$	volumetric activity of enzyme species E_{ij}	$[IUL^{-3}]$
e	enzyme volumetric activity	$[IUL^{-3}]$
e_0	initial enzyme volumetric activity	$[IUL^{-3}]$
F	bioreactor feed flow-rate	$[L^3T^{-1}]$
F_T	total flow-rate to downstream operations	$[L^3T^{-1}]$
F_{0i}	initial feed flow-rate to bioreactor "i"	$[L^3T^{-1}]$
H	number of half-lives of biocatalyst use	
h	film volumetric mass transfer coefficient for substrate	$[L3T^{-1}]$
K	Michaelis–Menten constant	$[ML^{-3}]$
k_{cat}	catalytic rate constant	
k_D	first-order inactivation rate constant	$[T^{-1}]$
$k_{i,j}$	transition rate constants	$[T^{-1}]$
$k_{i,0}$	transition rate constant in stage i of inactivation in the absence of modulator	$[T^{-1}]$
$k_{i,M}$	transition rate constant in stage i of inactivation in the presence of modulator	$[T^{-1}]$
M_{cat}	mass of biocatalyst	[M]
m_{cat}	concentration of biocatalyst	$[ML^{-3}]$
N_R	number of staggered reactors	
N_i	global modulation factor	
n	stoichiometric coefficient	
$n_{i,M}$	modulation factor by modulator M in stage i of inactivation	
p	molar product concentration	$[ML^{-3}]$
R_F	flow-rate variation tolerance (ratio of minimum to maximum allowable flow-rate to downstream)	
s	molar substrate concentration	$[ML^{-3}]$
s_0	molar substrate concentration in the bulk medium	$[ML^{-3}]$
s_i	molar initial (or inlet) substrate concentration	$[ML^{-3}]$
$s_{0,i}$	molar initial (or inlet) substrate concentration in bulk reaction medium	$[ML^{-3}]$
t	time	[T]
$t_{1/2}$	biocatalyst half-life	[T]
t_s	time interval between each rector start-up	

V_B	bed volume of packed-bed reactor	L^3
V_R	volume of reaction	L^3
v	initial reaction rate	$[ML^{-3}T^{-1}]$
X	conversion of limiting substrate into product	
X_i	conversion at initial steady-state	
z	variable height of biocatalyst bed	[L]
α	dimensionless number of Damkoehler	
$\alpha_{1,j}$	ratio of specific activities between the intermediate and initial enzyme species	
β	dimensionless substrate concentration	
β_0	dimensionless substrate concentration in bulk reaction medium (s_0/K)	
β_i	dimensionless initial (or inlet) substrate concentration (s_i/K)	
$\beta_{0,i}$	dimensionless initial (or inlet) substrate concentration in bulk reaction medium $(s_{0,i}/K)$	
γ_0	dimensionless product concentration in bulk reaction medium	
ε	void fraction	
Φ	dimensionless Thièle modulus for substrate	
η	effectiveness factor (local)	
η'	mean integral value of effectiveness factor (global)	
ρ_{ap}	apparent density of the packed biocatalyst	$[ML^{-3}]$
τ	fluid residence time	[T]
ν	dimensionless reaction rate	

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Chapter 6
Study Cases of Enzymatic Processes

6.1 Proteases as Catalysts for Peptide Synthesis

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Peptides are heteropolymers composed by amino acid residues linked by peptidic bonds between the carboxyl group of one amino acid residue and the α-amino group of the next one. The definition is rather vague in terms of chain length, peptides ranging from two residues to a few dozens residues. Its upper limit of molecular mass has been set rather arbitrarily in 6,000 Da. The size of the molecule determines the technology most suitable for its production. Recombinant DNA technology is particularly suitable for the synthesis of large peptides and proteins, as illustrated by the case of insulin and other hormones (Walsh 2005). Chemical synthesis is a viable technology for the production of small and medium size peptides ranging from about 5 to 80 residues (Kimmerlin and Seebach 2005). Enzymatic synthesis is more restricted and has been hardly applied for the synthesis of peptides exceeding 10 residues. Its potential relies on the synthesis of very small peptides and, in fact, most of the cases reported correspond to dipeptides and tripeptides (Kumar and

A. Illanes (ed.), *Enzyme Biocatalysis*.

Bhalla 2005). In this sense, the technologies for peptide production are not competitive with each other in most of the cases.

6.1.1 Chemical Synthesis of Peptides

The chemical route is often a better technological option than the biotechnological methods of recombinant DNA and biocatalysis for the synthesis of medium size peptides that comprise most of the pharmaceutically relevant molecules. The synthesis of peptides was originally performed in solution. However, since the introduction of solid-phase synthesis by Merrifield (1986), this technology has gained more relevance (Stewart and Young 1984).

Solid-phase peptide synthesis (SPPS) consists in the elongation of a peptidic chain anchored to a solid matrix by successive additions of amino acids which are linked by amide (peptide) bond formation between the carboxyl group of the incoming amino acid and the amino group of the amino acid previously bound to the matrix, until the peptide of the desired sequence and length has been synthesized (Nilsson et al. 2005). SPPS has many advantages over the classical system in solution: the reaction can be automated and the problem of solubilization of the peptide no longer exists since it remains attached to the solid matrix. The strategy of synthesis (Fmoc or t-Boc), the nature of the solid carrier, the coupling reagents and the procedure of cleavage of the peptide from the solid matrix are the most relevant variables in SPPS. A general scheme of the stepwise SPPS is presented in Fig. 6.1.1. The first step is the coupling of the C-terminal amino acid to the solid matrix. The Nα (A) group is then removed by treatment with trifluoroacetic acid (TFA) in the t-Boc strategy and with piperidine in the Fmoc strategy. The next (Nα protected) amino acid is coupled to the already synthesized peptide chain bound to the polymeric matrix and, once coupled, its Nα amino group is deprotected. This coupling–deprotection cycle is repeated until the desired amino acid sequence has been synthesized. Finally, the peptide–matrix complex is cleaved and side chain protecting groups are removed to yield the peptide with either a free acid or amide depending on the chemical nature of the functional group in the solid matrix. The cleavage reagent must remove the protecting groups of the side chains of the amino acids, which are stable at the conditions of Nα deprotection.

Research and development in SPPS has conducted to two main schemes of protection, which are known as t-Boc/Bzl and Fmoc/tBu strategies (Chan and White 2000). In t-Boc/Bzl, the t-Boc (tert-butoxycarbonyl) group is used for the protection of the Nα amino group and a benzyl or cyclohexyl for the side chains of several amino acids. In Fmoc/tBu, the Fmoc (9-fluorenyl methoxycarbonyl) group is used for the protection of the Nα amino group and the tert-butyl group for the protection of the side chains of several amino acids (Albericio 2000).

Solid matrices should meet several requirements: particles should be of conventional and uniform size, mechanically robust, easily filterable, chemically inert and chemically stable under the conditions of synthesis and highly accessible to the

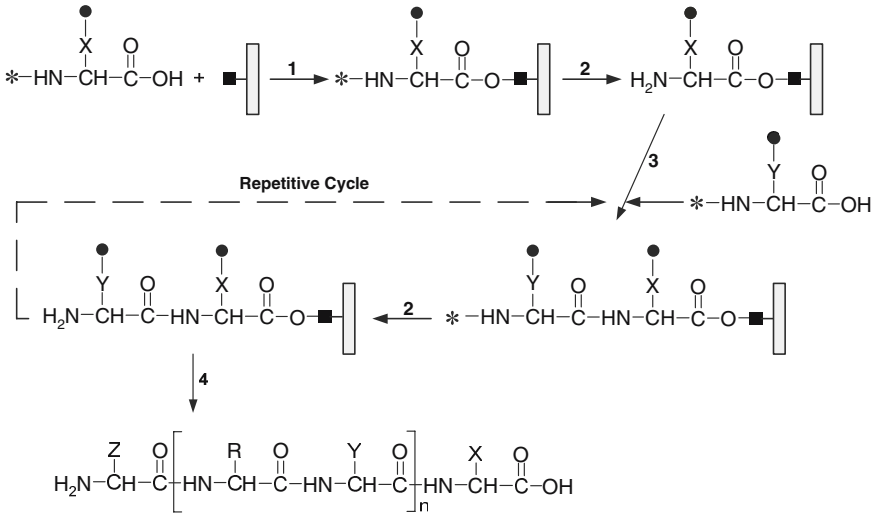


Fig. 6.1.1 Scheme for the stepwise solid-phase peptide synthesis: •: side-chain protecting group; ∗ : Nα amino protecting group; ■: functional group in the resin (Cl or NH_2); ▯: resin. 1: coupling of first residue; 2: Nα deprotection; 3: coupling of following residues (repetitive cycle) 4: cleavage, side-chain deprotection

solvents allowing the penetration of the reagents and the enlargement of the peptide chain within its microstructure. They must not interact physically with the peptide chain being synthesized and should be capable of being functionalized by a starting group. Several polymeric supports are now available which can be derivatized with functional groups to produce a highly stable linkage to the peptide being synthesized (Barlos et al. 1991).

Once the peptide synthesis of the desired sequence is finished, the protecting groups of the side chains are removed and the peptide freed from the support. In the t-Boc/Bzl strategy, the most popular method is the one developed by Tam et al. (1983). Deprotection is carried out with strong acids that may lead to unwanted secondary reactions of alkylation or acylation in certain amino acids that are promoted by the leaving protecting groups. To avoid such reactions, combinations of solvents acting as nucleophiles and acids that allow the process of deprotection have been pursued for decades. Different is the case of the Fmoc/tBu strategy, in which simpler solvents as TFA in combination with triisopropylsilane can be used.

The main SPPS strategies are sequential synthesis, convergent synthesis and chemical ligation. Sequential synthesis involves the stepwise addition of amino acids until the desired sequence is synthesized. This strategy is used for the synthesis of small to medium size peptides having up to 50 residues. However, larger size polypeptides can be constructed using sequential synthesis by the technique of cysteine polymerization, the construction of dendrimers using lysine matrices, or the construction of TASP (Template-Assembled Synthetic Protein) (Tuchscherer and Mutter 1996; Banfi et al. 2004). In convergent synthesis, peptides (up to 50

residues) are separately produced by sequential synthesis and then linked in solution or in solid phase to obtain the desired high molecular weight peptide or protein. The advantage of convergent synthesis is that each peptide fragment is purified and characterized before being linked. However, convergent synthesis has some drawbacks: the solubility of the protected fragments in the aqueous solvents used in the purification by HPLC and in the organic solvents used in the coupling reactions is usually low, reaction rates for the coupling of fragments are substantially lower than for the activated amino acid species in the conventional stepwise synthesis and, finally, the C terminal of each peptide fragment may be racemized during coupling. Some of these problems have been circumvented by using mixtures of solvents to increase the solubility, by using prolonged reaction times to increase the efficiency of coupling and by using glycine or proline in the C terminal to avoid the problem of racemization. In this way, convergent synthesis represents the best option for the chemical synthesis of large peptides and proteins so that a variety of large peptides have been successfully produced accordingly (Bray 2003). Chemical ligation is a particularly appealing strategy for the chemical synthesis of large peptides and proteins (Baca et al. 1995; Yan and Dawson 2001). It is based on the chemical linkage of short unprotected peptides which are easy to handle because of its high solubility in the solvents used for synthesis. These peptides are functionalized with groups that react chemoselectively with only one group of the acceptor peptide preserving the integrity of the unprotected side chains. Many proteins and peptides of biological interest have been synthesized by chemical ligation using a variety of ligands with the formation of thioester (Lu and Tam 2005), oxime (Nardin et al. 1998), disulfide or thiazolidine bonds (Tam et al. 1995).

SPPS can be performed in different ways. There are manual and automated systems available for small and large scale synthesis of only one peptide or several peptides at the time (multiple peptide synthesis). All operations in SPPS, namely coupling, deprotection and final removal, are conducted in the same pot so that several washing steps have to be considered; reagents are used in large excess to speed-up the reaction and drive it to completion. Manual synthesis of individual peptides can be performed in syringes of different sizes provided with a bottom sintered-glass or plastic filter. Multiple peptide synthesis at the micromolar level can be conducted in functionalized cellulose, polypropylene or polyvynilidene difluoride membranes, according to the spot-synthesis methodology developed by Frank (2002). Fmoc/tBu and t-Boc/Bzl multiple peptide synthesis at the millimolar level can be performed using the tea-bag methodology developed by Houghten (1985), in which up to 400 peptides of 20 residues or less can be synthesized at the same time. The tea-bag system of SPPS has been successfully employed to construct peptide libraries (Houghten et al. 2000). Several systems for the automated t-Boc/Bzl and Fmoc/tBu SPPS, going from 1.5 mg to 5 kg scale, are now available from various suppliers (Chan and White 2000).

The precise quantification of the amount of peptide synthesized is determined through amino acid analysis by HPLC after peptide hydrolysis in acid medium considering those acid-resistant amino acids. Although amino acid analysis is usually performed after cleavage from the resin, it can also be conducted to the peptide

still attached to the resin (Ambulos et al. 2000). The quantification of cysteinic SH groups in the peptide is performed according to the method of Ellman (1958). The determination of peptide structure can be done by circular dichroism (CD) (Brahms and Brahms 1980) and nuclear magnetic resonance (NMR) (Rance et al. 1983). The analysis determines if the peptide has a secondary structure and if that structure is α-helix or β-turn (Guzmán et al. 2003). The application of native peptides for pharmacological use may have some restrictions because of the degradation by endogenous proteases, hepatic clearance, undesirable side effects by interaction with different receptors, and low membrane permeability due to their hydrophilic character. The most straightforward approach to solve the problem is by introducing changes into the side chains of single amino acids or the modification of the peptide chain backbone (Ahn et al. 2002). There is great interest in this class of products due to their wide range of biological properties such as immunosuppressant, antibiotic, antifungal, antiinflammatory and antitumoral activities (Hamel and Cowell 2002; Sarabia et al. 2004). Another approach is the construction of constrained structure peptidomimetics in which the amino acids comprising a region of a defined structure in the natural peptide are synthesized and bound by its amino and carboxy terminals through a non-protein ligand (Lioy et al. 2001). In this way, better recognition by antibodies can be obtained (Calvo et al. 2003). Such peptidomimetics have also been produced to inhibit protein–protein interaction (Yin et al. 2006).

6.1.2 Proteases as Catalysts for Peptide Synthesis

Proteolytic enzymes comprise a group of hydrolases referred as peptidases (EC 3.4) which share the common feature of attacking peptide bonds. These enzymes are usually termed proteases. Proteases are classified in six families in which serine, threonine, cysteine, aspartic, glutamic or metallic groups play a primary catalytic role. Serine, cysteine and threonine proteases are quite different from aspartic, glutamic and metalloproteases. In the first three groups, the nucleophile in the catalytic center is part of an amino acid residue, while in the second three groups the nucleophile is an activated water molecule. In cysteine proteases the nucleophile is a sulfhydryl group and the catalytic mechanism is similar to the serine proteases in which the proton donor is a histidine residue.

Proteases are the most relevant enzymes in technological terms, sharing about one half of the world market of enzymes, with annual sales of about US$ 3 billion (Chellapan et al. 2006). Microbial and plant proteases are the most relevant and have been widely utilized in medicine and in different industrial processes for decades (see section 1.5). New sources of proteases are continuously being reported, especially from endogenous plant species (Priolo et al. 2000; Obregón et al. 2001) and exotic organisms that thrive in extreme environments, being their proteases abnormally stable and/or active at such extreme conditions (Chellapan et al. 2006). Proteases are active at mild conditions, with pH optima in the range of 6–8; they are robust and stable, do not require stoichiometric cofactors and are also highly stereo

and regioselective (Bordusa 2002). These properties are quite relevant to use them as catalysts in organic synthesis. This is possible because proteases can not only catalyze the cleavage of peptide bonds but also their formation (Capellas et al. 1997; Björup et al. 1999), as well as other reactions of relevance for organic synthesis, for instance: the regiospecific hydrolysis of esters and the kinetic resolution of racemic mixtures (Carrea and Riva 2000; Bordusa 2002). Subtilisin, chymotrypsin, trypsin and papain have been widely used proteases in the chemical synthesis of peptides. However, the broad specificity of proteases restricts their application in peptide synthesis, since the peptide product that accumulates during the reaction can be attacked by the proteases simultaneously with the reaction of synthesis (Bordusa 2002).

6.1.3 Enzymatic Synthesis of Peptides

Biocatalysis in non-conventional (non-aqueous) media has expanded the spectrum of application of proteases to those reactions that cannot proceed effectively in aqueous environments, this is, the synthesis of peptide bonds instead of their hydrolysis (Barberis and Illanes 1996; Kasche 1996). Organic solvents (Clark 2004), supercritical fluids (Mesiano et al. 1999), eutectic mixtures (Gill and Valivety 2002), solid-state (Halling et al. 1995) and ionic liquids (Park and Kazlauskas 2003; Lou et al. 2004) have been used as media for enzymatic synthesis of peptides, being the former the most relevant. Benefits of conducting reverse reactions of synthesis in such media have been thoroughly analyzed in section 1.6. As compared to chemical synthesis, a most important advantage of biocatalysis is the specificity of the reaction, which reduces the requirement of side-chain protection.

Enzymatically synthesized small peptides (usually di or tripetides) are being used successfully for human and animal nutrition and also as pharmaceuticals and agrochemicals. Some relevant examples are the synthesis of the leading non-caloric sweetener aspartame, the lysine sweet peptide, kyotorphin, angiotensin, enkephalin, cholecystokinin and dynorphin (Aso 1989; Clapés et al. 1989; Kimura et al. 1990a; Nakanishi et al. 1990), and some nutritional dipeptides and tripeptides (Kimura et al. 1990b). Several other examples of enzymatically synthesized biologically active peptides have been reported in the last decade (Zaks and Dodds 1997; Barberis et al. 2002; Liu et al. 2002; Hou et al. 2006 a,b).

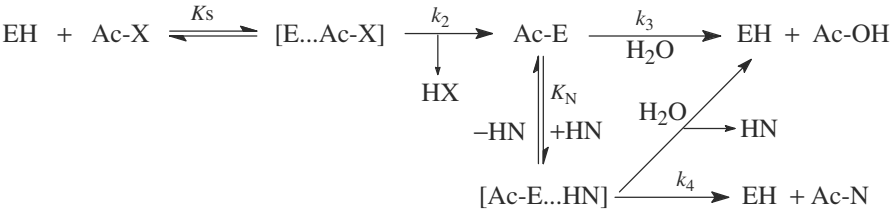
Enzymatic peptide synthesis can proceed by two mechanisms: thermodynamic and kinetic control (Kumar and Bhalla 2005). The thermodynamically controlled synthesis of peptides (TCS) with proteases represents the reverse of the hydrolytic breakage of peptide bond catalyzed by those enzymes, as shown in the scheme (Jakubke et al. 1985):

$$R'COO^- + H_3^+NR'' \overset{K\text{ion}}{\rightleftharpoons} R'CO_2H + H_2NR'' \overset{K\text{con}}{\rightleftharpoons} R'CO\text{-}NHR'' + H_2O$$

where K_{ion} is the equilibrium constant of ionization and K_{con} is the equilibrium constant of conversion. According to the principle of microscopic reversibility, both

the formation and the hydrolysis of the peptide bond proceed by the same mechanism and through the same intermediate. The formation of the acyl intermediate from a carboxylic acid is a very slow reaction and represents the rate limiting step in TCS (Bordusa 2002). Main issues of TCS are the use of an acyl donor with the free carboxylic group and the possibility of using any type of proteases, independent of their catalytic mechanism. Their main drawbacks are the low reaction rates and product yield (determined by the equilibrium constant of the reaction) attainable, the high amount of enzyme biocatalyst often required and the need of precise reaction conditions to displace the equilibrium towards synthesis. This in practice can produce severe compromises with enzyme activity and stability. From the above scheme, it is apparent that the equilibrium will be displaced to hydrolysis in an aqueous medium. The displacement of equilibrium towards peptide bond formation can be attained by manipulation of the equilibrium of ionization (i.e. pH change) and the equilibrium of the reaction (i.e. by product precipitation or by modification of medium composition) (Halling 1994). The addition of organic cosolvents and the use of aqueous-organic biphasic systems are good strategies to displace the equilibrium towards synthesis. The presence of organic solvents reduces the activity of water in the reaction medium, which favors the equilibrium, and also reduces the dielectric constant of the medium, which in turn reduces the acidity of the carboxylic group of the acyl donor and so increases the equilibrium constant K_{ion} promoting the reaction of synthesis. The use of cosolvents is a rather simple strategy but high concentrations of cosolvents are usually detrimental for enzyme activity (see section 1.6). In biphasic systems, the partition of the peptide products from the aqueous phase that contains the enzyme to the organic phase drives the equilibrium towards synthesis, with the additional benefit that the product is no longer subjected to hydrolysis. However, reaction rates in biphasic systems are low because of limitation by substrate diffusion; besides, proteases tend to denature in the water–solvent interface (Halling 1994). The use of neat hydrophobic solvents with very low water content can in principle be effective for peptide synthesis by reducing the backward hydrolytic reaction. However, proteases may exhibit very low activity and reduced stability, and substrates and products may be poorly soluble in this kind of media.

The kinetically controlled synthesis of peptides (KCS) with proteases can be represented by the following scheme (Bordusa 2002):



where EH is the free enzyme; Ac-X is the acyl donor substrate; [E…Ac-X] is the Michaelis–Menten acyl–enzyme complex; HX is the released group; Ac-E is the acyl–enzyme intermediate, HN is the acceptor substrate (nucleophile), Ac-N

is the product of synthesis (peptide) and Ac-OH is the product of hydrolysis of the acyl donor. As shown in the above scheme, the acyl donor, that requires being activated in the form of an ester or an amide, first binds to the enzyme to form a tetrahedric enzyme–substrate complex [E . . . Ac-X] that collapses to form the covalent acyl–enzyme intermediate [Ac-E]. This intermediate can be nucleophilically attacked by water and by the nucleophile (HN), which can be an amine, an alcohol or a thiol that will compete with water for the deacylation reaction. The success of the reaction of synthesis will depend on the kinetics of these nucleophilic reactions. Different than TCS, only serine or cysteine proteases can be used to perform KCS, because the enzyme acts in this case as a transferase and catalyzes the transference of an acyl group from the acyl donor to the amino acid nucleophile through the formation of an acyl–enzyme intermediate. Generally KCS proceeds faster and requires lower enzyme to substrate ratios than TCS because the acyl donor is now in the form of an activated carboxylic acid (Bordusa 2002). As in TCS, the decrease of water activity, by using an organic cosolvent, favors synthesis in KCS by reducing the hydrolysis of the acyl–enzyme intermediate and the final product, but, again, the reaction medium can be harmful to the enzyme (Barberis et al. 2002, 2006). However, mutant enzymes have been used in the KCS of peptides in alkaline medium containing organic cosolvents where the parent enzymes were denatured, obtaining high conversion yields (West et al. 1990). Biphasic systems are not adequate to perform KCS since in this case, the neutral esters commonly used as acyl donors partition poorly into the aqueous phase and therefore the concentration in that phase, where the enzymatic reaction occurs, is low.

Despite their good catalytic properties, proteases are not ideal catalysts for the synthesis of peptides. Its specificity and selectivity might limit their potential, particularly in the case of rather large peptides where unwanted hydrolytic reactions will occur over the formed product and the substrates. Besides, the use of non-conventional reaction media and the conditions of temperature and pH required for synthesis can be detrimental both for protease activity and stability (Barberis et al. 2002; Bordusa 2002; Quiroga et al. 2005, 2006). However, there are different strategies to overcome such problems, which comprise the engineering of the reaction medium, the biocatalyst and the substrate (Lombard et al. 2005).

Medium engineering refers to the rational manipulation of the reaction medium to positively influence the properties of the enzyme with respect to the reaction of synthesis (Clapés et al. 1990 a,b). This frequently implies the substitution of the usual aqueous medium for a non-conventional medium in which water has been replaced partially or almost totally by another solvent (see section 1.6). Organic cosolvents are usually detrimental for enzyme activity at moderately high concentrations; however, polyols and glymes are notable exceptions among cosolvents and there are several examples of proteases and other peptide bond forming enzymes which have been successfully employed in synthesis in such media (Guisán et al. 1997; Castro 2000; Illanes and Fajardo 2001; Illanes et al. 2004; Hou et al. 2006b). Biphasic systems have been extensively used for enzymatic peptide synthesis and represent a good strategy because they are highly flexible and can accommodate to the properties of substrates and products (Kimura et al. 1990a; Feliú et al. 1995; Murakami

et al. 2000; Barberis et al. 2002; Trusek-Holownia 2003). Suspension of proteases in nearly anhydrous hydrophobic solvents has also been applied to peptide synthesis. Despite its advantages (see section 1.6), protease activity can be drastically reduced in such media (Klibanov 1997; Quiroga et al. 2006). However, some improvements in activity have been obtained by the addition of water mimics, such as formamide or ethylene glycol, to the reaction medium or the addition of crown ethers during the preparation of the lyophilized enzyme (van Unen et al. 2001, 2002). A 425-fold increase in activity was observed by the addition of 18-crown-6 to chymotrypsin in the synthesis of a dipeptide in acetonitrile medium (van Unen et al. 1998). An alternative to the classic biphasic and micellar systems has been proposed by Clapés et al. (2001) as reaction medium for peptide synthesis with chymotrypsin by using water-in-oil (W/O) emulsion with a high water content (95%) which is the opposite as in reverse micelles. Such system has been occasionally used in organic synthesis (Manabe et al. 2000), but its application in biocatalysis is still in its early development.

Biocatalyst engineering refers to all strategies aimed to obtain biocatalysts well suited to perform under the conditions of synthesis and include approaches that range from chemical modification to genetic and protein engineering (Bordusa 2002; Adamczak and Hari Krishna 2004; Hudson et al. 2005). The insolubilization of the biocatalyst by immobilization to a solid carrier or by protein aggregation represents the most relevant biocatalyst engineering strategy for producing robust enzyme catalysts well suited to withstand the harsh conditions prevailing during the reactions of synthesis. Immobilization has been extensively used to produce protease biocatalysts for peptide synthesis (Guisán et al. 1997; Filippova and Lysogorskaya 2003; Lei et al. 2004). Insolubilization by protein cross-linking is a promising technology to produce biocatalysts for synthesis. Cross-linked enzyme crystals (CLECs) are robust biocatalysts produced by crystallization of the enzyme protein followed by cross-linking with the bifunctional reagent glutaraldehyde. Their specific activities are very high since there is no inert matrix and the whole mass of biocatalyst is essentially pure enzyme protein; however, they have the serious disadvantage of requiring the enzyme in a pure state to be able to crystallize it, which in practice means that the cost of the biocatalyst is very high. CLECs of subtilisin have been recently used successfully in organic synthesis both in repeated batch and continuous operation (Amorim Fernandes et al. 2005). Cross-linked enzyme aggregates (CLEAs) are similar to CLECs in their properties, with the additional advantage of their simplicity and low cost since the enzyme does not require to be purified to any extent (Cao et al. 2000, 2003). CLEAs prepared by co-aggregation with polyionic polymers (Wilson et al. 2004a) and CLEAs encapsulated into hydrophilic polyvynil alcohol hydrogel (Wilson et al. 2004b) have provided an adequate microenvironment for enzymatic peptide-bond formation in the synthesis of β-lactam antibiotics in non-aqueous environments (see section 6.2). However, in the specific case of peptide synthesis with proteases, autoproteolysis can play a role because of the close proximity and flexibility of the enzyme molecules. Other strategies of biocatalyst engineering consider the manipulation of the genes encoding the enzyme protein (see section 1.5) and proteases have been preferred models. In fact, subtilisin has

been extensively studied, and considerable progress has been made in engineering this protease and its substrates for peptide bond formation in aqueous media (Abrahamsen et al. 1991). In *Pseudomonas aeruginosa*, a disulfide bond between Cys-30 and Cys-58 played an important role in the organic solvent stability of the PST-01 protease (Ogino et al. 2001). The effect of a novel disulfide bond engineered in subtilisin E from *Bacillus* sp. based on the structure of a thermophilic subtilisin-type serine protease (aqualysin I) was examined and suggested that an electrostatic interaction between Lys 170 and Gly 195 is important for catalysis and stability in that protease (Takagi et al. 2000). Substitution of native amino acids by fluoroalkyl analogs in the commercially available proteases trypsin and α-chymotrypsin represents a new approach for the design of biologically active peptides with increased stability. (Thurst and Koksche 2003). Site-directed mutagenesis has also been employed to improve the properties of trypsin for performing peptide synthesis (Lombard et al. 2005). Other strategy for improving enzyme performance based on genetic manipulation is directed evolution (Arnold 2001) based on tandem random mutagenesis which has been successfully applied to improve the thermal stability of subtilisin (Adamczak and Hari Krishna 2004).

Substrate engineering refers to the modification of the substrate rather than the enzyme to improve reaction. Even though most proteases are able to recognize more than one amino acid, not all are able to couple any aminoacidic sequence. Due to the specificity of proteases to a particular amino acid, only those acyl donors that have a specific amino acid in the C-terminal position can be coupled without side reactions. For instance, trypsin requires arginine or lysine residues as carboxylic terminal components in the structure of the acyl donor (Bordusa 2002). In this way, the manipulation of the leaving group is generally useful to increase the specificity of the protease to a previously less specific amino acid, so increasing reaction rate (Miyazawa et al. 2001 a,b). The manipulation of the leaving group affects the aminolysis/hydrolysis ratio of an acyl donor and therefore the conversion yield, since the acyl–enzyme intermediate formed is the same regardless of the change produced in the leaving group. The influence of the structure of the acyl donor in the selectivity of α-chymotrypsin and the efficiency of nucleophiles on peptide synthesis in organic solvents have been reported by Cabezas et al. (1990) and Fischer et al. (1991). Another approach is the use of mimetic substrates. Contrary to the classic manipulation of the leaving group, focused on the increase in enzyme specificity, mimetic substrates are designed to bind to the active site of the enzyme. In this way, serine and cysteine proteases can react with non-specific amino acids or peptide sequences without altering the enzyme or the reaction medium (Thormann et al. 1999). The main advantage of this strategy is that mimetic substrates allow the formation of the acyl–enzyme and the nucleophile reaction to establish a peptide bond that cannot be further hydrolyzed because it does not correspond to the protease specificity (Bordusa et al. 1997; Lombard et al. 2005). However, this approach is limited to reactions with non-specific amino acid containing peptides, whereas the coupling of specific ones leads to unwanted cleavages due to the native proteolytic activity of the biocatalyst (Grunberg et al. 2000).

6.1.4 Process Considerations for the Synthesis of Peptides

Production of peptides on a productive scale represents the ultimate goal of technological development and presents several challenges that have to be addressed.

6.1.4.1 Chemical Synthesis

Since the pioneering works of Du Vigneaud (Du Vigneaud et al. 1953) and Merrifield (Merrifield 1963, 1996), SPPS has been developed and automated to a high degree in the last decades. Protocols amenable for scale-up have been well established to match the production levels required by the market. A key advantage of SPPS is that the peptide product can be easily separated from impurities and side products. Major drawbacks refer to the racemization during peptide bond formation, the requirement of protection of the side chains of the amino acids that increases the cost of the substrates and reduces the yield of product recovery during deprotection, the difficulty of recycling the coupling reagent and the acyl donor used in excess to achieve rapid and complete acylation of the nucleophile, the time consumed in protection and deprotection reactions that reduce the productivity of the process and the toxic nature of solvents and coupling reagents that may lead to health and environmental concerns (Nilsson et al. 2005). Despite these restrictions, SPPS can be considered now a mature technology and is the most appealing for large scale production of medium size peptides up to 100 amino acid residues, which comprises most of the peptides of therapeutic relevance (Patarroyo et al. 1988; Bruckdorfer et al. 2004). Commonly, peptides of less than 30 residues are produced entirely by sequential SPPS (Lloyd-Williams and Giralt 2000), while larger peptides (up to 60 residues) must be produced by convergent synthesis in which protected peptide fragments are synthesized by SPPS and then combined by liquid phase synthesis (Barlos and Gatos 1999). Larger size peptides and proteins are preferably produced by chemoselective ligation (Johnson et al. 2007), in which all the unprotected linked fragments have been previously synthesized by SPPS.

At productive scale, the high cost of materials and the release of environmentally aggressive and even toxic residues must be carefully considered. Costs of reagents are usually high; therefore, the use of large excess, which is sometimes practiced at laboratory scale and in the early stages of development, is inadmissible at large scale, where it is highly desirable to use reagents as close to stoichiometry as possible since large amounts of reagents cannot be wasted just to ensure the completion of the reaction. Aside from costs, environmental impact is also a matter of concern because of the surplus effluent produced. Therefore, the kinetics of the reactions must be thoroughly studied when scaling up to reduce the costs associated with reagents and waste treatment after reaction (Bruckdorfer et al. 2004; Patarroyo and Guzman 2004). Process validation should consider reproducibility in terms of yields of intermediate and final products and consistency in the profile of impurities of the product (Andersson et al. 2000). This is a complex task in the case of the synthesis of peptides because of the complexity and the number of operations involved

in the production process. The stringent requirements for validation, despite being costly and time-consuming, have to be appreciated as the adequate way to ensure the higher standards of quality and safety required by the final consumer, which is the recipient patient.

More than 40 therapeutic peptides are now in the market, which represents a considerable increase, since less than 10 were in the market by 1990. A much higher number of therapeutic peptides are in the different phases of approval. The therapeutic application of peptides has an enormous potential, which has been enhanced by the advances in the fields of formulation and administration of pharmaceuticals. Several of those peptides are now being produced at large scale (Guzmán et al. 2007).

6.1.4.2 Enzymatic Synthesis

The development of new methods suitable for the large-scale production of biologically active peptides with proteases has been actively pursued over the last decade. However, enzymes are in general labile catalysts, so that process engineering of enzymatic reactions should be designed carefully. This implies the optimization of most relevant operational parameters: pH, temperature, organic solvent concentration, and the assessment of the activity and stability of the biocatalyst under operation conditions, the solubility of reactants, the stability of reactants and products and the selection of conversion yield or productivity as an adequate objective function, or an economic objective functions comprising both (Illanes and Wilson 2003). In this sense, enzymatic synthesis of peptides is a less mature technology than chemical synthesis and no general protocols of synthesis are available, being each situation a particular case that has to be extensively studied and optimized to be technologically competitive.

Process development for the total enzymatic synthesis of a polypeptide requires the previous definition of a synthetic strategy, the proper selection of the proteases to be used for each coupling according to their specificity, the sequencing of peptide bond formation (one pot or consecutive reactions), the formulation of the reaction media and the selection of the amino acid and peptide derivatives acting as acyl donors and nucleophiles (Kumar and Bhalla 2005). Although there are some rules for a previous selection, most of the variables have to be experimentally determined for each step before optimization of the overall synthesis. The development of a practical process for the enzymatic synthesis of a target peptide needs to fulfil the following requirements:

- Optimization of the overall yield, by reducing the number of steps
- Integration of reaction and separation steps (i.e. the product from one reaction used as a substrate for the next one with minimum modifications)
- Minimal protection and use of alternative protecting groups easy to introduce and remove (by enzymatic catalysis if possible)

The large number of critical variables in an enzymatic process makes its optimization cumbersome. However, many of the limitations of chemical synthesis can be overcome by protease synthesis. The high specificity and high reactivity under mild operation conditions, which is characteristic of enzymatic processes, can have a

strong impact on process economics, since it will reduce the number of operations required for the synthesis, will have less stringent requirement for equipment, will reduce the energy input required and will produce a significantly milder environmental impact as a consequence of the lesser amounts and lower toxicity indexes of the wastes produced (Sinisterra and Alcantara 1993). This latter aspect is quite relevant since enzymatic processes can be considered as a clean technology, more in accordance with the concept of sustainable growth. Increasing health and safety regulations and the growing demand for biologically active peptides have prompted an intensive search for biotechnological alternatives to chemical synthesis of peptides of relevance in the medical and food areas. The size of the peptide is a major constraint for the enzymatic synthesis since no established and automated protocols have been yet developed and as the peptide chain growths, requirements of protection and modification of several operational variables at each step make the process cumbersome, so that in practice only small peptides of less than 10 residues have been synthesized enzymatically with moderate success. Of course, the strategy of convergence used in chemical synthesis is also an option for the enzymatic synthesis of peptides, as shown by the protease synthesis of the CCK octapeptide which is analyzed in more detail ahead (Fité et al. 2002). The great potential of the enzymatic synthesis of peptides is a powerful driving force for research in the design of the enzyme biocatalysts as well as in the substrate and the reaction medium and advances in those fields are already significant (see section 6.1.3) so that technological outcomes will certainly occur in the forthcoming decades. As for now, competition with the more established technologies of chemical synthesis is hard, except in particular niches where the outstanding properties of enzymes have profound process implications. This is illustrated by the case of the non-caloric sweetener aspartame, which is increasingly being produced by an enzymatic process with the protease thermolysin (Murakami et al. 2000; Schmid et al. 2001). Other outstanding examples of enzymatic technology for the production of biologically active peptides are the production of the neuroactive pain regulator dipeptide kyotorphin with immobilized and membrane-bound chymotrypsin (Floersheimer et al. 1989; Schwarz et al. 1992); the 10-step synthesis of the "delicious octapeptide" amide with a combination of serine and cysteine proteases with an overall yield of 39% (Gill et al. 1995); the thee step synthesis of the pain and nociception regulator pentapeptide enkephalin with Celite-immobilized proteinase, obtained with an overall yield of 30% (Richards et al. 1993); the production of di, tri and tetra bioactive peptides with Celite-deposited chymopapain and subtilisin, with overall yields of 73%, 74% and 67% respectively (Gill and Valivety 2002). An illustrative example that highlights the potentials and challenges of enzymatic peptide synthesis is the case of the cholecystokinin C-terminal octapeptide (CCK-8) that will be presented in more detail.

CCK-8 is the cholecystokinin C-terminal octapeptide (H-Asp-Tyr $[SO_3]$-Met-Gly-Trp-Met-Asp-Phe-NH_2) of cholecystokynin (CCK), a polypeptidic hormone of 33 amino acid residues, responsible for stimulating the digestion of fat and protein in the small intestine (Schwartz et al. 1991). CCK-8 ($CCK_{26–33}$) fragment is the minimum biologically active sequence with the same biological activity as CCK-33 (Villanueva et al. 1982) and has been suggested as potential therapeutic agent in

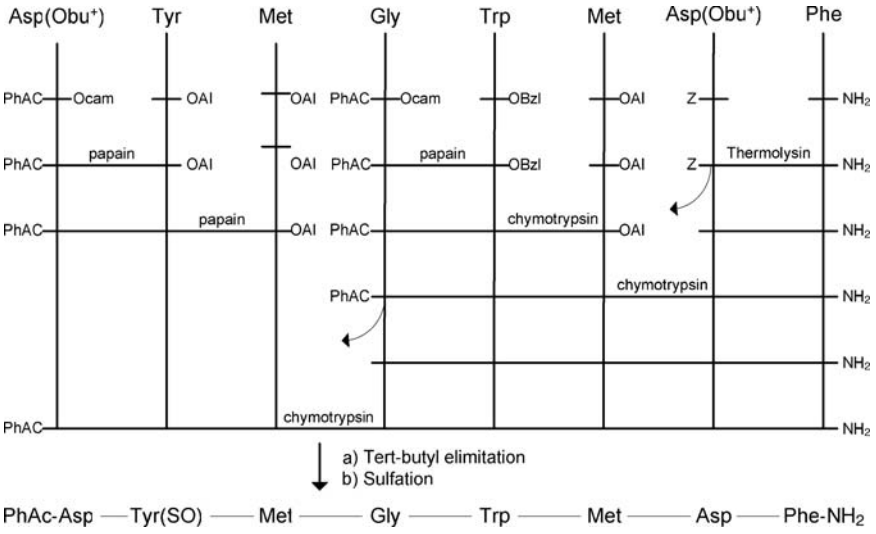


Fig. 6.1.2 Convergent enzymatic synthesis of the C-terminal cholecystokinin octapeptide (CCK-8)

the control of gastrointestinal function (Baile et al. 1986). The proposed enzymatic process involved the synthesis of two fragments of three and five amino acids, and their final condensation to obtain the desired octapeptide. This convergent synthetic strategy is presented in Fig. 6.1.2.

Papain and α-chymotrypsin were selected for the KCS of the peptide bonds, with the exception of the Asp-Phe coupling for which TCS with thermolysin was considered. All the enzymatic reactions were catalyzed by readily available inexpensive proteases immobilized by deposition onto solid supports (Celite and polyamide) (Clapés et al. 2000). The acyl donor N-terminal protecting group for both the pentapeptide and the tripeptide fragments was phenyl acetic acid due to its selective enzymatic introduction and removal using penicillin acylase. The selection of the nucleophile esters was made taking into account their reactivity as well as the fact that the product of one reaction is the acyl donor for the next one. Carboxamidomethyl (OCam) esters have been reported as the most reactive acyl donors but, as they are poor nucleophiles, benzyl and allyl esters were selected instead (Capellas et al. 1996). Taking into account the main objective of reducing the number of purification steps, the concentrations and molar ratio nucleophile/acyl donor was selected to achieve good yields and minimize the presence of by-products, which are undesirable if the product of one reaction is to be used in the next with minimum purification.

The preparative synthesis took place in stirred batch reactors of 200–500 mL volume, with a sintered plate at the bottom, allowing the retention of the immobilized enzyme after discharge of the reacted medium. The reactions were performed in organic solvent (ethyl acetate or acetonitrile) at controlled initial water activity $(a_w = 0.1)$. For KCS reactions, only a very small amount of water is produced so that its concentration can be considered constant during the reaction. After addition of the biocatalyst (previously equilibrated) and substrates, the water content was

determined by Karl-Fisher titration and the necessary amount of buffer added to the organic solvent in the reactor in order to obtain a water content corresponding to a_w around 0.1 (0.2% v/v in ethyl acetate). Overpressure was maintained by N_2 to avoid further humidification of the reaction medium. In all the steps of synthesis the solid biocatalyst was retained in the reactor after filtration. When the desired product was soluble, the solid was washed to recover as much product as possible. When the product precipitated, it was separated from the solid phase by solvent extraction.

Process yields were calculated on the basis of the limiting reagent, including both reaction and purification steps. For instance, for the final coupling between the pentapeptide and the tripeptide, a reaction yield of 82% was obtained and, after removal of the immobilized enzyme, the solution was concentrated and the precipitated product recovered by filtration as a solid with a yield of 72%. The sulfated octapeptide was obtained then with an overall yield of 59% (moles of PhAc-CCK8/100 mole of initial pentapeptide). The final overall yield of the whole process was 15%, calculated from the initial amount of limiting reagent (H-Trp-OBzl). This totally enzymatic process of synthesis constitutes an illustrative example of the application of biocatalysis to the synthesis of biologically active peptides, involving seven protease-catalyzed reactions and the corresponding separation steps. Following the above approach, this peptide has been successfully synthesized at gram scale (Fité et al. 2002). This strategy of convergent enzymatic synthesis with inexpensive readily available proteases may prove useful for other high-value biologically active peptides (Capellas et al. 1997; Xiang et al. 2004). Nevertheless, the global yield decreases sharply with the number of amino acid residues which is still a major obstacle to go into full scale operation when the product exceeds three to four residues.

It is important to point out that the use of organic solvents usually required for performing enzymatic peptide synthesis imposes additional restrictions on process design and engineering (Gill et al. 1996) and contradicts the clean technology concept, so major efforts are being directed toward the replacement by greener systems, among which ionic liquids and solid or semisolid reaction media are prominent (see section 1.6). Recently, Meng et al. (2006) reported the enzymatic synthesis of the cholecystokinin pentapeptide (CCK-5) where the coupling was conducted in aqueous buffer and no hazardous chemical were necessary. However, these systems are still in a stage of technological development.

Despite the technological advances in peptide synthesis by biocatalysis, a general approach for the process remains to be formulated and low productivity, low conversion yield and high cost of enzymes are problems yet to be solved to make it competitive in a broad spectrum of cases. More specific, active and stable enzymes are intensively being pursued and the trend should be to improve existing proteases by the use of the modern technologies of genetic and protein engineering and automated screening of proteases from novel microbial strains, mainly extremophiles.

6.1.5 Concluding Remarks

Chemical synthesis, especially in solid-phase, can be considered now as the most mature technology for peptide production, being especially suited for medium

size peptides among which are the most relevant pharmaceutical and health-care products. However, lack of specificity and environmental burden are drawbacks of chemical processes that can be successfully overcame by enzyme biocatalysis, since many of the present constraints of enzymatic processes for peptide synthesis are being solved through research and development in different areas of enzyme biocatalysis. The combination of chemical and enzymatic synthesis is probably the way to go as already suggested (Hou et al. 2005), since the good properties of each technology can be synergistically used in the context of one process objective.

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6.2 Synthesis of β-Lactam Antibiotics with Penicillin Acylases

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6.2.1 Introduction

The industrial production of β-lactam antibiotics (βLA) is a landmark of biotechnology: antibiotics produced by fermentation (Demain 1991) and molecules derived from them by biocatalysis (Wegman et al. 2001) are prominent examples of its industrial impact and potential. Penicillins and cephalosporins are the most relevant βLA, with annual sales of about 15 billion US dollars (Elander 2003), which represents 60% of the total antibiotic market (http://www.biz-lib.com/ZKC78659.html). The first-generation βLA: i.e. penicillin G, penicillin V and cephalosporin C, have been produced by submerged fermentation with suitable fungal strains since World War II. Penicillin G was the first important commercial product of aerobic, submerged fermentation and it represents the onset of the fruitful encounter between biological sciences and process engineering that lies beyond the impact and success of modern biotechnology. On the other hand, it was a remarkably effective life-saving drug and its industrial production pave the way for the production of a succession of important antibiotics and other valuable products (Mateles 1998). In the mid-1950s cephalosporin C was discovered (Newton and Abraham 1955; Abraham and Newton 1961) and went quickly into industrial production (Elander 2003) because it was effective against some Gram negative bacteria, broadening the spectrum of action of penicillin (Marín and Gudiol 2003). Later on, a new family of β-lactam antibiotics, known as cephamycins, was discovered. These antibiotics are chemically related to cephalosporins, but produced by prokaryotes (Aharonowitz and Cohen 1981) and went also into the market because of its increased spectrum of action with respect to cephalosporins and penicillins (Stapley et al. 1972). First generation βLA have been superseded by their semi-synthetic derivatives because of their improved properties, but mainly because of the problem of induced antibiotic resistance (Marín and Gudiol 2003; Janssen 2006). Therefore, they are considered today mostly as lead molecules (Michels et al. 1998) and most relevant products are now second and higher generation semi-synthetic βLA (SSβLA).

6.2.2 Chemical Versus Enzymatic Synthesis of Semi-Synthetic β-Lactam Antibiotics

SSβLA are derived either from the penicillinic nucleus 6-aminopenicillanic acid (6APA) or the cephalosporinic nuclei 7-aminocephalosporanic acid (7ACA) and 7-amino3-desacetoxicephalosporanic acid (7ADCA) (Vandamme 1981; Pan and Syu 2004). Most relevant SSβLA are the 6APA derived ampicillin and amoxicillin, the 7ADCA derived cephalexin, cefadroxil and cefaclor and the 7ACA derived cefazolin and cefotaxime (Bruggink 2001).

Fifty years have elapsed since the discovery of 6APA (Rolinson and Geddes 2007). 6APA was confirmed as an intermediate in the synthesis of penicillin and detected in the fermentation when no side chain precursor was added (Batchelor et al.

1959), but the level was not enough for production despite the efforts for increasing it by environmental and genetic manipulations. However, screening of deacylase producing microorganisms render enzymes from actynomicetes and bacteria that efficiently remove the side-chain of penicillin V and penicillin G, respectively (Rolinson and Geddes 2007). In spite of these findings, 6APA was originally produced chemically by the Delft cleavage process because of higher conversion yields and productivities (Verweij and de Vroom 1993), but nowadays the chemical process has been almost completely replaced by a biocatalytic process with immobilized penicillin acylase (Shewale et al. 1990; Parmar et al. 2000) that considerably reduces environmental burden (van de Sandt and de Vroom 2000). Enzymatic production of 6-APA is a mature technology but further technological improvements are still underway, like the hydrolysis in the presence of organic solvents to combine reaction with product recovery (Abian et al. 2003), the use of ionic liquids as reaction media (Zhang et al. 2006; Jiang et al. 2007), the use of auxiliary phase to remove the inhibitory side-products (Wang et al. 2007a) and the use of novel carrier-free biocatalysts (Rajendhran and Gunasekaran 2007a).

7ACA is produced by the chemical cleavage of the α-aminoadipyl side chain of cephalosporin C but, as opposed to penicillin G, no acylase works efficiently on the removal of the side chain of cephalosporin C. Therefore, a chemo-enzymatic three-step process has been developed in which cephalosporin C is oxidatively deaminated to 7-β-(carboxybutanamido) cephalosporanic acid that is then chemically decarboxylated to glutaryl-7ACA and finally the side chain is removed by glutaryl-7ACA acylase (López-Gallego et al. 2004). An acylase from *Pseudomonas* sp. deacylates cephalosporin C to 7ACA, but the activity is low and no production technology has been developed yet for the one step hydrolysis of cephalosporin C into 7ACA (Parmar et al. 1998).

7ADCA is produced by the chemical ring expansion of penicillin G to deacetoxycephalosporin G, which is then deacylated according to the Delft cleavage reaction (Wegman et al. 2001). Complex chemical strategies for ring expansion of penicillins to cephalsporins have been envisaged that require several steps of synthesis to protect reactive groups (van der Klein et al. 1996). Biotechnological options are increasingly competitive since an enzyme termed *expandase* has been discovered and produced that can expand the dethiazolidine ring of penicillins into the dehydrothiazine ring of cephalosporins. Unfortunately, the *Acremonium chrysogenum* and the *Streptomyces clavuligerus* expandases only work efficiently on penicillin N and not on other penicillins (Kohsaka and Demain 1976; Kupka et al. 1983; Maeda et al. 1995; Báez-Vásquez et al. 1999), which precludes their utilization. It has been claimed that by proper adjustment of reaction conditions, *S. clavuligerus* expandase can convert penicillins other than penicillin N, including penicillin G, into the corresponding cephalosporins (Demain et al. 2002). Also claimed is the increased reactivity towards penicillin G of mutated *S. clavuligerus* expandase (Hsu et al. 2004). However, the industrial application of this system has not been reported. Cephalosporin G is a suitable substrate for penicillin acylase, so that 7ADCA can be efficiently produced enzymatically displacing the former chemical route. Metabolic engineering has also been proposed for the synthesis of 7-ADCA: an engineered *Penicillium*

chrysogenum strain fed with adipic acid, produced adipyl 7ADCA that upon hydrolysis with glutaryl acylase yielded 7ADCA (Crawford et al. 1995).

The production of β-lactam nuclei triggered the development of processes for SSβLA, mostly based on chemical synthesis. However, the situation began to change as a consequence of the advances in biocatalysis and the increasing environmental regulations imposed to the production companies (Bruggink 2001).

6.2.3 Strategies of Enzymatic Synthesis

An early breakthrough for the production of SSβLA by biocatalysis was the demonstration that the same enzyme that hydrolyzes the C—N bond in penicillin G (Rolinson et al. 1960), namely penicillin acylase, can form it between a β-lactam nucleus and a suitable side chain (McDougall et al. 1982). Penicillin acylase (penicillin amidohydrolase, EC 3.5.1.11) is a flexible enzyme able to catalyze several reactions of organic synthesis, like the resolution of racemic mixtures of amino acid derivatives (Rosell et al. 1993; Lummer et al. 1999; Basso et al. 2001; Liu et al. 2006) and other chiral nucelophiles (Fuganti et al. 1986; Švedas et al. 1996), the acylation of amines (van Langen et al. 2000), the kinetic resolution of esters and amides (Ebert et al. 1996; Roche et al. 1999; Rocchietti et al. 2002), the *N*-protection of amino acid derivatives during peptide synthesis (Fité et al. 1997) and even transesterification reactions (Lindsay et al. 2004). Many of these reactions require to be conducted in non-aqueous media so that penicillin acylases active and stable on those media are required (Abian et al. 2002; Yang et al. 2002; Lindsay et al. 2004; Liu et al. 2006; Koreishi et al. 2007). Amongst those reactions, the synthesis of derived penicillins and cephalosporins from the corresponding β-lactam nuclei and suitable acyl donors is of paramount importance for the pharmaceutical industry (Wegman et al. 2001).

The enzymatic synthesis of SSβLA can be conducted under thermodynamic (Fernández-Lafuente et al. 1996a; Nierstrasz et al. 1999; Schroën et al. 1999) or kinetic control (Hernández-Justiz et al. 1999; Schroën et al. 2001a).

Thermodynamically controlled synthesis (TCS) considers the displacement of equilibrium from hydrolysis to synthesis, this is, the direct condensation of the nucleophile (β-lactam nucleus) and the acyl donor (Kurochkina and Nys 2002). In most SSβLA, the zwitterionic nature of the side chains (D-phenylglicine or D-hydroxyphenylglycine) precludes its application (Ulijn et al. 2002a). However, it has been tried in some cases because it is simple and do not require activated acyl donors (Fernández-Lafuente et al. 1991, 1996b). Condensation reaction requires that the substrates be non-ionized, so that pH is a critical variable (Ferreira et al. 2004; Guranda et al. 2004). In TCS, conversion yield is determined by the thermodynamic equilibrium of the reaction and drastic conditions are usually required to displace it in favor of synthesis. The use of organic solvents (Rosell et al. 1998), in-situ product removal (Wei et al. 2003), ionic liquids (Zhang et al. 2006; de los Ríos et al. 2007) and the use of biphasic systems (Hernández-Justiz et al. 1998; Terreni et al. 2005) have been tried to increase conversion yield. Organic solvents, which reduce water activity and displace the equilibrium toward synthesis and favor the ionic equilibria

to the reactive non-ionized substrates species, have been the most thoroughly studied reaction media for performing TCS (Diender et al. 1998; Schroën et al. 1999). TCS has not been considered as a technological option for the enzymatic synthesis of β-lactam antibiotics: harsh conditions are required to displace the equilibrium toward synthesis that are detrimental for enzyme activity, the system is not flexible since conversion yield is determined by the equilibrium constant of the reaction and is independent on biocatalyst properties, and productivity is considerably lower than in kinetic control

In the kinetically controlled synthesis (KCS) of βLA, the reaction of synthesis (synthetase activity) occurs simultaneously with the hydrolysis of the activated acyl donor (esterase activity) and the antibiotic product (amidase activity) (Fernández-Lafuente et al. 1998). KCS requires an activated acyl donor, in the form of an ester (phenylglycinemethyl ester, PGME) (Kim and Lee 1996; Illanes and Fajardo 2001) or an amide (phenylglycineamide) (Schroën et al. 2001b) and it is a much better strategy when product yield is the main issue, since product concentration is not limited by the equilibrium of the reaction (Diender et al. 1998). This is the case with pharmaceuticals where high yields significantly reduce the cost of downstream operations (Baldaro 1991). As in TCS, conversion yields can be improved in KCS by using organic solvents (Illanes et al. 2002), precipitation-driven (Yang and Wei 2003; Zhang et al. 2007) and biphasic systems (Hernández-Jústiz et al. 1998; Wei et al. 2002).

As a consequence of several developments both in the field of biocatalyst and medium engineering, the enzymatic production of SSβLA by KCS is becoming increasingly competitive with current chemical processes. As a token, an industrial facility for producing cephalexin by a totally enzymatic process has entered into operation recently (Janssen 2006); reduction of 50 to 15 kg waste/kg product is a major factor for success (Gavrilescu and Chisti 2005). Because of its relevance, KCS of SSβLA will be analyzed in depth in section 6.2.5.

6.2.4 Penicillin Acylase Biocatalysts

Designation of the enzyme as penicillin acylase (penicillin amidohydrolase) is based on its technological potential, but certainly not in its biological function in the producing organisms where it is supposed to act as a scavenger of phenylacetylated compounds (Valle et al. 1991) or be involved in the assimilation of aromatic compounds as carbon source (Duggleby et al. 1995). Penicillin acylase was first isolated from *Penicillium chrysogenum* (Matsumoto 1993) but penicillin acylase activity was soon detected in a wide range of bacteria, actinomycetes and fungi (Chisti and Moo-Young 1991). According to its preferred substrate, penicillin acylases have been grouped in three classes: penicillin G acylases, penicillin V acylases and ampicillin acylases (Shewale et al. 1990). Penicillin G acylases are produced by bacteria and are the most technologically relevant (Parmar et al. 2000). Penicillin V acylases are mainly produced by actinomycetes but also by bacteria and yeast (Shewale and Sudhakaran 1997). Enzymes from a wide range of microorganisms

have been isolated and among them. *Escherichia coli* and *Bacillus megaterium* are the most important sources for industrial production of penicillin acylase. Other enzymes of potential interest are the penicillin G acylases from *Kluyvera citrophila* (Alvaro et al. 1992; Liu et al. 2006) and *Alcaligenes faecalis* (Wang et al. 2006), the recombinant penicillin G acylase from *Providencia rettgeri* (Senerovic et al. 2005) and the penicillin V acylases from *Streptomyces* sp. (Shewale and Sudhakaran 1997; Arroyo et al. 2000; Koreishi et al. 2007).

Most available information refers to the enzyme from *E. coli*, which has been studied in depth and fully characterized (Ospina et al. 1992; Sudhakaran et al. 1992; Duggleby et al. 1995). That enzyme is intracellular (mostly periplasmic) so that cell extraction is required. It is a heterodimer composed by two subunits, α and β, of 19,500 and 60,000 Da respectively and has a isoelectric point of 6.8 (Novella et al. 1994). The enzyme from *E. coli* has been improved both by genetic and protein engineering. Genetic engineering of producing strains has been focused mainly in increasing productivity by proper enzyme induction and manipulation of fermentation conditions (Lin et al. 2001; Ospina et al. 1995; Ramírez et al. 2004). Hybrid penicillin acylases from *E. coli* and other organisms have been produced by family gene shuffling obtaining variants with improved performance (higher conversion yields and higher ratio of synthesis to hydrolysis rates) in the synthesis of ampicillin (Jager et al. 2007). Detailed information on enzyme production can be found in extensive reviews on the subject (Shewale and Sivaraman 1989; Shewale et al. 1990). By contrast, information on the enzyme from *B. megaterium* is scattered and mostly protected by industrial secrecy (Ishimura and Seijo 1991). The gene encoding its synthesis is composed of 2,406 nucleotides, its deduced amino acid sequence bearing significant similarity with other β-lactam acylases (Martín et al. 1995). The enzyme from *B. megaterium* has been reported as extracellular (Matsumoto 1993). We have found that the enzyme is in fact excretable, but a substantial fraction remains cell associated if phosphate in the medium is kept low (Illanes et al. 1994). The enzyme, as the one from *E. coli*, is induced by phenylacetic acid (Babu and Panda 1991; Illanes et al. 1994; Rajendhran et al. 2003) and inhibited by it competitively and non-competitively by 6-aminopenicillanic acid (Lee and Ryu 1982; Ospina et al. 1992). The enzyme compares quite favourably in terms of its kinetic parameters with the one from *E. coli* (Illanes et al. 1993). Penicillin acylase genes from *Bacillus* species have been cloned and expressed in *E. coli* with moderate success (Meevootisom and Saunders 1987; Rajendhran and Gunasekaran 2007b). Homologous recombinant *B. megaterium* produced increased levels of synthesis and excretion of the enzyme (Yang et al. 2006). Protein engineering has also been used employing site-directed mutagenesis for improving penicillin acylase performance in the synthesis of SSβLA (higher synthesis to hydrolysis ratio) (Wang et al. 2007b) and also biocatalyst stability in organic media (Yang et al. 2002). Surprisingly, information on its use for the production of SSβLA is scarce and only recent (Zhang et al. 2007). Despite the above mentioned advantages, the enzyme from *E. coli* is still the choice in most cases.

Enzymes are labile catalysts that require to be stabilized under operating conditions. Penicillin acylase is a moderately expensive enzyme so that its efficient

utilization is crucial for industrial success. Substantial improvements in penicillin acylase stabilization have been obtained by directed immobilization to solid supports (Fernandez-Lafuente et al. 1999; Terreni et al. 2001; Basso et al. 2003; Bryjak and Trochimczuk 2006; Montes et al. 2007), aggregation (Mateo et al. 2004; Roy and Abraham 2004; Rajendhran and Gunasekaran 2007a), derivatization (Özturk et al. 2002; Mislovičova et al. 2006), activation (Lindsay et al. 2004), site-directed mutagenesis and directed evolution (Rajendhran and Gunasekaran 2004; Wang et al. 2006) and functional screening from environmental gene pools (Gabor et al. 2005). Immobilization represents the most meaningful strategy at production scale since enzyme reuse and stabilization are important to reduce the impact of biocatalyst cost in total production cost (Illanes et al. 2000). A myriad of methods for penicillin acylase immobilization have been developed (Kallenberg et al. 2005), most of them for 6APA production. Therefore, they are not necessarily adequate for reactions of synthesis, for which a thorough characterization of the biocatalysts in terms of their efficiency parameter (synthesis to hydrolysis ratio) is advisable (Janssen 2006). Multi-point covalent attachment to activated agarose gels and cross-linked enzyme aggregates (CLEA) are particularly promising. The former is one of the more effectives in terms of enzyme stabilization (Mateo et al. 2005) and penicillin acylase biocatalysts have been obtained accordingly exhibiting an increased stability in harsh conditions, like elevated temperatures and high concentrations of organic solvents (Alvaro et al. 1990; Guisan et al. 1990; Abian et al. 2002). CLEAs are non-supported insoluble biocatalysts particularly promising for organic synthesis (Cao et al. 2000–2003; López-Serrano et al. 2002). CLEAs of penicillin acylase have been successfully produced (Cao et al. 2001) and used in the synthesis of ampicillin, its performance being comparable in terms of yield and better in terms of productivity than carrier-bound penicillin acylase (Illanes et al. 2006). Combination of CLEAs with polymeric co-aggregates (Wilson et al. 2004a) and encapsulation into hydrophilic gels (Wilson et al. 2002; Wilson et al. 2004b) have also been proposed as suitable forms of penicillin acylase biocatalysts. They have the benefits of simple production and high specific activities by being devoid of an inert matrix. They are, however, mechanically weak, prone to mass transfer limitations and hard to recover, which are constraints to be taken into consideration when scaling-up to production level (Illanes et al. 2007a). Even though in some instances soluble penicillin acylases exhibit better kinetic parameters than their immobilized counterparts (Janssen 2006), it is foreseeable that only immobilized biocatalysts will make enzymatic processes competitive with existing chemical technologies.

6.2.5 Synthesis of β-Lactam Antibiotics in Homogeneous and Heterogeneous Aqueous and Organic Media

KCS is the best technological option for the enzymatic synthesis of SSβLA: conversion yield is not limited by the equilibrium of the reaction and high productivities

are attainable (Schroën et al. 1999). The main drawbacks of KCS are the high cost of the active acyl donor and the precise control of reaction required since in this case conversion yield exhibits a maximum after which it sharply decreases (especially in fully aqueous media) when product hydrolysis outweighs its synthesis (Illanes et al. 2004). In KCS, the reaction of synthesis competes with the reactions of hydrolysis of the product and the activated acyl donor (Fernández-Lafuente et al. 1995; Giordano et al. 2006). KCS is a more flexible option since conversion yield results from the balance between the synthetic and hydrolytic activities and is therefore dependent on the biocatalyst properties that can then be optimized for synthesis (Aguirre et al. 2002). However, higher yields are not easily attainable and several strategies have been proposed to increase it. The reduction of water activity in the reaction medium is beneficial for KCS, since it depresses the competing hydrolytic reactions in favor of synthesis (Hyun et al. 1993; Wei and Yang 2003). Water activity can be depressed by using cosolvents, or high concentrations of substrates, or both. Organic cosolvents have proven to be suitable media for the synthesis of SSβLA with immobilized penicillin acylase (Rosell et al. 1998) and higher conversion yields have been obtained than in fully aqueous media (Arroyo et al. 2000; Illanes and Fajardo 2001), actually approaching stoichiometric values (Illanes et al. 2004). KCS at high substrates concentration is beneficial by Michaelian considerations and also for reducing water activity (Illanes et al. 2005a; Illanes et al. 2007b). Aqueous solution precipitate, consisting in keeping a saturated concentration of the nucleophile throughout the reaction by repetitive additions, has been successfully applied to the synthesis of ampicillin (Youshko and Švedas 2000; Youshko et al. 2000) and conversion yields over 97% have been obtained (Youshko et al. 2001). Working under substrate supersaturation, significant increases in yield have been obtained for ampicillin and cephalexin with respect to heterogeneous systems (Youshko et al. 2004). Synthesis in solid sate (Erbeldinger et al. 1998) has also been applied to SSβLA, although with a moderate success (Diender et al. 2000; Ulijn et al. 2001, 2002a; Basso et al. 2006); however, 70% conversion yield was reported for ampicillin (Youshko and Švedas 2002), which is encouraging. Other strategies to improve KCS by alleviating product hydrolysis are in-situ product removal (Kemperman et al. 1999; Schroën et al. 2002a; Wegman et al. 2002; Wei et al. 2003; Zhang et al. 2007) and partition to a second phase (Hernández-Justiz et al. 1998; Wei et al. 2002; Yang and Wei 2003). All these strategies have succeeded in obtaining high conversion yields; however, not much attention has been paid to productivity which is a very relevant process parameter. Productivity can be significantly enhanced by working at very high substrates concentrations, as shown in Table 6.2.1. A tenfold increase in productivity, without sacrifice in conversion yield, was obtained in the KCS of cephalexin with glyoxyl agarose immobilized PA when the concentration of substrates was increased from 30 to 200 mM 7ADCA. To assess global productivity, sequential batch operation at 200 mM 7ADCA was performed for one biocatalyst half-life, which occurred after 30 batches in the case of the commercial penicillin acylase PGA-450 and 24 batches in the case of penicillin acylase CLEA. Global productivity and the amount of product per unit mass of biocatalyst after that period were 3.67 mmol/h/g and 40.1 (g/g) respectively for PGA-450 and 3.15 mmol/h/g and

Table 6.2.1 Conversion Yield (Y) and Productivity (Pr) of Enzymatic Synthesis of β-Lactam Antibiotics with Penicillin Acylase (PA) at Increasing Substrates Concentrations

Antibiotic	Enzyme	[Nucleophile] mM	Y (%)	Pr (mM/h)	Refs.
Ampicillin	PGA-450	30	64	13	Yang and Wei (2003)
Cephalexin	PGA-450	30	98	29	Illanes et al. (2004)
Cephalexin	Nylon IPA	40	ND	9	Travascio et al. (2002)
Cefaclor	Epoxy resin IPA	50	86	8	Wei et al. (2003)
Cephalotin	PGA-450	100	60	18	Illanes et al. (2005a)
Cephalexin	Assemblase	100	60	45	Schroën et al. (2001a)
Cephalexin	Glyoxyl agarose	150	93	167	Illanes et al. (2005a,b)
	IPA	180	85	229	
Ampicillin	Soluble PA	300	75	130	Youshko and Švedas (2000)
		450	93	167	
Ampicillin	Soluble PA	600	90	220	Youshko et al. (2001)
		Saturated (fed-batch)	97	300	
Ampicillin	Soluble PA	650	98	ND	Youshko et al. (2004)
Amoxicillin		650	91	60	
Cephalexin		600	92	ND	
Cephalexin	PGA-450	150	100	129	Illanes et al. (2007a)
	CLEA PA	200	99	298	
		250	91	250	
		200	99	63	
Cephalexin	PGA-450	200	99	297	Illanes et al. (2007b)
Ampicillin	Eupergit IPA	Solid-state	70	300	Youshko and Švedas (2002)

IPA: immobilized PA; ND: not determinable

135.5 (g/g) respectively for penicillin acylase CLEA (Illanes et al. 2007a). These results are encouraging and there is still room for further improvement to make biocatalysis of SSβLA competitive. Actually, better results have been recently obtained with PGA-450 in a fully aqueous medium under a green chemistry concept; specific productivity was 7.6 mmol/h/g at almost stoichiometric conversion yield (Illanes et al. 2007b).

Despite its advantages, KCS has some drawbacks that have to be properly addressed. The use of activated acyl donors is an additional requirement and phenylglycine, produced from benzaldehyde by conventional Strecker synthesis, or *p*-hydroxy phenylglycine, produced from phenol by Mannich condensation, must be activated by esterification or amidation to produce ampicillin, cephalexin, amoxicillin and cefadroxil respectively. Amides are considered better since they can be synthesized from a precursor of phenylglycine, while the esters must be synthesized from phenylglycine requiring an extra step (Wegman et al. 2001); however, amides generate ammonia during synthesis which upon neutralization produces undesirable salt. To circumvent this problem, a process considering both the amide and the ester has been proposed (Sheldon et al. 2001). In-situ production of phenylglycine amide from the corresponding nitrile by nitrile hydratase has been claimed as the best option for the one-pot synthesis of cephalexin (Wegman et al. 2002).

Excess acyl donor is required to increase conversion yield (Schroën et al. 2001a; Illanes et al. 2007b), so that copious amounts of phenylglycine are produced in KCS that hamper downstream operations and increase costs, making recycle necessary. This is not an easy task, since the poorly soluble phenylglycine will be mostly as a precipitate which must be separated from the biocatalyst by differential screening (Bruggink et al. 1998). Contrary to TCS, maximum conversion yield in KCS is transient since after that point product hydrolysis outweighs synthesis (Illanes et al. 2004); reaction must be carefully monitored then to harvest the reactor at or close to that point. Product decay rate can be substantially reduced by using organic cosolvents in the reaction medium (Illanes et al. 2003). However, the use of solvents is rather contradictory since it pushes enzyme technology away from the concept of green chemistry, which is a definite advantage of going enzymatic. In this sense, the use of high substrates concentrations, beyond the limit of solubility (Youshko et al. 2000–2004), or even in solid-state (Diender et al. 2000; Ulijn et al. 2002b; Youshko and Švedas 2002; Basso et al. 2006) should be the way to go. At such conditions, no cosolvent is required to attain high conversion yields steadily. Product recovery and biocatalyst and by-product recycling are complex, but strategies based on differential solubilization and screening have been developed to tackle this problem (Bruggink et al. 1998).

Enzymatic production of SSβLA is a very appealing option from a process engineering perspective (Giordano et al. 2006). The same biocatalyst that produces the corresponding β-lactam nuclei can produce the SSβLA from them simply by shifting operating conditions within the reactor. This is an efficient way of handling it and certainly a very interesting process to optimize. The option even exists to conduct both reactions simultaneously at optimally balanced operation conditions (van der Weilken et al. 2001).

Advances both in biocatalyst and process engineering, together with more stringent environmental regulations, will certainly pave the way for enzyme processes in the production of SSβLA as well as in other applications in the pharmaceutical and fine-chemical industries.

6.2.6 Model of Reactor Performance for the Production of Semi-Synthetic β-Lactam Antibiotics

Kinetics of penicillin hydrolysis of penicillin G with penicillin acylase has been extensively studied. An example of reactor design and performance evaluation based on such kinetics was presented in section 5.4.4.

The kinetic mechanism for the KCS of SSβLA has been thoroughly studied since the pioneering work of Kasche (1985, 1986). Several variants of that mechanism have been proposed since then (Nam et al. 1985; Kheirolomoom et al. 2001; Youshko et al. 2002, 2003; Alkema et al. 2003). Based on the mechanism proposed by Nam et al. (1985), which is shown in Fig. 6.2.1, we developed a parametric expression for the synthesis of ampicillin whose parameters were determined by initial rate studies of the synthesis of ampicillin, hydrolysis of ampicillin, and

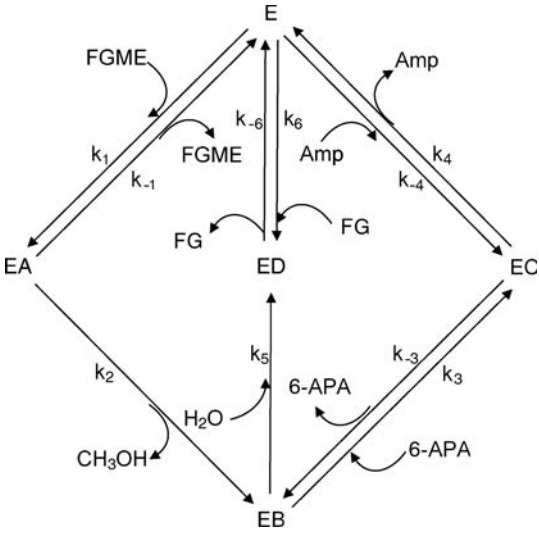

Fig. 6.2.1 Proposed mechanism for the kinetically controlled synthesis of ampicillin

hydrolysis of the acyl donor, PGME, represented by Eqs. 6.2.1, 6.2.2 and 6.2.3:

$$v_{Amp} = \frac{V_{6APA}\,[6APA]\cdot[FGME]}{1+\frac{[6APA]}{K_{6APA}}+\frac{[FGME]}{K_{FGME}}+\frac{[6APA]\cdot[FGME]}{C_1\cdot K_{6APA}\cdot K_{FGME}}} \tag{6.2.1}$$

$$v'_{Amp} = \frac{V_{Amp}\,[Amp]}{1+\frac{[6APA]}{K_{6APA}}+\frac{[6APA][Amp]}{C_2\cdot K_{Amp}}+\frac{[Amp]}{K_{Amp}}} \tag{6.2.2}$$

$$v_{FGME} = \frac{V_{FGME}\,[FGME]}{1+\frac{[FGME]}{K_{FGME}}+\frac{[FG]}{K_{FG}}} \tag{6.2.3}$$

Kinetic parameters were determined by proper linearization of the initial rate data and are presented in Table 6.2.2 (Gorziglia et al. 2002).

Models derived from such kinetic mechanisms have been used for describing reactor performance in SSβLA synthesis (Ospina et al. 1996; Gonçalves et al. 2000, 2002; Youshko and Švedas 2000; Schroën et al. 2001 a,b, 2002b; Kurochkina and

Table 6.2.2 Kinetic Parameters of the KCS of Ampicillin with Penicillin

Parameter	Value
V_{6APA}	0.0003 min^{-1}
V_{Amp}	0.0344 min^{-1}
V_{FGME}	0.0174 min^{-1}
K_{6APA}	16.5 mM
K_{Amp}	83.7 mM
K_{FGME}	160.8 mM
K_{FG}	–
C_1	0.28
C_2	8.4 mM

Nys 2002; Pan and Syu 2005; Giordano et al. 2006). We developed a model for describing batch reactor performance with immobilized penicillin acylase for the KCS of cephalexin from 7ADCA and PGME based on the kinetic mechanism proposed by Nam et al. (1985). The rate of cephalexin synthesis can be expressed as:

$$\frac{d[Ceph]}{dt} = \frac{V_{7ADCA} \cdot [7ADCA] \cdot [PGME] - V_{Ceph} \cdot [Ceph]}{N} \tag{6.2.4}$$

$$N = 1 + \frac{[7ADCA]}{K_{7ADCA}} + \frac{[PGME]}{K_{PGME}} + \frac{[7ADCA] \cdot [PGME]}{C_1 \cdot K_{7ADCA} \cdot K_{PGME}} + \frac{[PG]}{K_{7ADCA} \cdot K_{PG}} + \frac{[7ADCA] \cdot [Cef]}{C_2 \cdot K_{Ceph}} + \frac{[Ceph]}{K_{Ceph}}$$

Eq. 6.2.4 can be expressed in terms of substrate conversion (X) in parametric form as:

$$\frac{d[Ceph]}{dt} = v_{Ceph} = \frac{A \cdot X^2 + B \cdot X + C}{D \cdot X^2 + E \cdot X + F} \tag{6.2.5}$$

Where the lumped kinetic parameters A to F are:

$$A = V_{7ADCA} \cdot [7ADCA]_0^2$$

$$B = -\begin{pmatrix} V_{7ADCA} \cdot [7ADCA]_0 \cdot [PGME]_0 + V_{7ADCA} \cdot [7ADCA]_0^2 \\ + V_{Ceph} \cdot [7ADCA]_0 \end{pmatrix}$$

$$C = V_{7ADCA} \cdot [7ADCA]_0 \cdot [PGME]_0$$

$$D = \frac{[7ADCA]_0^2}{C_1 \cdot K_{7ADCA} \cdot K_{PGME}} - \frac{[7ADCA]_0^2}{C_2 \cdot K_{Ceph}}$$

$$E = \frac{[7ADCA]_0^2}{C_2 \cdot K_{Ceph}} - \left(\frac{[7ADCA]_0^2 + [7ADCA]_0 \cdot [PGME]_0}{C_1 \cdot K_{7ADCA} \cdot K_{PGME}} \right) + \frac{[7ADCA]_0}{K_{Ceph}} - \frac{[7ADCA]_0}{K_{7ADCA}} - \frac{[7ADCA]_0}{K_{PGME}}$$

$$F = 1 + \frac{[7ADCA]_0}{K_{7ADCA}} + \frac{[PGME]_0}{K_{PGME}} + \frac{[7ADCA]_0 \cdot [PGME]_0}{C_1 \cdot K_{7ADCA} \cdot K_{PGME}}$$

and the equation for batch reactor operation is:

$$\int \frac{D \cdot X^2 + E \cdot X + F}{AX^2 + B \cdot X + C} dX = \frac{1}{[7ADCA]_0} \int dt \tag{6.2.6}$$

Eq. 6.2.6 was solved analytically to obtain the operation curve of the reactor (X vs t). Lumped kinetic parameters were determined by non-linear regression of experimental data using the numerical method of Newton–Raphson with first-order Taylor series expansion. Lumped parameters were smooth functions of temperature; all parameters were adequately fitted to second order polynomials except for D that required a fourth order polynomial. The model can be used for reactor temperature optimization and can be extended to prolonged sequential batch operation provided that a sound model for enzyme inactivation is validated (Illanes et al. 2005b).

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6.3 Chimioselective Esterification of Wood Sterols with Lipases

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Esterases (EC 3.1) are a subclass of enzymes that catalyze the hydrolysis of esters to carboxylic acids and alcohols. Carboxylic ester hydrolases (EC 3.1.1)

are a subgroup of esterases to which lipases (triacylglycerol acylhydrolases; EC 3.1.1.3) belong (http://www.chem.qmul.ac.uk/iubmb/enzyme/). The term lipase can be somewhat misleading since in a very broad sense lipases are enzymes that hydrolyze lipids, which are amply defined in physicochemical terms as fat-soluble molecules. More strictly, lipases are considered as enzymes that catalyze the hydrolysis of long chain fatty acids from acylglycerols, usually performing at oil–water interfaces and having a particular structure surrounding its active site, as shown in section 6.3.1 (Jensen and Hamosh 1996; Nardini et al. 2000). This property actually distinguishes lipases (sometimes termed as "true lipases") from other esterases, since lipases are barely active on soluble substrates in aqueous medium and require a lipid–water interface to act (Martinelle et al. 1995; Louwrier et al. 1996; Jaeger et al. 1999). This mechanism of action can be traced back to some particular structural features that will be described below. The definition of lipases as hydrolases of long chain acylglycerols is rather physiological, because lipases can also hydrolyze carboxylic acids from a variety of compounds of different chemical nature. More importantly, in low water activity systems lipases can catalyze the reverse reactions of esterification from fatty acids and glycerol (and other alcohols as well) (Yadav and Devi 2004), interesterification from two esters (Bloomer et al. 1990; Osório et al. 2005) or from carboxylic acids and glycerol (and other alcohols as well) (Reyes et al. 1994), and transesterification from an ester and an alcohol (Houssam et al. 2004). This intended definition of lipases highlights their technological potential. In fact, lipases are at present subject of intense study and no less than one third of all the published papers on enzyme biocatalysis are related to lipases. With the advent of biocatalysis in non-aqueous media (Koskinen and Klibanov 1996), lipases have come to play a central role since by its own nature these enzymes are particularly well suited to perform in such otherwise deleterious media (Hari Krishna and Karanth 2002; Reetz 2002), where most enzymes are poorly active and unstable (Klibanov 1997).

6.3.1 Sources and Production of Lipases

6.3.1.1 Lipase Sources

Lipases are widely distributed in nature, being synthesized by plants, animals and microorganisms. Lipases from microorganisms, mainly bacterial and fungal, are the most used as biocatalysts in biotechnological applications and organic chemistry. Fungal lipases from *Candida rugosa, Candida antarctica, Thermomyces lanuginosus and Rhizomucor miehei* and bacterial lipases from *Burkholderia cepacia, Pseudomonas alcaligenes, Pseudomonas mendocina* and *Chromobacterium viscosum* are examples of commercially available lipases widely used in biotechnology (Jaeger and Reetz 1998). Microbial enzymes are more useful than enzymes derived from animals or plants because their bulk production is simpler, cheaper and safer (Wiseman 1995; Hasan et al. 2006). Only about 2% of the world's microorganisms have been tested as enzyme sources so they offer a huge biodiversity as lipase sources (Hasan et al. 2006). Nowadays, 38 distinct bacterial

sources of common lipase producers have been referenced (Gupta et al. 2004). In 1998, a search of available data banks revealed 217 entries of lipolytic enzymes from bacteria (Jaeger et al. 1999).

Depending on their sources (bacterial, fungal, plant or animal), lipases have a wide range of properties like positional specificity, enantioselectivity, temperature tolerance and pH dependence (Saxena et al. 2003). Screening and protein engineering techniques are powerful tools for the selection of the most adequate biocatalyst by searching or modulating their catalytic properties (such as substrate specificity or selectivity). The extracellular nature of most lipases and recombinant DNA technology make possible to produce them in large quantities by over-expression in an adequate microbial host. Many microbial lipase genes have been cloned, including those of important commercial lipases (Schmidt-Dannert 1999). Other lipases from very different sources, like plants (Hong et al. 2000) and extremophilic microorganisms (Demirjian et al. 2001), have been cloned and expressed in *Escherichia coli*. Also, *Bacillus subtilis* has been used frequently as a bacterial host for recombinant lipase expression (Misset et al. 1994). When the target lipase is a glycoprotein, it should be expressed in a host that has appropriate metabolic pathways for protein glycosylation in the host cells. Thus, lipase from different sources have been overexpressed and produced in *Pichia pastoris* (Cos et al. 2005) and in *Saccharomyces cerevisiae* (Schmidt-Dannert 1999).

6.3.1.2 Production of Microbial Lipases by Fermentation

Microbial lipases are produced mostly by submerged fermentation. Many studies have been undertaken to define the optimal culture and nutritional requirements for lipase production. Lipase production is strongly influenced by a wide range of fermentation parameters such as nitrogen and carbon sources, pH, temperature, agitation, dissolved oxygen concentration and presence of lipids (Elibol and Ozer 2001). A survey of fermentation conditions for the production of bacterial lipases has been reported by Gupta et al. (2004).

Carbon source is the most important factor for lipase expression. Lipases are induced by the presence of oil, fatty acids, triacylglicerols, tweens, bile salts and glycerol (Bradoo et al. 1999; Rathi et al. 2001). Other carbon sources, such as sugars, whey, polysaccharides and casamino acids, strongly influence lipase production (Ghanem et al. 2000; Rashid et al. 2001). The type of nitrogen source also influences lipase production. Organic nitrogen is preferred, such as peptone or tryptone and yeast extract, which have been used for lipase production by *Bacillus* spp., *Pseudomonads and Staphylococcus haemolyticus* (Oh et al. 1999; Ghanem et al. 2000). A medium containing corn steep liquor and peptone has been used as nitrogen source for *Rhizopus oryzae* (Hiol et al. 2000). Inorganic nitrogen sources such as ammonium chloride or diammonium hydrogen phosphate have been reported to be effective for some microorganisms (Bradoo et al. 1999; Rathi et al. 2001). Divalent cations, mainly Ca^{2+} and Mg^{2+}, stimulate lipase production. Sharon et al. (1998) observed stimulation in lipase production from *Pseudomonas*

pseudoalcaligenes by the presence of Mg^{2+}. Lipase production by *Bacillus* sp. A-301 required a complex medium that contained different divalent cations such as Ca^{2+}, Mg^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+}, and Mo^{2+} (Wang et al. 1995). Sharma et al. (2002) observed stimulation in lipase production from *Bacillus* sp. RSJ1in presence of Ca^{2+}, but inhibition by other metal ion salts.

Other fermentation parameters, such as temperature, pH, agitation and aeration rate are important in microbial lipase production. These parameters are strain dependent: bacteria prefer neutral pHs and the optimum temperature for lipase production corresponds with the growth temperature of the respective organism, which is in the 20–45 °C range, although in some cases optimum temperatures outside that range have been reported Sharma et al. (2002).

Fermentation strategy is a key factor for high lipase productivity. Lipase production is linked to cell density and high density cultures allow improving lipase productivity. Fed-batch is the preferred operational mode for getting high lipase productivity. Ferrer et al. (2001) reported a high cell density fermentation to produce heterologous protein in *Pichia pastoris* using a fed-batch strategy. The basal salt defined medium contained methanol as carbon source which is the responsible to induce the heterologous expression of the foreign gene. The optimal temperature for growth and production was 30 °C, with a pH between 5 and 6 to reduce degradation by endogenous proteases. The fermentation was aerobic and high agitation and aeration rates were necessary (1,000 rpm; 1–3 vvm).

6.3.1.3 Purification Strategies for Microbial Lipases

Most commercial applications, such as enzyme preparations for detergents, do not require pure lipases, but a certain degree of purity simplifies their successful usage as biocatalysts because reduces side-product formation and simplifies product downstream. Extensive lipase purification should be considered when structural studies are going to be performed or when it will be used as biocatalyst in a synthetic reaction for the pharmaceutical industry. The main drawbacks of traditional purification strategies are low yields and productivities. The extent of purification varies with the number and the order of purification steps (see section 2.2.3); the importance of designing optimal purification schemes has been highlighted in several comprehensive reviews on this topic (Taipa et al. 1992; Aires-Barros et al. 1994; Palekar et al. 2000; Saxena et al. 2003).

Most of microbial lipases are extracellular and the fermentation process is followed by the removal of cells from the culture broth, either by centrifugation or by filtration. Pre-purification steps involve concentration of cell-free culture broth by ammonium sulfate precipitation, ultrafiltration or extraction with organic solvents. Pre-purification steps are included in most purification schemes (Saxena et al. 2003) and precipitation often has the highest average yield (Aires-Barros et al. 1994). Pre-purification steps are usually followed by chromatographic techniques in the purification process. A single chromatographic step may be not enough for getting the level of required purity and combination of these techniques should be applied. Ion

exchange chromatography, followed by gel filtration are the most used techniques in lipase purification protocols (Saxena et al. 2003). Lipases are hydrophobic in nature having large hydrophobic surfaces around the active site (Gupta et al. 2004); thus, hydrophobic interaction chromatography is also frequently used in lipase purification (Hong and Chang 1998; Queiroz et al. 2001). Octyl and phenyl are the most popular functional groups of the hydrophobic matrices, although they may be too expensive for large-scale purification processes. Immobilized lectins, such as Concanavalin A, are used as affinity chromatography matrices for the purification of lipases from fungal and mammalian sources when the enzyme is a glycoprotein (Tombs and Blake 1982; Aires-Barros and Cabral 1991).

Some novel purification techniques have been applied for lipase purification that include aqueous two phase systems (Terstappen et al. 1992; Queiroz et al. 1995), reversed micellar system (Vicente et al. 1990) and immunopurification (Bandmann et al. 2000). They take advantage of lipases unusual properties: hydrophobic nature and interfacial activation. Aqueous two phase and reversed micellar systems are based on the partitioning properties of proteins in a biphasic system. Selective solubilization of a mixture of proteins can be achieved by manipulating the parameters of the biphasic systems, both in the micellar and aqueous phases, the most important parameters being the pH and ionic strength of the aqueous phase (Gupta et al. 2004). Immunopurification is one of the most selective protein-purification techniques because of the high specificity of the antibody–antigen recognition. This technique has the drawback of the very high cost of the monoclonal antibodies.

6.3.2 Structure and Functionality of Lipases

6.3.2.1 Definition and Activity Measurement

The definition of lipase is not well established. Lipolytic reactions take place at the lipid–water interface of the biphasic system formed by the lipolytic substrate and the aqueous medium. A criterion has been to classify a lipolytic enzyme as a "true" lipase (EC 3.1.1.3) when it catalyzes the hydrolysis of long chain fatty acids from acylglycerols (Jaeger et al. 1999). Lipases are activated by the presence of emulsion interfaces and they contain a polypeptide chain, called *lid*, covering the active center, which moves away in contact with interfaces leaving the active center available for substrate binding and processing. However, there are exceptions, such as enzymes that contain the lid but do not exhibit interfacial activation (Verger 1997). Lipases may be defined as esterases catalyzing the hydrolysis and synthesis of long-chain fatty acid esters from glycerol. But, there is not a strict definition for "long-chain fatty acid" and, furthermore, most lipases hydrolyze ester substrates (Jaeger et al. 1999) with an acyl chain length of less than 10 carbon atoms with tributyrylglycerol (tributyrin) as the standard substrate.

Lipases hydrolyze triglycerides giving rise to free fatty acids and glycerol. Assays for lipase activity include a wide range of techniques: spectrophotometry,

fluorimetry, titrimetry, turbidimetry, surface tension method and estimation of free fatty acids by HPLC (Thompson et al. 1999). The most widely used assays to test lipase activity are based on hydrolytic reactions. Tributyrin (tributyrylglycerol) and triolein (trioleylglycerol) have been used as substrates in Petri dish assays to identify lipase-producing bacteria. Also, lipase activity has been assayed by the spectrophotometric detection of *p*-nitrophenol at 410 nm, which is released by the hydrolysis of *p*-nitrophenylesters of fatty acids with various chain lengths. This assay must be taken with caution since esterases can also hydrolyze fatty acid monoester substrates. This problem can be overcome by using a triglyceride derivative (1,2-*O*-dilauryl-*rac*-glycero-3-glutaric acid resorufin ester) yielding resorufin, which can be determined spectrophotometrically at 572 nm or fluorometrically at 583 nm. Also, the "true lipases" can be determined by titration of fatty acids released from a triglyceride, usually trioleoylglycerol (Jensen 1983). Since lipases are used for stereoselective synthetic reactions, lipase stereoselectivity is relevant to be determined which is usually done by esterification of an alcohol with a carboxylic acid. Esterification rate and stereoselective product formation are monitored by gas chromatography (GC) and high performance liquid chromatography (HPLC) with chirally modified columns.

6.3.2.2 Lipase Families

Lipases have been classified into families. Search of different available data banks (e.g. Swiss Protein Sequence Database) revealed different results based on amino acid sequence homology. Jaeger et al. (1999) identified 47 different bacterial lipases and grouped them into six families. Homologous serine hydrolases were assigned to 32 homologous families and 15 superfamilies by Pleiss et al. (2000). Determination and comparison of specific activities and substrates specificities of different lipases are absolutely required to investigate lipase functions as well as to identify their usefulness for biotechnological applications. However, comparison of results is not always possible because no standard substrates are used to determine specific lipase activities or to distinguish lipases from esterases.

6.3.2.3 Three Dimensional Structure of Lipases and Lipolytic Mechanism

Crystallization of lipases from several sources had been reported (Misset et al. 1994; Jaeger et al. 1999). Mammalian, fungal and bacterial lipases had been crystallized and since 1990 lipase structure has been determined by X-ray; all of them, except pancreatic lipase, where of bacterial origin (Jaeger and Reetz 1998). These enzymes have molecular weights which vary between 20 and 60 kDa. They have very similar folds despite a lack of sequence similarity (Smith et al. 1992). A comparison with X-ray structure of other hydrolytic enzymes revealed that all these enzymes share the same folding patter. Because all of them are hydrolytic enzymes, the common folding pattern was named α/β hydrolase fold (Ollis et al. 1992). The α/β

hydrolase fold is composed of a central β sheet consisting of up to eight different β strands connected by up to six α helices (Jaeger et al. 1999). The active site of lipases is composed by a catalytic triad consisting of serine, aspartic (or glutamic) and histidine residues. Serine residue is located in a highly conserved pentapaptide GXSXG forming a nucleophilic elbow where the nucleophilic attack by serine oxygen on the carbonyl carbon atom of the ester bond of the substrate starts to yield an acyl-enzyme intermediate stabilized by hydrogen bonds with residues that belong to the oxyanion hole. The alcohol is released and the acyl–lipase complex is finally hydrolyzed to yield the fatty acid and the free enzyme to close the catalytic cycle.

Lipolytic enzymes are characterized by their increased activity in the presence of lipid–water interfaces of micellar or emulsified substrates. This increase in enzyme activity is triggered by structural rearrangements of the lipase active site region (Brzozowski et al. 1991; Derewenda et al. 1992; van Tilbeurgh et al. 1993). In the absence of lipid–water interfaces, the active site is covered by the so called *lid*. Upon binding to the interface, this lid moves away, turning the *closed* form of the enzyme into an *open* form, exposing a large hydrophobic surface and making the catalytic residues accessible to the substrate. This hydrophobic surface is presumed to interact with the lipid interface. The presence of a lid-like structure is not necessarily correlated with interfacial activation (Jaeger and Reetz 1998): lipases from *Pseudomonas aeruginosa, Burkholderia glumae* and *Candida antartica* do not show interfacial activation despite having lids covering their active sites. Other lipases, like cutinase from *Bacillus subtilis* and guinea pig pancreatic lipase, despite having interfacial activation lack a lid covering their active site (Jaeger et al. 1999). These observations led to the conclusion that the presence of a lid domain and interfacial activation are unsuitable criteria to classify an enzyme as a lipase. However, the presence of the lid has been suggested to play an important role in modulating activity, stability, specificity and enantioselectivity of lipases (Lowrier et al. 1996; Secundo et al. 2006).

Lipases from several microorganisms have been studied extensively being their properties (molecular weight, pH and temperature optima, stability, substrate specificity) source dependent. Generally, microbial lipases have neutral or alkaline pH optima (Lee et al. 1999; Sunna et al. 2002; Gulati et al. 2005) with the exception of lipase from *P. fluorescens* which has an acidic optimum pH (Anderson et al. 1999). Other lipases, as those from *Bacillus*, are active over a broad pH range. Bacterial lipases are stable over a wide range, from pH 4 to pH 11 (Dong et al. 1999) and generally have temperature optima in the 30–60 °C range (Litthauer et al. 2002). However, bacterial lipases with optima in both lower and higher ranges have been reported (Lee et al. 1999; Sunna et al. 2002). Thermostability is another relevant property for lipases. Most of the studies conducted on thermal stability have been carried out on mesophiles, being many of their lipases stable at elevated temperatures (Sugihara et al. 1991). Thermostable lipases have been isolated from many sources and thermal stability data are available from *Bacillus, Chromobacterium, Pseudomonas* and *Staphylococcus* (Gupta et al. 2004). A highly thermostable lipase was isolated from a *Bacillus* strain that retained 100% of activity after 30 minutes at 75 °C (Wang et al. 1995). Cofactors are generally not required for lipase activity, but divalent cations such as calcium often stimulate enzyme activity. This has

been suggested to be due to the formation of calcium salts of long chain fatty acids. Calcium stimulated lipases have been reported in the case of *B. subtilis* 168, *B. thermoleovarans* ID-1, *P. aeruginosa* EF2, *S. aureus* 226, *S. hyicus, C. viscosum* and *Acinetobacter* sp. (Gupta et al. 2004). Lipase activity is drastically inhibited by heavy metals like Co^{2+}, Ni^{2+}, Hg^{2+} and Sn^{2+}, and slightly inhibited by Zn^{2+} and Mg^{2+} (Patklar and Bjorkling 1994).

Based on substrate specificity, microbial lipases may be divided into three categories: non specific, regiospecific and fatty acid specific. Examples of lipases belonging to these three categories have been well described by Gupta et al. (2004). Lipases in the first group catalyze the complete breakdown of triacylglycerols to glycerol and free fatty acids together with diacylglycerols and monoglycerol as intermediates in the reactions. These intermediates do not accumulate since they are hydrolysed faster than the triacylglycerol. Regiospecific lipases hydrolyze fatty acids from the primary ester bonds in the 1 and 3 positions of acylglycerols. These lipases hydrolyze triacylglycerol to give free fatty acids 1,2 (or 2,3)-diacylglycerols and 2-monoacylglycerols. Partial stereospecificity in the hydrolysis of triacylglycerols has been observed in *Rhizopus arrhizus, Rhizopus delemar* and *Candida cylindracea*. Owing to this property, lipases can be used to isolate optically pure esters and alcohols. The third group is fatty acid-specific lipases. Some of them show preference for hydrolyzing triacylglycerols composed by long chain fatty acids. However, lipases can be found that also show preference for triacylglycerides with medium and small-chain fatty acids. Enantiospecific lipases are able to discriminate between the enantiomers from a racemic mixture. These lipases have a great biotechnological value because they can be used for the production of enantiomerically pure compounds, which are gaining importance in the chemistry of pharmaceutical, cosmetic, agricultural, organic synthetic and natural products (Reetz 2001).

Lipase selectivity is the property that allows obtaining mostly one of the possible reaction products from the same substrate. Lipases have been employed by organic chemists for a long time to catalyze a wide variety of chemo, regio and stereoselective transformations (Koeller and Wong 2001; Klibanov 2001; Gotor 2002; Muralidhar et al. 2002), being stereoselectivity highly appreciated for synthetic chemists. Alcohols and carboxylic-acid esters are the main classes of lipase substrates in hydrolysis and transesterification reactions (Petschen et al. 1996; Takagi et al. 1996; Schulz et al. 2000), but the range of compounds has expanded rapidly to include diols, α- and β-hydroxyacids, cyanohydrins, chlorohydrins, diesters, lactones, amines, diamines, amino-alcohols and α- and β-amino acid derivatives (Faber 1997; Rubin and Dennis 1997; Kazlauskas and Bornscheuer 1998).

6.3.3 Improvement of Lipases by Medium and Biocatalyst Engineering

Lipases can be used as biocatalysts in hydrolytic and synthetic reactions. These enzymes are the most widely used biocatalysts in organic chemistry and they have

been successfully exploited for racemic mixtures resolution and for the synthesis of complex drug intermediates, specialty chemicals and even commodity chemicals in the pharmaceutical, chemical, food, pulp and paper, and detergent industry. Applications of lipases are reviewed in section 6.3.4. As outlined above, the two types of organic transformations catalyzed by lipases are reactions of prochiral substrates and kinetic resolution of racemates. All of these lipase catalyzed reactions can be exploited because of the valuable lipase properties: specificity, selectivity and organic solvent resistance. Lipases can be improved by tuning their properties according to their particular applications by engineering either the biocatalyst and/or the reaction medium.

6.3.3.1 Medium Engineering

Lipases catalyze the hydrolytic cleavage of triglycerides into fatty acids and glycerol, or into fatty acids and mono or di-glycerides, at oil interfaces in nature. However, this hydrolytic reaction can be reversed and transformed into reactions of esterification (inter or transesterification), alcoholysis or aminolysis by engineering the medium polarity or the water content of the medium. Therefore, substrates for lipases can be esters, like the natural triglyceride substrates in hydrolytic reactions or, if the reaction is reversed, carboxylic acids, alcohols, amines or esters. The reaction medium not only determines the direction of the reaction (hydrolytic or synthetic), but also determines the solubility and stability of lipase substrates. Therefore, lipase activity and selectivity are strongly influenced by reaction medium.

Lipases are able to work in very different media. They work in biphasic systems and in monophasic (in the presence of hydrophilic or hydrophobic solvents) systems where the water content can vary significantly between aqueous and anhydrous media. They have been tested also in ionic liquid media (Lau et al. 2000; Wasserscheid and Keim 2000; Kamal and Chouhan 2004; Ha et al. 2007), in supercritical fluids (Laudani et al. 2007) and in gaseous media (Cameron et al. 2002). The different media for enzymatic catalysis has been outlined before (see section 1.6) and it will not be further discussed here. However, some examples of modulation of activity and selectivity of lipases by medium engineering will be described in this section.

Numerous papers have published on lipase-catalyzed reactions in organic solvents (Cui et al. 1997; Matsumoto et al. 2001; Plou et al. 2002; Alcántara et al. 2004; Li et al. 2007). When lipase reactions are performed in non aqueous solvents, the nature of the solvent plays a crucial role on lipase activity. A direct correlation between solvent hydrophobicity, evaluated as the logarithm of the 1-octanol-water partition coefficient (log P), and enzyme activity has been found (Berglund 2001; Matsumoto et al. 2001; Laane et al. 1987); in most cases activity is enhanced by the solvents with higher hydrophobicity (Zaks and Klibanov 1988). However, in most cases no satisfactory correlation exists between solvent log P and enantioselectivity. Enantioselectivity of *Candida rugosa* lipase in the hydrolysis of phenoxypropionate derivatives was enhanced by the addition of 30–70% dimethyl sulfoxide (DMSO); for transesterification reactions, similar effects were observed by adding only 0.05–0.3% DMSO to the organic solvent system (Watanabe and Ueji 2001).

Water activity of the reaction medium plays a central role in lipase catalyzed reactions (Berglund 2001). Different authors have described and demonstrated the usefulness of controlling water activity on lipase performance. In esterification reactions on cyclohexane media, the reaction rate increased with water activity in the low activity range; however, it reached a maximum at a value of 0.84 and a subsequent increase in water activity led to a decrease in the reaction rate (Matsumoto et al. 2001). Results on the effect of water activity on enantioselectivity of lipases are rather contradictory (Berglund 2001). However, very good papers have been published in tuning lipase enantioselectivity by reaction medium engineering (Wehtje and Adlercreutz 1997; Matsumoto et al. 2001; Bornscheuer 2002).

6.3.3.2 Biocatalyst Engineering

6.3.3.2.1 Genetic and Protein Engineering

Recent advances in recombinant DNA technology, high-throughput technologies, genomics and proteomics, have fuelled the development of new biocatalysts and biocatalytic processes. In particular, site directed mutagenesis, directed evolution or metagenome approach are very valuable tools to enhance lipase properties.

Recombinant DNA and genetic engineering technologies allow the alteration of the amino acid sequence, and thus the properties of an enzyme. These very precise modifications of the genetic material can be translated into very specific modifications within the protein structure. This ability to focus genetic changes makes genetic engineering a powerful tool for productive protein alteration. However, the potential of site directed mutagenesis cannot be fully exploited in the absence of 3D enzyme structure knowledge. Fortunately, knowledge of the structural and catalytic chemistry of lipases has been generated very fast since the publication of the first 3D structure in 1990 (Brady et al. 1990). Recent mechanistic and molecular modeling studies allow insight into the origin of enantioselectivity in lipase catalyzed reactions (Kovac et al. 2000; Svendsen 2000; Berglund 2001; Ottonson et al. 2001) and provide guidelines for improving activity and selectivity by rational protein engineering (Magnusson et al. 2001; Rotticci et al. 2001). Villeneuve et al. (2000) have reviewed the alteration of activity and substrate specificity, the alterations in the ratio of activities toward cholesterol esters and triglyceride esters and the alteration of lipase stability by using site directed mutagenesis. Mutants addressing lid function, positional specificity or chain length specificity, have been reviewed by Svendsen (2000).

Directed evolution proved to be a rapid yet powerful method to alter enzyme properties or to develop enzymes with novel properties, without requiring knowledge of the enzyme structure and catalytic mechanism. Arnold (2001) has an impressive review on enzyme performance improvement by a combination of rational design and directed evolution. Directed evolution applied to lipases has been reviewed by different authors (Petrounia and Arnold 2000; Tobin et al. 2000; Jaeger et al. 2001). Directed evolution has been employed for the creation of

enantioselective catalysts or lipases with enhanced thermal stability (Gupta et al. 2004) and to increase activity (Fujii et al. 2005; Nakagawa et al. 2007). A bacterial lipase from *Pseudomonas aeruginosa* was evolved toward a model substrate to yield a lipase mutant showing $> 90\%$ enantiomeric excess, as compared with 2% for the wild type lipase (Jaeger and Reetz 2001). In another study, directed evolution was applied in order to convert a staphylococcal lipase into a phospholipase (van Kappen and Egmond 2000).

Microbial diversity is a major resource for biotechnological products. In the biosphere, the majority of live beings are microorganism and it is estimated that less than 1% has been identified because the vast majority of microorganism are incapable of being cultured (Lorenz et al. 2002). The metagenome approach takes advantage of the genetic diversity of the microorganisms, in a certain environment as a whole, in order to find new or improved genes for biotechnological purposes. Henne et al. (2000) screened environmental DNA libraries prepared from three different soil samples for genes conferring lipolytic activity to *Escherichia coli* clones and identified four clones harboring lipase and esterase activities.

6.3.3.2.2 Lipase immobilization

One of the bottlenecks of enzyme technology is enzyme availability. When the biocatalyst is commercial, the price may be too high, but in most cases there is no commercial source available so that the enzyme must be produced by means of an overproducing strain and finally the enzyme should be purified. Enzyme purification (discussed in section 6.3.1) is a time consuming process and may represent up to 80% of the enzyme production cost. The usual procedures for lipase purification are sometimes troublesome, time consuming and result in low final yields (Gupta et al. 2004). Enzyme immobilization overcomes this handicap because it allows its reuse and can also enhance enzyme stability and activity (Sharma et al. 2001); furthermore, enzyme immobilization facilitates bioreactor design and final product downstream from reaction medium (see section 4.1).

Lipases used in laundry detergents and in other bulk applications do not require enzyme immobilization; however, an increasing number of applications in synthesis and biotransformation demand an immobilized biocatalyst for efficient use. It has been claimed that the success of a lipase catalyzed biotransformation for the production of certain pharmaceuticals depends on immobilization. For example, in the industrial preparation of the chiral intermediate used in the synthesis of Diltiazem, the lipase from *Serratia marcescens* was supported in a spongy matrix, which was used in a two-phase membrane bioreactor (Cowan 1996).

Methods to immobilize enzymes are numerous, and they have been reviewed in section 4.1. There are many reports of lipase immobilization, using all of the different techniques: adsorption, inclusion, encapsulation, covalent attachment and aggregation (Jaeger and Reetz 1998; Jaeger et al. 1999; Villeneuve et al. 2000; Dosanjh and Kaur 2002; López-Serrano et al. 2002; Palomo et al. 2002, 2003, 2005; Soares et al. 2002; Hung et al. 2003; Salis et al. 2003; Hsu et al. 2004;

de Lathouder et al. 2005; Petkar et al. 2006). Lipase immobilization by physical adsorption was well reviewed by Balcao et al. (1996). Many different types of carriers have been used, like glass, silica, alumina, diatomaceous earth. . . . More recently, the most used supports for lipase immobilization are ion exchange resins, Celite and biopolymers (Villeneuve et al. 2000). Hiol et al. (2000) reported immobilization of lipase from *Rhizopus oryzae* onto Amberlite IRC 50; this ion exchange resin offered a high adsorption capacity and good long-term stability for the immobilized lipase. Generally, hydrophobic materials are better supports than hydrophilic for lipase immobilization (see section 6.3.5). Interfacial adsorption of bacterial lipases on hydrophobic supports, as octyl-agarose, has been reported (Bastida et al. 1998). When lipases are immobilized by inclusion, the enzyme is brought into solution in a monomeric phase which, upon polymerization, leads to its entrapment. A number of relevant publications dealing with entrapment of lipases have appeared (Jaeger and Reetz 1998; Krishnakant and Madamwar 2001). Microencapsulation is very similar to entrapment, although in this case, it is the enzyme and its environment that are immobilized. A recent example of immobilization of lipase from *Candida antarctica* is based on encapsulation in hydrophobic sol–gel materials (Reetz et al. 1996; Hsu et al. 2004). In order to facilitate immobilized lipase separation, nanoparticles of iron oxide are included in the encapsulation (Reetz et al. 1996). Use of hollow fiber and flat membrane reactors for biotransformations with immobilized lipases have been reported (Balcao et al. 1996; Xu et al. 2000; Xin et al. 2001). Lipase immobilization by covalent attachment onto activated matrix presents the advantage of avoiding catalyst desorption and frequently the thermal stability of the immobilized lipase is significantly enhanced. Enzyme desorption may not be a problem though when working with organic solvents at low water activity. Arroyo et al. (1999) covalently immobilized *Candida antarctica* lipase B on Sepharose, alumina and silica yielding immobilized catalysts with an increased thermal stability. However, lipase immobilization by covalent attachment may produce significant activity losses (Villeneuve et al. 2000) as compared to other immobilization methods. Manjon et al. (1991) compared the behavior of *Mucor miehi* lipase adsorbed on Celite and covalently bound to nylon and observed that covalent binding of the lipase to nylon showed much lower activity than the enzyme adsorbed onto Celite. Lipase immobilization has been reported also using different cross-linking reagents such as polyacrylamide, polyaminopolystyrene or glutaraldehide (Lieberman and Ollis 1975). Glutaraldehyde is the most popular cross-linking reagent (Villeneuve et al. 2000) and it has been used for lipase immobilization with different solid supports. Omar et al. (1988) immobilized lipase from *Humicola lanuginosa* on amberlite by cross-linking with hexamehylenediamine and glutaraldehyde.

One interesting technology uses lipases in the form of cross-linked enzyme crystals (CLECs) (Margolin 1996). This immobilization method does not use any solid support and the lipase specific activity (units of activity/g of immobilized catalyst) of the immobilized lipase derivative can be enhanced by 10-fold because there is no inert support, that usually represent more than 90% of the catalyst weight in the case of carrier-bound enzymes. These cross-linked crystals have been used for the chiral resolution of commercially important organic compounds, such as ibuprofen,

naproxen or menthol (Lalonde et al. 1995). An even more interesting form of non-supported biocatalyst is cross-inked enzyme aggregates (CLEAs) that have similar characteristics than CLECs with the advantage of not requiring a pure enzyme protein. Lipase CLEAs have demonstrated very good catalytic properties (López-Serrano et al. 2002; Schoevaart et al. 2004; Yu et al. 2006) and used successfully in combination with membrane retention (Hilal et al. 2004) and gel entrapment (Wilson et al. 2006). CLEAs are promising forms of immobilized lipases that are being tested for the production of biodiesel (Kumari et al. 2007) and ibuprofen, where the enantioselectivity of the CLEA was higher than the free lipase counterpart (Yu et al. 2004).

6.3.4 Applications of Lipases

Lipases are robust and versatile enzymes that can catalyze a myriad of chemical reactions, many of which being of technological present impact or potential (Straathof et al. 2002). Because of its extracellular nature in most producing systems, lipases can be conveniently produced in large quantities so that, as a whole, they are now considered as the most promising biocatalysts for biotechnological applications (Schmidt-Dannert 1999); even so, they are still third to amylases and proteases in total sales volume (Hasan et al. 2006). Current applications of lipases extend from its more conventional uses as hydrolases to the more sophisticated uses as catalysts for organic synthesis, where their stereo and regioselectivity is highly appreciated (Saxena et al. 1999).

Acting as hydrolases, the most traditional applications of lipases are related to the food industry (Seitz 1974), where they are used as flavor boosters in dairy products by the selective hydrolysis of fat triglycerides to release the corresponding fatty acids which, by themselves or acting as precursors, are responsible for the developments of special flavors, which is particularly relevant in cheese-making (Saxena et al. 1999). They are used in other products as well, like butter, margarine and milk chocolate (Tombs 1995; Jaeger and Reetz 1998), and also in bakery products (Hasan et al. 2006). The most relevant application of lipases acting as hydrolases is in detergent manufacturing. More than 30% of the present market for lipases comes from this sector (Sharma et al. 2001). Enzymes are now considered as key ingredients in detergents, since they reduce environmental load by saving energy and reducing the content of offensive chemicals; they are also biodegradable and harmless to the sewage treatment processes and aquatic ecosystems. More than three decades ago, alkaline proteases were the first enzymes used as ingredients in detergents (Maurer 2004), but finding and developing lipases able to withstand the harsh conditions of laundry was much challenging and only in the mid 1990s a recombinant alkaline lipase went into the market (Jaeger and Reetz 1998). Several improvements have been introduced since then to solve the problems associated with narrow substrate specificity and instability at laundering conditions (high pH, moderately high temperature, presence of strong chemical oxidants, surfactants and even proteases).

They include screening of novel sources (Wang et al. 1995; Cardenas et al. 2001; Gulati et al. 2005) and protein engineering techniques (Cernia et al. 2000) as well as genetic engineering techniques to overexpress the tailored lipases into suitable hosts (Schmidt-Dannert 1999). Closely related to the use in detergents is the use of lipases in the formulation of liquid dishwashers and contact lens cleaners, in textiles to remove size lubricants and to improve the wettability and absorbance of synthetic fibers, in leather degreasing, in fat removal in clogged pipes from domestic and industrial effluent treatment plants and in wastewater treatment for the removal of fat from the surface of activated sludge tanks and other aerobic systems to aid in oxygen transfer and also in the treatment of fat-rich industrial effluents (Hasan et al. 2006). Another bulk application of lipases as hydrolytic enzymes is in the pulp and paper industry for the removal of pitch, the hydrophobic components of wood (mainly waxes and triglycerides). Pitch deposition during pulping is a nuisance that reduces the quality of pulp and may cause mill shutdown (Allen 2000). Biotechnological control of pitch with lipases is an already mature technology being used in Japan for over a decade now (Jaeger and Reetz 1998) and the performance of lipases has been improved by screening enzymes with broader substrate spectrum and by protein engineering techniques (Gutiérrez et al. 2001).

Despite their numerous and relevant applications as hydrolases, lipases have been employed for long in organic synthesis to catalyze a large number of chemo, regio and stereoselective transformations (Kazlauskas and Bornscheuer 1998). It is in organic synthesis were most of the potential of lipases lies and in fact a myriad of reactions of organic synthesis has been conducted with lipases (Faber 1997) being the most relevant of all enzymes in this respect, as already highlighted in section 1.6. Since many such reactions require to be conducted in non-aqueous media, lipases are the enzymes of choice because they are particularly well suited to perform in such media. It is no simple task to cover all applications (actual or potential) of lipases in organic synthesis, but rather comprehensive and updated reviews have been published (Sharma et al. 2001; Houde et al. 2004; Hasan et al. 2006). Some of the most relevant applications of lipases in organic synthesis from an industrial perspective are analyzed.

Lipases have a great potential in oleochemistry where, beyond the hydrolysis of triglycerides, they can catalyze reactions of esterification, interesterification and transesterification. Energy savings, reduction of thermal degradation of substrates and products and savings in chemical reagents are the driving forces for replacing the current chemical technologies by biotechnology (Vulfson 1994). However, there is a long way to go for enzyme technology to overcome, because the oleochemical industry is a conservative high-investment sector, the cost of enzymes remained for many years prohibitive for bulk production and still enzymatic processes are regarded as hard to validate. As mentioned in section 1.6, the situation is changing slowly but steadily as can be deduced for the commercialization of several lipase-based technologies. The modification of the fatty acid composition of fats with regiospecific lipases is of commercial significance since high priced fats can be produced from cheap and plentiful oils, as illustrated by the case of the production of cocoa butter analogue by the interesterification of palm oil mid-fraction

(Jandacek et al. 1987; Undurraga et al. 2001). This strategy is applicable to the synthesis of other structured triglycerides of dietetic or nutritional value, which are products of increasing demand (Soumanou et al. 1997). Enrichment of natural oils with polyinsaturated fatty acids such as arachidonic and eicosapentaenoic, which have proven effective in the treatment of atherosclerosis (Ando et al. 1999), has been carried out successfully with lipases (Fregapane et al. 1991; Li and Ward 1993).

Production of biodiesel deserves a special consideration because of the urgency of replacing fossil fuels for renewable sources of energy and reducing CO_2 emission. Biodiesel is a diesel fuel substitute produced from renewable triglyceride sources, like vegetable oils, animal fats and even recycled cooking-oils (Knothe et al. 2005). Recently, the use of algal biomass as a source of lipids for biodiesel production has been proposed (Li et al. 2007). Besides its renewable nature, biodiesel is neutral with respect to CO_2 emission and its combustion produce less offensive gases and particulate matter; therefore, biodiesel can be considered as a plausible diesel substitute (Iso et al. 2001). Biodiesel has been conventionally produced by chemical transesterification of triglycerides with primary alcohols using base catalysts to yield the corresponding esters (biodiesel) and glycerol (Ma and Hanna 1999; van Gerpen 2005). Even though this is for the moment the technology of choice, there are problems like the requirement of catalyst removal by neutralization and the difficulty of recovering the glycerol. Besides, chemical catalysts are less specific and produce unwanted side reactions (Fukuda et al. 2001; Soumanou and Bornscheuer 2003). Enzyme catalysis is a more specific and environmentally benign process so that enzymatic transesterification with lipases is being under intense research. Methanol has been the most used alcohol because it is cheap and readily available (Shimada et al. 2002; Royon et al. 2007); however, as a petroleum-derived product, it is rather contradictory to use it as a raw material for biodiesel. Ethanol is in this sense more appropriate because it is derived from renewable resources and has been also frequently used (de Oliveira et al. 2004; Yamada et al. 2007). Other acyl donors, like methyl acetate, have also been tested (Du et al. 2004). The main problem to overcome is the high price of available lipases that are to be required in large quantities for this large-scale process. Even though most research has been conducted with commercial lipases, considerable attention is given to obtain improved lipase biocatalysts by screening (Luo et al. 2006), genetic manipulations (Matsumoto et al. 2001; Shibamoto et al. 2004; Hatti-Kaul et al. 2007) and immobilization (Park et al. 2006; Al-Zuhair 2007), and also to optimize operation conditions (Soumanou and Bornscheuer 2003; Sunitha et al. 2007; Yamada et al. 2007) to reduce costs. The current state of the art for the production of biodiesel with lipases has been recently reviewed (Salis et al. 2007). It is premature to assess the real impact of enzymatic production of biodiesel; moreover, the mere idea of producing fuel from crops is arguable and certainly a matter of debate. Biodiesel will hardly represent a significant contribution to the energy bill in the foreseen future, but it might be an interesting local answer to energy needs in particular niches.

Lipases are being used in several reactions of synthesis for the production of valuable compounds. Biodegradable polymers, like butyl oleate and some polyesters, have been synthesized by esterification and transesterification reactions with lipases

(Linko et al. 1998). Lipases have been extensively used in the production of surfactants of different chemical nature: by esterification of alkyl glycosides and fatty acids, by transesterification of natural oils and alcohols, by transesterification of phospholipids and alcohols, and also by esterification of amino acids and amides (Saxena et al. 1999). Personal care products, like isopropyl myristate and isopropyl palmitate, have been produced in solvent-free media with immobilized lipase and wax esters (esters from long-chain fatty acids and fatty alcohols) are also being produced with lipases (Hasan et al. 2006).

One of the most appealing properties of lipases for organic synthesis is their enantioselectivity and enantiospecificity; it has been claimed that most functionalized organic compounds can be produced accordingly by lipase catalysis (Jaeger and Reetz 1998). There is a large number of synthetic pharmaceuticals and agrochemicals which are *chiral* molecules (ones that cannot be superimposed on their mirror images) and only one of its *enantiomers* (a pair of molecules that are mirror images of each other) is the active principle (*eutomer*), being the other (*distomer*) not functional or even harmful (Faber 1997). Therefore, there is a strong pressure, especially from the pharmaceutical industry, to produce chiral drugs as pure enantiomers as opposed to the *racemates* (equimolar mixture of both enantiomers) produced by chemical synthesis, that are still in the market. An impressive number of potentially useful reactions have been studied taking advantage of the enantioselectivity of lipases in the transformation of prochiral substrates and their enantiospecificity in the kinetic resolution of racemates (Kirchner et al. 1985; Archelas and Furstoss 1997; Faber 1997; Roberts and Williamson 1997; Rubin and Dennis 1997; Jaeger and Reetz 1998; Roberts 1998; Ghanem and Aboul-Enein 2004, 2005). In the first case, stoichiometric conversion yields can be obtained while in the second case maximum attainable molar conversion yield is 50%. Chiral intermediates are now in high demand both for the production of pharmaceuticals and agrochemicals. Some illustrative examples of the use of lipases in commercially relevant processes are:

- The synthesis of phenoxypropionate herbicides form 2-halopropionic acids, based on the specific esterification of S-isomers with butanol catalyzed by porcine pancreatic lipase (Hasan et al. 2006)
- The production of 2(R),2(S) methyl methoxyphenyl glycidate, a key intermediate in the manufacture of the optically pure cardiovascular drug Diltiazem (Houde et al. 2004)
- The production of one of the leading anti-inflammatory drugs S-ibuprofen [S-2(4-isobutylphenyl) propionic acid] by kinetic resolution of the racemic ester (Ducret et al. 1998). This is illustrated in Fig. 6.3.1 for a lipase enantiospecifically acting on the S methyl ester in isooctane organic medium in a membrane bioreactor (1), followed by chromatographic separation of the product S-ibuprofen from the unreacted R-methyl ester then racemized (3) and recycled back into the bioreactor. Later on, the same reaction was conducted in ionic liquids (Yu et al. 2004) obtaining a higher enantiospecificity and somewhat higher operational stability in 1-butyl-3-methylimidazolium hexafluorophosphate (BMIMPF6) medium than in isooctane. The process is quite relevant since the S eutomer is 160 times more potent than the R distomer (Sharma et al. 2001)

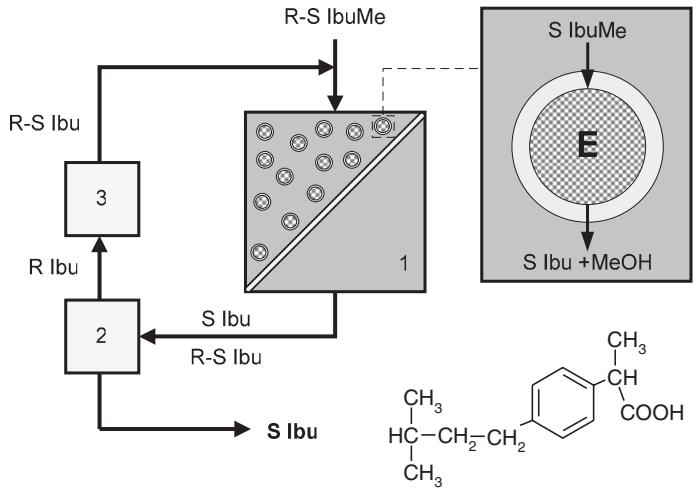

Fig. 6.3.1 Production of S-ibuprofen (S-Ibu) by kinetic resolution of the ester racemate (R-S IbuME) with lipase (L) in organic medium in a membrane bioreactor (1), with product separation (2) and racemization (3) of the unreacted R-ibuprofen methyl ester (R-IbuME)

- The production of other profens as well, like the enantiospecific esterification of racemic ketoprofen in non-aqueous solvent under reduced pressure (De Crescenzo et al. 2000; Ong et al. 2005) and the anti-inflammatory drug naproxen by kinetic resolution of the racemic ester in a in an aqueous-organic biphasic system in a stirred tank membrane bioreactor (Xin et al. 2001)

Some other miscellaneous applications of lipases have bee reported, including their use in diagnostics and biosensors (Hasan et al. 2006). A comprehensive analysis of lipase applications can be found in http://www.au-kbc.org/beta/bioproj2/index. html.

New applications of lipases are expected to develop vigorously in the forthcoming years in view of its remarkable versatility, selectivity and robustness, which are well appreciated attributes for performing chemical synthesis. In the next section, a novel application of lipase for the selective transesterification of wood sterols will be described.

6.3.5 Development of a Process for the Selective Transesterification of the Stanol Fraction of Wood Sterols with Immobilized Lipases

Pulp and paper industry is quite relevant to Chilean economy. Kraft process for cellulose production is based on the chemical digestion of wood chips with sodium hydroxide and sodium sulfide at high pressure to extract the lignin and recover the

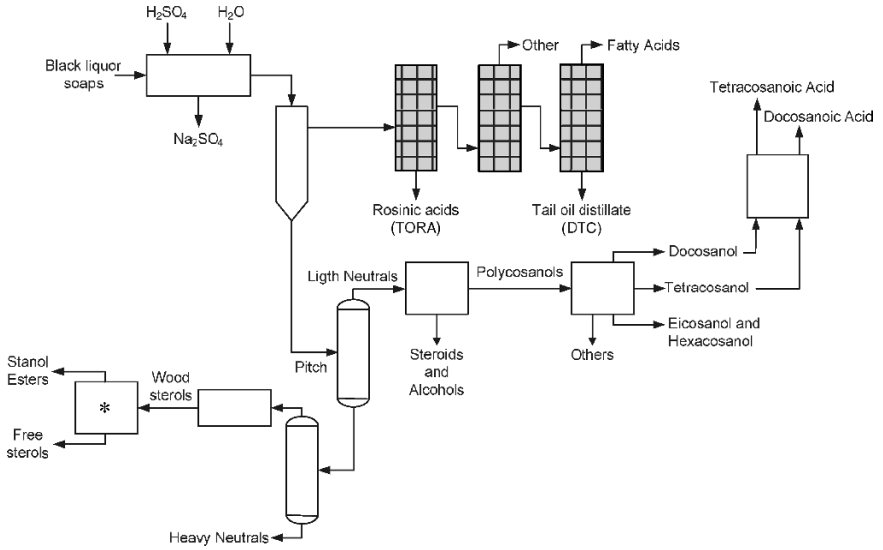

Fig. 6.3.2 Technological platform for the reclamation and upgrading of black liquor from the Kraft process

cellulose fibers. Residues like black liquor soap and turpentine oil are produced in huge amounts that need to be recovered or treated to alleviate environmental pollution (Johansson 1982). A technological platform has been envisaged to reclaim and upgrade the black liquor from Kraft pulping process, as shown in Fig. 6.3.2.

Black liquor soap is treated with sulfuric aid to produce tall-oil. Tall-oil is a potentially valuable intermediate and it has been evaluated as a source of biodiesel (see section 6.3.4), even though it is far from ideal for that purpose and several problems remain to be solved (Lee et al. 2006). Subsequent vacuum fractional distillation of tall-oil produces a light fraction containing tall-oil rosinic acids (TORA), tall-oil fatty acids (TOFA) and tall-oil distillate that are further separated by sequential distillations, and a heavy fraction called *pitch*, according to a well established technology. TORA and TOFA are used as adhesives, rubbers, inks, emulsifiers, soaps and lubricants, an also as a source of volatile fatty acids. On the other hand, pitch has been used as a binder for cement and as emulsifier for asphalt; however, it contains a rich spectrum of potentially useful products of significantly higher added value. Distillation of pitch renders a light fraction from which long-chain aliphatic alcohols (polycosanols) are recovered that can in turn be subjected to short-path distillation to obtain at least three fractions rich in docosanol, tetracosanol and higher fatty alcohols respectively. Fractionation of polycosanols can provide substantial added value since those long-chain fatty alcohols have several pharmaceutical applications as anti-inflammatory and anti-hyperproliferative cells (keloids) agents and are also effective for the treatment of herpes and other infections caused by membrane viruses (Pope et al. 1999; http://www.pslgroup.com/dg/1d434e.htm; http://www.medscape.com/viewarticle/529346). Docosanol and tetracosanol can be chemically oxidized to produce the corresponding acids that are potentially valuable

Table 6.3.1 Composition of Wood Sterols

Compound	Weight Percentage
β-Sitosterol	75
β-Sitostanol	15
Campesterol	7
Campestanol	2
Stigmasterol	<1

products for personal care. An alternative to chemical oxidation is enzymatic oxidation, but no enzymes active enough on long chain fatty alcohols are available, being this an interesting challenge for screening or genetically improving existing dehydrogenases.

Heavy fraction after pitch distillation is rich in phytosterols (wood sterols in this case), which is a mixture of saturated sterols (stanols) and mono-unsaturated sterols. This fraction is highly valuable since their esters have proved to reduce total and LDL (low density lipoprotein) cholesterol by inhibiting cholesterol absorption from the intestine in humans (Nguyen 1999; Lichtenstein and Deckelbaum 2001). Products based on such esters, produced by chemical esterification of phytosterols, have been in the market for some years now (www.benecol.com). However, stanol esters have been considered superior than sterol esters, so that its separation can be beneficial from that perspective but, moreover, because phytosterols can be used as raw material for steroid drug production. Many commercially important steroid drugs are in fact produced by bioconversion from soy sterols (Pérez et al. 2006); however, soy prices have risen so that alternative sources have been explored (Beaton 1978) and, among them, wood derived sterols are an interesting option (Donova et al. 2004), mainly if derived from low price byproducts, as is the present case. Wood sterols have the advantage over soy sterols of having a more defined composition. In fact, as seen in Table 6.3.1, 90% of wood sterols is represented by β-sitosterol and β-sitostanol, whose chemical structures are shown in Fig. 6.3.3.

The fractionation of wood sterols into a sterol-rich fraction oriented to the pharmaceutical industry for the production of steroid hormones, and a stanol-rich fraction oriented to the food industry for producing health-foods is certainly quite appealing and has been the motivation behind the work that is now presented.

Non-specific esterification of wood sterols can be performed chemically (www.freshpatents.com/Phytosterol-esterification-product-and-method-of-make-same-dt-20070628ptan20070148311.php); however, enzymatic esterification with lipases has the potential advantages of higher specificity and mild reaction conditions which are desirable, both from process and environmental perspectives. More than 20 lipases were previously screened for their ability to catalyze the transesterification of wood sterols and fatty acid esters (Martínez et al. 2004). The goal was now to screen among them those specific for stanol esterification, so as to obtain a product consisting in mostly esterified stanols and mostly free sterols (see Fig. 6.3.4) amenable for separation through short-path distillation,

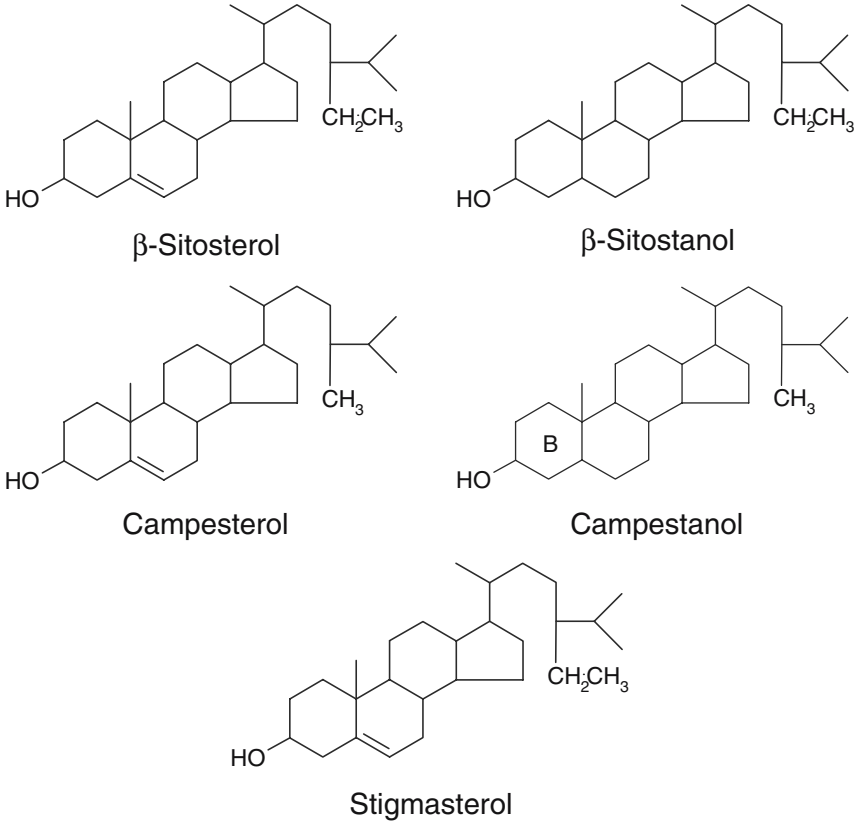

Fig. 6.3.3 Chemical structure of main components of wood sterols

according to the process scheme in Fig. 6.3.5. Two of them: soluble lipase QL and Cellite G-immobilized lipase QLG from *Alcaligenes* sp. were specific enough on stanol esterification so that the reaction was optimized in terms of enzyme to substrate ratio, ratio between substrates, temperature, pressure (vacuum) and initial water activity (a_w) (Fuenzalida et al. 2006).

The acylating agent was first selected at pre-established conditions of such variables (0.05 g enzyme/g wood sterols; 5 mol acyl agent/mol wood sterols, 50 °C, 20 mbar and 0.75 a_w) among eight different esters of commercial oils. Vacuum was an absolute requirement to remove the alcohol (methanol or ethanol) that strongly inhibits the enzyme. Criterion for selection was the one that gives the highest productivity at higher than 80% stanol esterification and lower than 30% sterol esterification. Methyl ester of sunflower oil was selected and optimal conditions were then determined for that oil with lipase QL, being: 0.03 g enzyme/g wood sterols; 5 mol acyl agent/mol wood sterols, 70 °C, 50 mbar and 0.75 a_w. Similar results were obtained with lipase QL, except that the esterification of both stanols and sterols was slightly lower: 88% and 20% with QL and 93% and 23% with QLG, respectively (Markovits et al. 2004). Time-course of sterification with

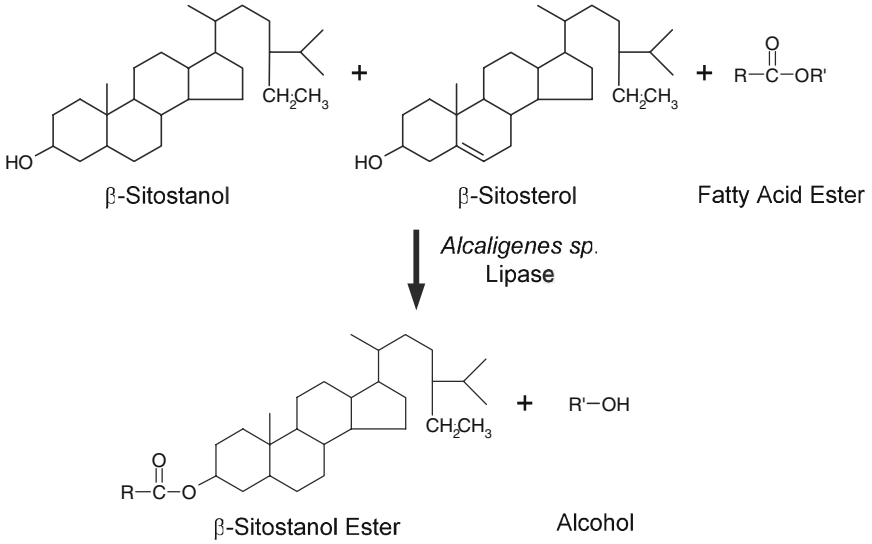

Fig. 6.3.4 Enzymatic selective transesterification of β-sitostanol from wood sterols with fatty acid esters

QLG at the optimized conditions is presented in Fig. 6.3.6. The specificity of the enzyme for stanols was not absolute and it is in fact a kinetic resolution: given enough time, a substantial fraction of the sterols (up to 40%) can be esterified (data not shown).

Even though results were satisfactory and fulfill the established requirements, operational stability of the enzyme was tested in sequential batch operation, where the biocatalyst was recovered after each batch by in-situ filtration. Results were disappointing since most of the enzyme (about 90%) was desorbed during operation

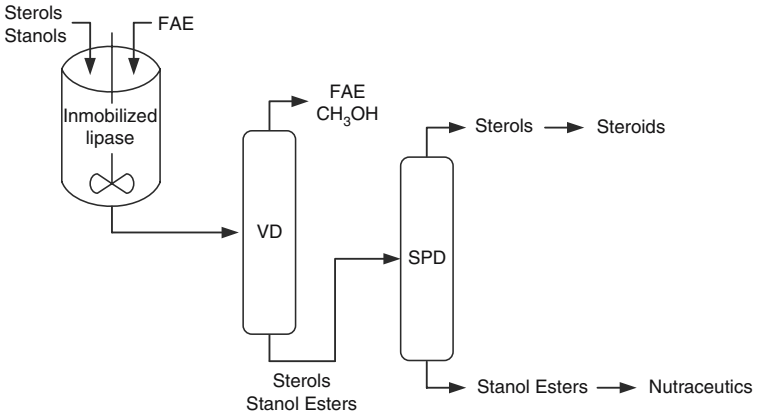

Fig. 6.3.5 Conceptual design for the production process of sterols and esterified stanols from wood sterols. FAE: fatty acid esters; VD: vacuum distillation; SPD: short path distillation

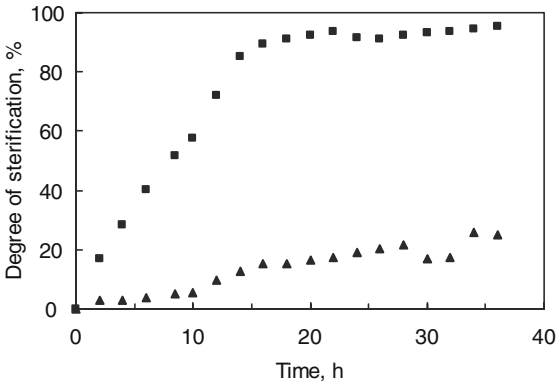

Fig. 6.3.6 Esterification of wood sterols with QLG at optimized conditions: 0.03 g enzyme/g wood sterols; 5 mol acyl agent/mol wood sterols, 70 °C, 50 mbar and 0.75 a_W. ■: stanols; ▲: sterols

so that in the second batch productivity was severely reduced and conversion yield was significantly lower even at prolonged operation. SDS-PAGE electrophoresis revealed that most of the desorbed protein had a molecular weight of 31,000 Da, which corresponds to that of *Alcaligenes faecalis* lipase. Most of the activity lost from batch to batch corresponded to protein desorption from the matrix, enzyme inactivation being quite low. Therefore, it made very little sense to use QLG instead of QL, but the free enzyme was not recoverable from the reaction medium and cost estimates indicated that the enzyme should be used at least five times to make the process economically attractive. Therefore, the next goal was to construct an immobilized lipase biocatalyst from soluble QL. The hydrophobic nature of the active site and the requirement of a hydrophobic interface for lipase action made reasonable to use hydrophobic supports; however to test the validity of this hypothesis several immobilization systems were tested. The results obtained are summarized in

Table 6.3.2 Immobilization of Lipase QL in Different Systems

Support	R_P (%)	R_E (%)	SA (IU*/g)
Cellite 580	80	55	12
Glyoxyl agarose	37	10	16
Polyethyleneimine-glyoxyl agarose	56	47	10
Eupergit C	52	38	8
Eupergit C250L	56	39	9
Chitosan	44	33	7
Accurel MP-1000	80	104	22
Octyl agarose	100	165	35
Butyl Sepabeads	88	169	36
Octadecyl sepabeads	96	149	32

R_P: protein immobilization yield (mg immobilized enzyme/mg contacted protein); R_E: enzyme immobilization yield (units of enzyme activity expressed in the biocatalyst/unit of enzyme activity contacted); SA: specific activity of the biocatalyst (units of enzyme activity expressed/unit mass of biocatalyst)

*1 IU was defined as the amount of enzyme that catalyzes the formation of 1 μmol of *p*-nitrophenol per minute from 0.24 mM *p*-nitrophenyl acetate at pH 7.0 and 30 °C

Table 6.3.3 Immobilization of Lipase QL in Octyl Agarose and Butyl Sepabeads at Increasing Protein Loads (P) (mg of Protein Offered/Unit Mass of Support)

	R_P(%)		R_E(%)		SA (IU/g)	
P (mg/g)	OIL	BIL	OIL	BIL	OIL	BIL
0.25	100	88	165	169	35	36
0.75	68	57	78	72	33	29
1	60	46	77	64	42	35
1.25	57	48	71	62	47	39
1.5	51	47	67	62	54	47
1.75	57	47	66	64	55	64
2	50	46	52	54	55	58
2.5	52	40	41	24	61	36
3	50	43	35	21	63	38
5	34	28	21	16	64	40
10	20	16	11	7	64	41

R_P: protein immobilization yield (mg immobilized enzyme/mg contacted protein); R_E: enzyme immobilization yield (units of enzyme activity expressed in the biocatalyst/unit of enzyme activity contacted); SA: specific activity of the biocatalyst (units of enzyme activity expressed/unit mass of biocatalyst). OIL: octyl agarose immobilized lipase; BIL: butyl Sepabeads immobilized lipase

Table 6.3.2. As seen lipase immobilized on hydrophobic supports (octadecyl Sepabeads, butyl Sepabeads and octyl agarose) were far better than the rest of the biocatalysts and, interestingly, interfacial activation was observed in such cases. Butyl Sepabeads and octyl agarose were selected for further studies.

Both hydrophobic supports were challenged at higher protein loads. As seen in Table 6.3.3, specific activity of the biocatalyst could be substantially increased, but no interfacial activation was revealed at higher loads. Butyl Sepabeads at 1.75 mg protein/g support was considered the best biocatalyst for having the highest specific activity at a still high enzyme immobilization yield; besides, the biocatalyst is easily

Table 6.3.4 Results of Sequential Batch Reactor Operation with Lipase QL Immobilized in Butyl Sepabeads at: 0.03 g enzyme/g wood sterols; 5 mol acyl agent/mol wood sterols, 70 °C, 50 mbar and 0.75 a_W

	Esterification (%)		Stanol/Sterol	Biocatalyst Recovery	
Batch No.	Stanols	Sterols	Esterification Ratio	In Batch	Cumulative
1	95	32	3.0	93	93
2	93	30	3.2	96	89
3	96	28	3.4	96	86
4	92	31	3.0	93	80
5	100	27	3.7	91	73
6	73	19	3.8	95	69
7	74	23	3.2	89	61
8	70	11	6.4	91	56
9	66	12	5.5	94	53
10	56	7	8.0	89	46

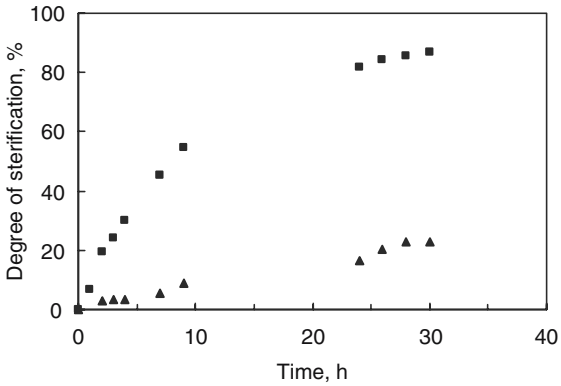

Fig. 6.3.7 Sterification of wood sterols with lipase QL immobilized in butyl Sepabeads at optimized conditions: 0.03 g enzyme/g wood sterols; 5 mol acyl agent/mol wood sterols, 70 °C, 50 mbar and 0.75 a_W. ■: stanols; ▲: sterols

handled and robust to withstand the stringent conditions of reaction. At the previously optimized conditions for QLG, sequential batch operation with lipase QL immobilized in butyl Sepabeads was conducted; results are summarized in Table 6.3.4 (Alvarez 2005). Results of the second batch are presented in Fig. 6.3.7 to illustrate the time-course of esterification.

As seen in Table 6.3.4, recovery of the biocatalyst activity after each batch was very high and protein desorption from the support was negligible. After five batches, reactor behavior remained almost unchanged and the levels of esterification met the pre-established requirements. From batch 6 on, the percentage of esterification of stanols was significantly reduced (it occurred the same with sterols) dropping below the level of acceptance. Results were satisfactory and the sequential batch process was validated through several runs. The process has been scaled up to pilot level and the technology transferred, even though there is still room for improvement, since conditions of operation remain to be optimized for the produced lipase biocatalyst.

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6.4 Oxidoreductases as Powerful Biocatalysts for Green Chemistry

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One of the most relevant goals in green chemistry is the development of mild and highly selective reductions and oxidations of complex substrates to yield key intermediates for the production of fine chemicals. The use of conventional chemical

synthesis is very often quite difficult because of the lack of highly effective and selective catalysts, so that the use of enzymes appears as a suitable alternative. Redox reactions, catalyzed by oxidoreductases, are vital in all living systems but, beyond this fundamental physiological role, such enzymes may also be used as process catalysts acting both on natural and non-natural substrates (Devaux-Basseguy et al. 1997; Hummel 1999). There are different groups of oxidoreductases (see section 1.4) that can be obtained from microorganisms, like oxidases (Kawakami et al. 1986; Maekawa 1995; Malherbe et al. 2003; Wilson et al. 2003) that use molecular oxygen as oxidizing agent but usually exhibit a quite narrow substrate specificity, and oxygenases, monooxygenases and dehydrogenases (Hummel and Kula 1989; Hummel 1997) that require the use of complex soluble coenzymes but have the interesting ability to modify non-natural substrates. These enzymes are involved in synthetic routes that may be important in fine and pharmaceutical chemistry in relation with the highly efficient synthesis of optically active compounds and chiral building blocks. The biotechnological opportunities of both types of enzymes will be discussed in this chapter.

6.4.1 Mild and Selective Oxidations Catalyzed by Oxidases

A wide spectrum of applications have been explored so far for this group of enzymes, including regioselective oxidized compounds, preparation and modification of polymer biosensors for a variety of analytical and clinical applications and degradation of organic pollutants. However, oxidases use to be quite specific enzymes, recognizing a very narrow range of substrates.

There are many examples of regioselective oxidation of different compounds (drugs, vitamins, etc.). One relevant example is the oxidation of cephalosporin C by D-amino acid oxidase, to produce 7-ACA (see section 6.2.2), the β-lactam nucleus of many semisynthetic cephalosporins (Pilone and Pollegioni 2002). Other interesting example is the regioselective oxidations of cholesterol derivatives by cholesterol oxidase (Lin and Yang 2003). Gluconic acid may be produced from glucose using glucose oxidase, being this compound interesting for food applications and building materials (Godjevargova et al. 2004).

Oxidases are among the most used enzymes for biosensors, the consumption of oxygen and the production of hydrogen peroxide allowing the simple determination of the enzyme activity. Some examples of compounds detected by oxidases are glucose (glucose oxidase), ethanol (alcohol oxidase), phenol (phenol oxidase) and cholesterol (cholesterol oxidase).

Regarding the production of polymers (Kobayashi and Higashimura 2003) oxidases have been used to catalyze the chemoselective polymerization of phenolic monomers having a reactive functional group like methacryloyl and also the induced polymerization of syringic acid that cannot be polymerized by conventional metal catalysis. Other example is the use of tyrosinase to produce the coupling of phenoxy radicals from 2- and/or 6-unsubstituted phenols. For example, crystalline

poly(1,4-phenylene oxide) was produced from 4-phenoxyphenol via oxidative polymerization by using this catalyst.

One of the main problems of oxidases is the equimolar production of hydrogen peroxide as a side-product that can inactivate them or alter its substrate. Thus, the elimination of this compound seems to be a must. Although some metals may destroy hydrogen peroxide, they can also produce some undesired modifications of enzymes or compounds, therefore milder and more selective agents should be preferred. In this sense, the use of catalase (EC 1.11.1.6) is an adequate solution since this enzyme specifically destroys the hydrogen peroxide without producing any other side-reaction. The combined use of oxidases and catalase is then a good solution, as illustrated in Fig 6.4.1. When the enzyme D-aminoacid oxidase is used alone (a), the reaction mainly produces undesired phenylacetic acid, but when used

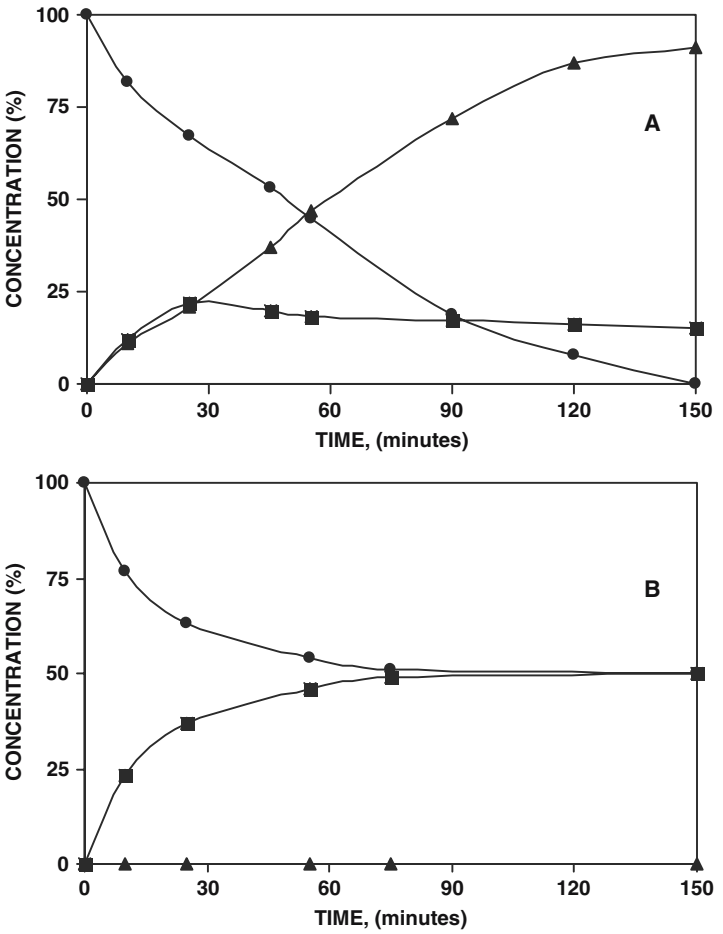

Fig. 6.4.1 Deamination of D-phenylalanine by immobilized DAAO. (•): phenylalanine; (■): phenylpyruvic acid; (▲): phenylacetic acid

together (b) with catalase, the rapid elimination of hydrogen peroxide allows the accumulation of pyruvic acid.

When two enzymes are used simultaneously, the enzymes may be immobilized separately or co-immobilized. In general, the use of individually immobilized enzymes is advantageous, because it allows using and optimizing specific immobilization techniques for each enzyme (i.e. the oxidase and the catalase) and if one enzyme becomes inactivated, only that enzyme needs to be replenished. However, co-immobilization may bring some kinetic benefits; if the substrate to be converted produces enzyme inactivation or modification of the other substrate, co-immobilization seems to be the optimal solution to prevent it (to prevent, in this case, the exposure of the oxidase to a high concentration of hydrogen peroxide). A recently reported example of this is the production of alpha-keto-acids by D-aminoacid oxidase and catalase. In this case, hydrogen peroxide produced the oxidative decarboxylation of the keto-acid when it was not readily destroyed, and the use of catalase and oxidase immobilized in different particles did not allow to completely avoiding the destruction of the desired product, even using large excess of catalase. However, using co-immobilized biocatalysts, this could be easily achieved, permitting not only to produce keto-acids, but also opening a new route for the production of 7ACA free of hydrogen peroxide when using co-immobilized catalase and D-aminoacid oxidase and glutaryl oxidase (López-Gallego et al. 2005).

6.4.2 Redox Biotransformations Catalyzed by Dehydrogenases

Dehydrogenases are extremely useful enzymes in organic chemistry. They can be divided into different groups according to the substrates used (Hummel 1999).

The first group is represented by the alcohol dehydrogenases (ADHs) that can be used for the synthesis of chiral alcohols. There are several commercially available ADHs isolated from yeast or horse liver (NADH dependent), or *T. brockii* (NADPH-dependent) that can be used for different types of substrates. *Lactobacillus kefir* produces an (*R*)-ADH that accepts a broad variety of ketone substrates (ring halogenated, aliphatic, open-chain ketones, 2- and 3-ketoesters, and cyclic ketones), producing, for example, enantiomerically pure *R*-1-(2-pyridyl ethanol), *R*-(1-trimerhylsylyl)-1-butyn-3-ol or *S*-phenylbutan-2-ol (Hummel 1990).

The second group is represented by the hydroxy-acid dehydrogenases. They are useful catalysts for obtaining chiral hydroxy acids from keto-acids. Commercially available lactate dehydrogenases (LDHs) are well-known representatives of this group. There are Land D-LDH available: L-LDH from several animal tissues and *Bacillus stearothermophilus*, and D-LDH from *Leuconostoc mesenteroides*. Theses enzymes exhibit a high specific activity and produce some enantiomerically pure compounds; for example, epoxyacrylic acid can be synthesized from (*S*)-chlorolactic acid, with L-LDH (Kim and Whitesides 1988). However, these enzymes are also highly specific, accepting only pyruvate, 2-oxobutyrate and some pyruvate derivatives as substrates. Other examples of this group are the D-hydroxyisocaproate

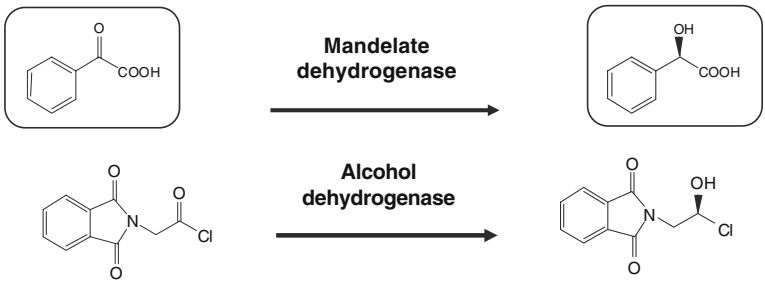

Fig. 6.4.2 Asymmetric reductions catalyzed by mandelate dehydrogenase and alcohol dehidrogenase

dehydrogenases and D-mandelate dehydrogenases, having very high specific activities and enantioselectivities (Hummel et al. 1985; Vasic-Racki et al. 1989). They have been used to convert a broad variety of 2-oxo acids (e.g. phenylpyruvate to produce D-mandelate).

Finally, amino acid dehydrogenases have been used to perform the reductive amination of 2-keto acids into L-amino acids (Bommarius et al. 1994).

Two examples of very interesting asymmetric reductions catalyzed by dehydrogenases are represented in Fig 6.4.2.

Dehydrogenases catalyze very interesting redox reactions by utilizing complex coenzymes as reducing or oxidizing reagents, mainly NAD^+, $NADP^+$, NADH or NADPH whose chemical structures are in Fig. 6.4.3.

In this way, to obtain a high substrate conversion, it is necessary to use a large excess of reduced coenzyme (Liu and Wang 2007). The requirement of coenzymes is a serious drawback for the implementation of a chemical processes catalyzed by dehydrogenases. However, these coenzymes are almost universally used by all living beings that employ them to accumulate reducing power. For this reason the same coenzymes are utilized by many different enzymes and that may permit to find a solution to perform redox processes catalyzed by dehydrogenases without the requirement of stoichiometric quantities of coenzymes. In fact, there are many dehydrogenases that utilize a given coenzyme to oxidize or reduce cheap substrates. These dehydrogenases may be used as auxiliary redox enzymes of high selectivity so that using a low concentration of coenzyme and a high concentration of the substrate of the auxiliary enzyme (in the redox suitable form), it will be possible to design oxidation or reduction processes using two enzymatic catalysts as shown in Fig. 6.4.4. Auxiliary substrate (B^{red} in the scheme) should be cheap and inert with respect to E_1. Reduction of the oxidized coenzyme can be conveniently performed by the enzyme formate dehydrogenase ($HCOOH + NAD(P)^+ \rightarrow CO_2 + NAD(P)H + H^+$) which is readily available uses a cheap co-substrate (formic acid) and produces CO_2 as by-product which is volatile and helps to displace the equilibrium of the main reaction in the right direction (reduction) even without a large excess of formate (Tishkov and Popov 2004). Glutamate dehydrogenase is another enzyme used for coenzyme regeneration; this enzyme may be used in both, reduction (D-glutamate as substrate) and oxidation reactions (α-keto glutarate and ammonium as substrates). ADHs may

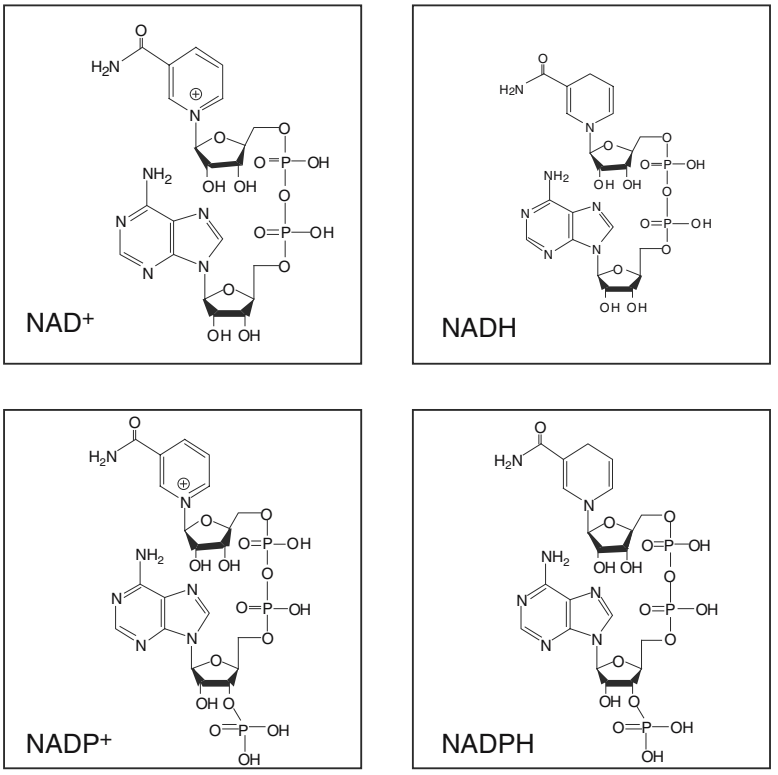

Fig. 6.4.3 Chemical structure of main coenzymes of dehidrogenases

also be used for coenzyme regeneration (Chenault et al. 1988; van der Donk and Zhao 2003); these enzymes may be used for reductions (using isopropanol) or oxidations (using acetone) reactions.

It is very important to find active and stable auxiliary enzymes in a broad range of conditions (e.g. from thermophilic microorganisms). Moreover, it is possible to further increase the enzyme stability by molecular biology techniques (random

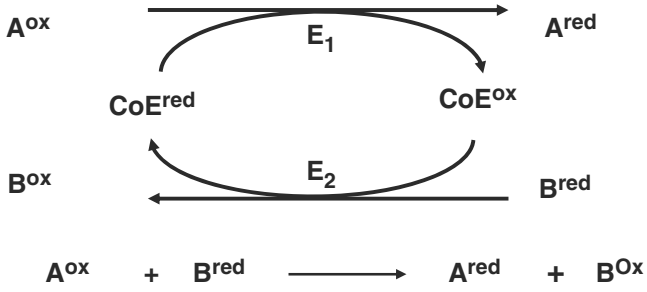

Fig. 6.4.4 Scheme of a coupled reaction catalyzed by dehydrogenases with cofactor regeneration

mutagenesis with selection pressure, site-directed mutagenesis) or physical–chemical techniques (multi-subunits or multi-point immobilizations). In that way, it is possible to find very active and stable immobilized catalysts of auxiliary enzymes under a wide range of experimental conditions (broad range of pH and temperatures, presence of cosolvents).

Thermophilic microorganisms (moderate or extreme) may be a good source of industrial enzymes. Dehydrogenases are key metabolic enzymes in all living beings so that the search of dehydrogenases from thermophilic microorganisms is an interesting objective in organic synthesis. These enzymes should be very stable at pH 7.0, but there is an increasing interest in obtaining enzymes stable and active at alkaline and acidic pH values, where the coenzymes are more stable and perhaps substrates may be more soluble or stable. Moreover, the selectivity and activity should be maintained when using non-natural substrates, that may be the most interesting from the point of view of the production of high added value products.

6.4.3 Immobilization-Stabilization of Dehydrogenases

Dehydrogenases are usually multimeric enzymes. Thus, under certain conditions even thermophilic dehydrogenases may be easily inactivated by subunit dissociation, being these enzymes an excellent target for the development of stabilization strategies by immobilization and post-immobilization modification techniques as shown in Fig. 6.4.5.

As an example, the dehydrogenase from *Thermus thermophilus* was highly stabilized, as seen in Fig. 6.4.6.

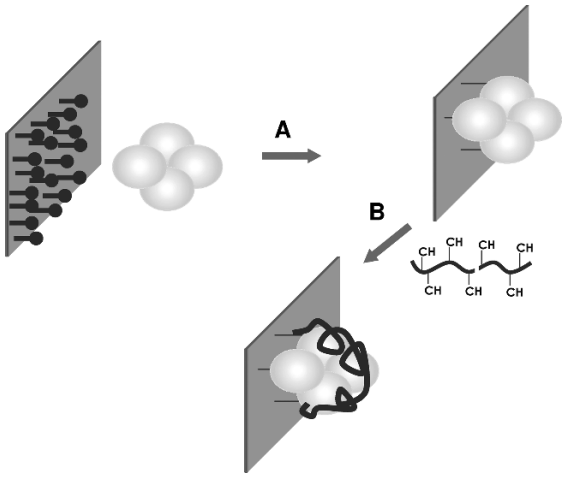

Fig. 6.4.5 Stabilization of multimeric enzymes against dissociation. A: multipoint covalent attachment of multisubunit enzyme; B: stabilization by polyaldehyde cross-linking

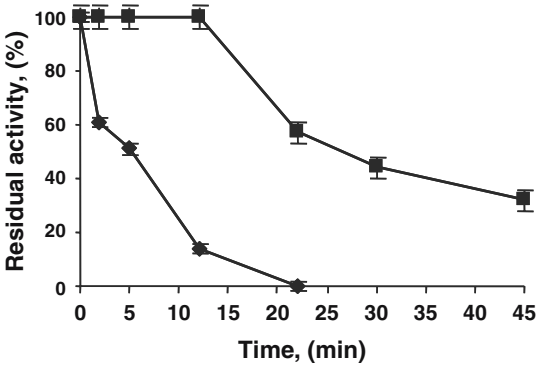

Fig. 6.4.6 Inactivation of a dehydrogenase from *Thermus thermophilus* at pH 7 and 83 °C. (♦): soluble enzyme; (■): immobilized-stabilized enzyme

It is also possible to distort the three-dimensional structure of the enzymes during the multi-point covalent immobilization step with the purpose of altering (improving) its selectivity towards non-natural substrates.

6.4.4 Reactor Engineering

Even having very active and stable immobilized preparations of the main dehydrogenase and the auxiliary enzyme used for coenzyme regeneration, a careful design of the reactor is needed to retain and reuse both enzymes and the coenzyme. The enzymes may be immobilized in solid supports, and in that way may be easily reused. However, during the design of the reactor it is necessary to pay special attention to the reuse of the coenzyme, which is a small and soluble molecule that can leave the reactor together with substrates and products (Liu and Wang 2007). There are several options to solve this problem.

If an ultrafiltration reactor is used, whose membranes only allow the passage of low molecular weight substances, the enzymes may be immobilized in macroporous supports and the coenzyme attached to a high molecular weight polymer (dextran, poly-ethyleneglycol, ...), so that enzymes and coenzymes may be used for many reaction cycles. The main and auxiliary dehydrogenases can be each immobilized inside a porous support. These immobilized enzymes will not interact with detrimental gas bubbles or organic solvent drops and will not aggregate. Their multipoint and multi-subunit covalent immobilization will also most certainly increase its stability against other inactivating agents like extreme pHs, high temperature and high concentration of organic cosolvents. The coenzymes (now polymeric) will be trapped within the reactor. For example, in a reduction reaction, the coenzyme will be oxidized by the main enzyme and then reduced by the auxiliary enzyme so closing the catalytic cycle. This kind of reactors have been already used for soluble enzymes, that are also retained by the membrane (Liu and Wang 2007), but the proposal of using immobilized-stabilized enzymes represents a significant improvement in reactor engineering.

Some future perspectives in biocatalysis with dehydrogenases include the immobilization of enzymes and cofactors into non-porous magnetic nano-particles (El-Zahab et al. 2008) where both enzymes can interact with the coenzyme and, at the end of the reaction, the particles containing the enzyme and the derivatized coenzyme can be retained with a magnet, while recovering the product. An alternative is the co-immobilization of both enzymes and the polymeric coenzyme onto a porous support. The co-immobilization of the main and the auxiliary enzymes, stabilizing both dehydrogenases by multipoint and multi-subunit immobilization, and the further co-immobilization of an excess of the polymeric coenzyme will permit the coenzyme molecules to interact with the active site of both dehydrogenases. In that way, conventional reactors could be used.

The technological potential of dehydrogenases in biocatalysis very much relies upon having active and stable enzymes (both the main and the auxiliary) and coenzymes, good regio and enantioselectivity, if required, and adequate solubility of substrates in the reaction medium. These requirements are not easy to fulfill, so that there is still a long way to go to have a significant technological impact. However, the impressive advances both in biocatalyst and medium engineering allow being optimistic about the implementation of redox enzymatic processes at industrial level in the near future.

6.4.5 Production of Long-Chain Fatty Acids with Dehydrogenases

To illustrate some of the challenges of using dehydrogenases in organic synthesis, the oxidation of polycosanols will be shortly reviewed.

As already mentioned in section 6.3, distillation of pitch (heavy fraction in tall-oil distillate) produces long-chain aliphatic alcohols (polycosanols) that can be recovered and fractionated to obtain mainly docosanol and tetracosanol. These alcohols can be oxidized to the corresponding long-chain fatty acids which have very interesting potentials as personal care (Nielsen et al. 2005) and pharmaceutical products (Yoshiro 2001; Clymer 2006). Chemical oxidation has been studied in depth using hydrogen peroxide as oxidant and different catalysts, like quaternary ammonium peroxotungstophosphate (Bi et al. 2001); as for now it is the technology of choice. The alternative oxidation by biocatalysis offers the usual potential benefits of higher specificity and milder operation conditions. However, the biocatalytic route is plagued with difficulties that we are currently addressing.

Long chain alcohol dehydrogenases (EC 1.1.1.192) have been described (Brenda Enzyme Database) that can oxidize long-chain aliphatic alcohols (up to 18 carbon atoms in length) to the corresponding long-chain fatty acids requiring two moles of oxidized coenzyme (usually NAD^+) per mole of acid produced. Several cellular systems have been reported that exhibit such activity (Hirakawa et al. 2004) but the enzyme is intracellular, complex and unstable and from the many sources, very few are of technological value. At present, the only readily available dehydrogenases that have been reported active on aliphatic alcohols of more than 16 carbon atoms

are from *Candida tropicalis* and *Candida lypolytica*. We have screened most of the available dehydrogenases and found that few of them, including the most used horse liver ADH, exhibit activity on docosanol (22 carbon atoms) and tetracosanol (24 carbon atoms) even though at a much lower level than on small chain aliphatic alcohols. We have succeeded in immobilizing that enzyme with good yield and increased stability. Another problem that has to be tackled is the low solubility of substrates, so that thermophilic organisms are being screened to obtain thermostable dehydrogenases that allow conducting the process at higher temperatures where the solubility of substrates is greatly enhanced. Should reasonably active and stable long chain alcohol dehydrogenases be obtained, a quite appealing process can be developed since, as opposite to most redox reactions of interest (i.e. production of enantiopure chiral intermediates by reduction of pro-chiral substrates), in this case the coenzyme is obtained in reduced form, so that their available electron pairs can be used in a high added value auxiliary reaction to close the catalytic cycle.

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6.5 Use of Aldolases for Asymmetric Synthesis

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Aldolases are a group of C−C bond forming enzymes with widespread applications. The stereoselective aldol addition reaction catalyzed by aldolases represents an attractive alternative to conventional chiral organic chemistry methods for chemical and pharmaceutical industries. Aldolases are classified according to both their proposed catalytic mechanism and the structure of the donor substrate, their sources and microbial production processes being presented in this chapter. To design appropriate bioreactors for aldol synthesis, the characteristics of aldolase biocatalysts obtained after purification procedures in free and immobilized form are discussed,

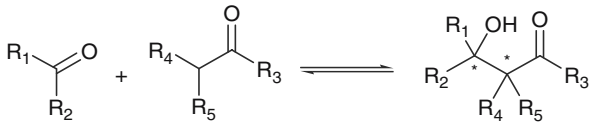
followed by the catalytic mechanism of action, kinetic behavior of aldolase-catalyzed reactions and medium reaction engineering. Finally, the synthetic applications of the different aldolases leading to products of interest for the chemical and pharmaceutical industry, is described in detail.

6.5.1 Aldolases: Definitions and Classification

Carbon–carbon bond formation is one of the most important reactions of organic synthetic chemistry (Wasserman et al. 1999). It is a strategic reaction in the synthesis of enantiopure molecules such as complex carbohydrates, sugar derivatives and analogues and other polyhydroxylated products. Among carbon–carbon coupling methodologies, the aldol reaction is one of the simplest and most powerful strategies (Mahrwald 2004). In this reaction, streoselective catalytic methods that enable a precise control over the stereochemistry of the newly formed stereogenic centers are of paramount importance and one of its most important and challenging goals. Efforts towards this goal have been recently reported from two approaches considering chiral chemical catalysts, such as Lewis acids and bases, and biochemical catalysts, such as aldolases (Machajewski and Wong 2000). Both methods have advantages and drawbacks; however, according to the scope of the present book the biochemical methods using aldolases will be explored in detail.

Aldolases are a specific group of lyases (aldehyde-lyases EC 4.1.2) catalyzing the reversible stereoselective addition of a donor compound (nucleophile) to an acceptor compound (electrophile).

The scheme of the general reaction catalyzed by aldolases is:



It involves the attack by the deprotonated α-carbon atom of an aldehyde or ketone on the carbonyl atom of another aldehyde or ketone, resulting in a β-hydroxyaldehyde or a β-hydroxyketone. While the donor compound for aldolases is usually invariable, the acceptor may vary, allowing the use of these biocatalysts in asymmetric synthesis (Takayama et al. 1997; Samland and Sprenger 2006).

Aldolases are usually classified according to their catalytic mechanism as:

Class I aldolases: From a mechanistic point of view, class I aldolases form a Schiff base intermediate with the substrate at the active site.

Class II aldolases: These enzymes use a divalent metal cofactor (Zn^{2+}), acting as a Lewis acid in the active site. The catalysis proceeds via the formation of an enediolate intermediate. These enzymes are often more stable than class I aldolases.

Aldolases can also be classified according to the structure of the donor substrate (see Fig. 6.5.1).

a) DHAP-dependent aldolases
The four enzymes of the family of dihydroxyacetone phosphate (DHAP)-dependent aldolases: fructose-1,6-bisphosphate aldolase (FruA, EC 4.1.2.13), fuculose-1-phosphate aldolase (FucA, EC 4.1.2.17), rhamnulose-1-phosphate aldolase (RhuA, EC 4.1.2.19) and tagatose-1,6-bisphosphate aldolase (TagA, EC 4.1.2.40), catalyze in vivo the reversible asymmetric addition of DHAP to D-glyceraldehyde-3-phosphate (G3P) or L-lactaldehyde, leading to four complementary diastereomers. DHAP-dependent aldolases create two new stereogenic centers, with excellent enantio and diastereoselectivity in many cases. These enzymes are quite specific for the donor substrate DHAP, but accept a wide range of aldehydes as acceptor substrates. There are only two fructose-6-phosphate aldolase isoenzymes reported to be able to use dihydroxyacetone (DHA) as donor substrate (Schürmann and Sprenger 2001).
b) Pyruvate and phosphoenolpyruvate-dependent aldolases
These aldolases catalyze the aldol addition between pyruvate or phosphoenolpyruvate (PEP) and different aldehydes giving products with a new stereogenic center.
c) 2-Deoxy-D-ribose 5-phosphate aldolase (DERA)
It is the only known member of the group of acetaldehyde-dependent aldolases. In vivo, DERA catalyzes the reversible aldol reaction of acetaldehyde and G3P. The donor substrate specificity of this enzyme is not as strict as with the other aldolases.
d) Glycine-dependent aldolases
Glycine-dependent aldolases are enzymes using pyridoxal-5-phosphate (PLP) as cofactor and catalyze the aldol addition of glycine with different acceptor aldehydes, rendering β-hydroxy-α-aminoacids with two new stereogenic centers (at carbons α and β). There are two types of enzymes: threonine aldolases (TA), catalyzing in vivo the reaction between threonine and glycine and serine hydroxymethyltransferases (SHMT), catalyzing in vivo the reaction between serine and glycine.

6.5.2 Preparation of Aldolase Biocatalysts

6.5.2.1 Sources and Production

Most organisms contain aldolases and more than 30 of such enzymes are known already. Class I aldolases occur mainly in higher eukaryotes. Nevertheless, there is a variety of class I enzymes found in prokaryotes. On the other hand, class II aldolases are present in prokariotes and lower eukariotes.

DHAP-dependent aldolases constitute the most important group concerning biocatalytic applications. Until now, class I fructose-1,6-bisphosphate aldolase from

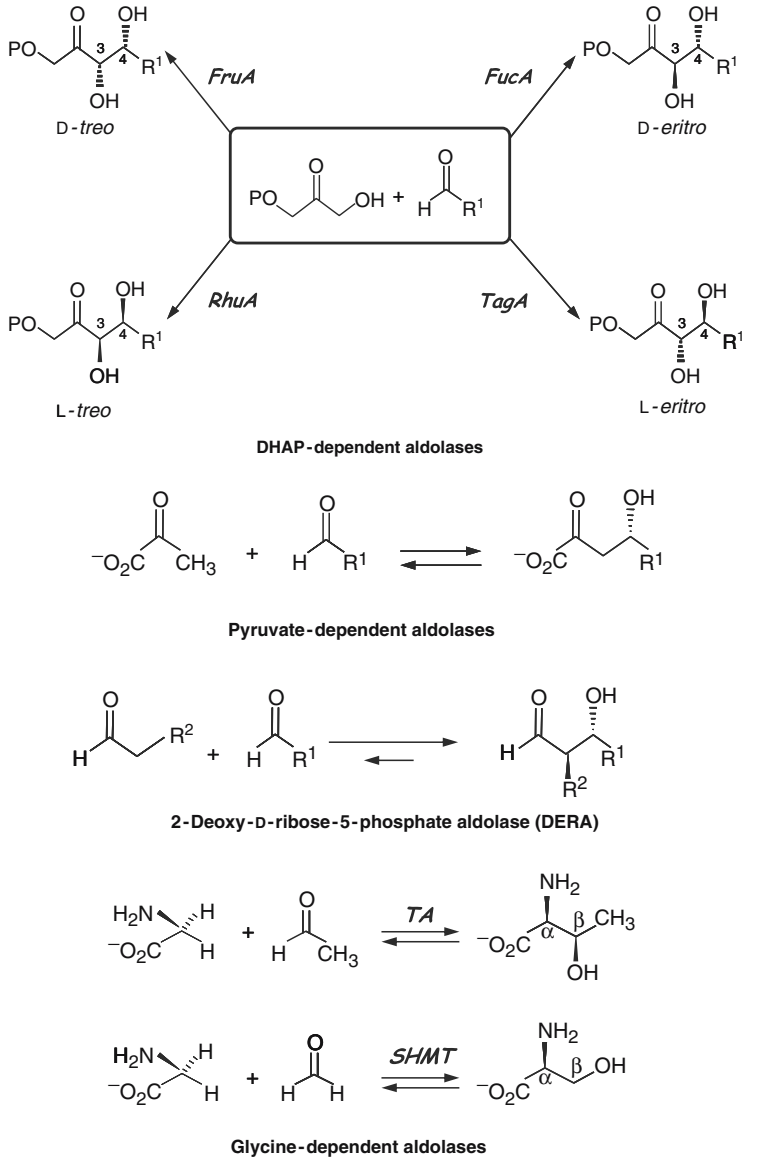


Fig. 6.5.1 Aldolases classified according to the donor substrate

rabbit muscle (RAMA) is the most extensively studied aldolase because of its commercial availability.

The four DHAP-dependent aldolases have been obtained from microbial and animal sources, determined their sequence, and cloned and overexpressed in *Escherichia coli* (Fessner et al. 1991; Henderson et al. 1994; Garcia-Junceda et al. 1995). DERA has also been found in microorganisms and cloned in *E. coli* (Chen

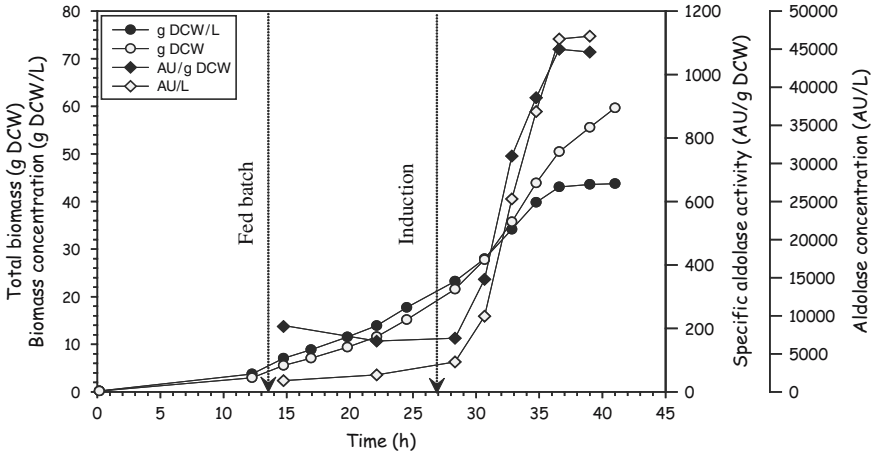


Fig. 6.5.2 Biomass and aldolase concentration profiles for the fed-batch production of FucA

et al. 1992). Aldolases which use pyruvate or PEP are almost exclusively from microbial origin. Concerning the threonine-aldolases group, there are six subclasses defined by different stereochemistry of the chiral carbons α and β (see Fig. 6.5.1). Different enzymes belonging to four of these subclasses have been identified and purified, but only some of them have been cloned and overexpressed. The two remaining subclasses have not been found in nature already (Liu et al. 2000a).

Recombinant protein production processes have been developed to get aldolases available for synthetic purposes. Thus, DHAP-dependent aldolases are being produced by recombinant *E. coli* strains in different expression systems (Durany et al. 2005; Vidal et al. 2005a). High cell density cultures, allowing high productivities, can be obtained by employing fed-batch growth strategies. In a typical process, as presented in Fig. 6.5.2, an IPTG (isopropylthiogalactoside) inducible expression system is employed. A fed-batch culture at constant specific growth rate (μ) is performed by employing either a predetermined exponential feed addition profile or a feedback growth control method based on macroscopic mass balances and exhaust gas analysis (Pinsach et al. 2006). After pulse induction, the intracellular production of the recombinant aldolase follows a growth associated pattern. When the expression of the foreign protein reaches a pre-defined level (between 30% and 50% of the total intracellular protein content), specific growth rate decreases and finally the growth stops due to metabolic burden. The same strategy has been applied for efficient production of DERA and glycine-dependent aldolases, employing improved expression systems (Vidal et al. 2005b).

After recovery of biomass by centrifugation, aldolase-containing cell lysates are obtained by mechanical or ultrasound cell disruption. A purification procedure is usually required to prepare a pure catalyst for synthetic purposes and to avoid the presence of any enzyme producing undesirable side-reactions. Usually, aldolases are purified from cell lysates by a combination of conventional purification steps: gel filtration, ammonium sulphate precipitation, ionic exchange chromatography,

hydrophobic interaction chromatography, heat treatment (Sauve and Sygusch 2001; Ramsaywak et al. 2004). However, this is a time consuming process and purification yields dramatically decrease when the number of purification steps is increased (see section 2.2.3).

Alternative purification strategies have been developed to overcome these problems. Thus, differential dye-ligand chromatography was used for purifying 2-keto-3-deoxy-6-phosphogluconate aldolase from different sources (Shelton and Toone 1995). When aldolases are produced as histidine tagged recombinant proteins, one-step purification can be performed by IMAC (immobilized metal-chelate affinity chromatography) and high yields are obtained. Thus, different DHAP-dependent (Garcia-Junceda et al. 1995; Ardao et al. 2006) and glycine-dependent aldolases (Vidal et al. 2005b) have been purified employing this method. However, when class II aldolases, which have a divalent metal cofactor, are purified by IMAC, a metal exchange between aldolase and the affinity matrix may take place. This metal exchange has been reported for purification of FucA from *E. coli* by IMAC, being the purification yields depending on the nature of the metal bound to the affinity matrix (Ardao et al. 2006). This fact is due to the dependence of metal-enzymes activity on the nature of the cofactor. For instance, FucA from *E. coli* shows higher activity when Zn^{2+} in the wild enzyme is replaced by Co^{2+} (Dreyer and Shulz 1996).

6.5.2.2 Immobilization of Aldolases

Enzyme immobilization allows a wider use of enzymes in fine chemistry because it facilitates catalyst reuse and downstream processing of the product and, sometimes, it improves enzyme stability. In spite that enzyme immobilization techniques have been used widely during the last 30 years, very few information can be found about aldolases immobilization.

Some results indicate that different attempts of FucA immobilization by covalent attachment provoked severe enzyme inactivation (Fessner and Walter 1997). FucA and DERA from *E. coli* and SHMT from *Streptococcus thermophilus* have been immobilized by multipoint covalent attachment to glyoxyl-agarose. Although this immobilization method had been very successful with many different enzymes (Guisán et al. 1993), results obtained with these aldolases were dissimilar. For FucA, in spite of an immobilization yield of 80–90%, enzyme inactivation occurred during immobilization process and only 10–20% of activity was retained (Suau et al. 2005). On the other hand, SHMT immobilization yield was 100%, but the immobilized activity was lost during the sodium borohydride reduction step, probably due to the reduction of the Schiff base established between the cofactor (pyridoxal phosphate) and the aldolase. Finally, 100% of immobilization yield and 65% of retained activity in the immobilized derivative was achieved with DERA.

On the other hand, recombinant FucA from *E. coli* expressed as a fusion protein with a hexa-histidine tag has been immobilized by using metal-chelate supports (Ardao et al. 2006). IMAC supports allowed FucA purification and immobilization in one step in order to obtain an immobilized FucA catalyst for aldol

addition reactions. The best results were obtained with high density supports containing Co^{2+}. The immobilization yield was 100% and the immobilized derivative retained 63% of activity. The activity retained on immobilized FucA is dependent on the metal nature of these metal-chelate supports, due to the already mentioned metal exchange between the enzyme and the affinity matrix. Class II RhuA from *E. coli* showed a similar behaviour when it was immobilized by using metal-chelate supports.

6.5.3 Reaction Performance: Medium Engineering and Kinetics

6.5.3.1 Mechanism and Kinetics of Aldol Addition Reactions

As pointed out before, the mechanism of aldolase-catalyzed aldol addition is different for class I and class II enzymes.

For class II DHAP-dependent aldolases, an ordered two-substrate mechanism has been derived from structural studies (Dreyer and Shulz 1996). DHAP binds the zinc ion with its hydroxyl and keto oxygen atoms, conducting to an enediolate intermediate, before the entrance of the acceptor aldehyde. For instance, FucA and RhuA are homotetramers with a Zn^{2+} atom in each subunit and the formed enediolate is linked to the carbonyl group of the acceptor aldehyde by either the *si* face (in the case of FucA) or the *re* face (in the case of RhuA). Thus, aldol addition products with 3R, 4R configuration are obtained in the case of FucA, and 3R, 4S configuration in the case of RhuA (Fig. 6.5.3).

As mentioned before, these enzymes are strictly dependent of DHAP as donor substrate, and DHAP is usually unstable at the reaction conditions (Fessner and Walter 1997). In aqueous medium, DHAP suffers decomposition and isomerization reactions as shown in Fig. 6.5.4.

The reactions proceed through the formation of an enediolate intermediate and it has been demonstrated that there are only two final reaction products: inorganic phosphate and a small amount of methylglyoxal due to its polymerization (Richard 1993). The irreversible DHAP decomposition has been shown to be of first order in a pH range between 7 and 9 (Phillips and Thornalley 1993), and becomes almost negligible for temperatures lower than 4 °C. On the other hand, as the decomposition

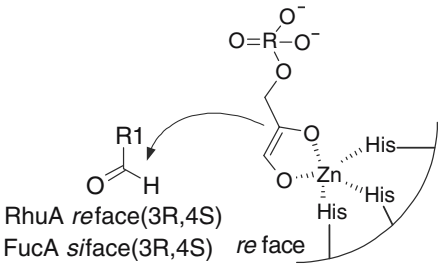


Fig. 6.5.3 Mechanism of type II aldolases illustrated for FucA and RhuA

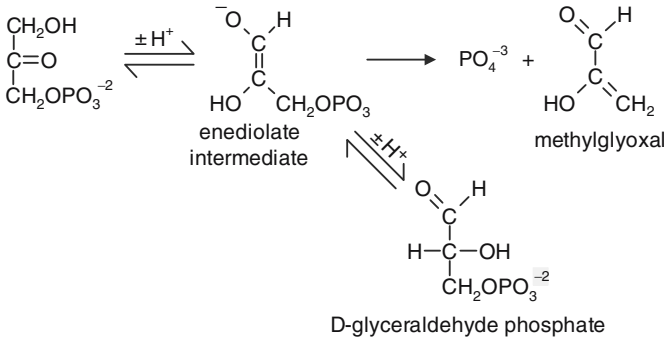


Fig. 6.5.4 DHAP isomerization and decomposition reactions

involves the same enediolate intermediate than the aldol addition, the presence of class II aldolase enzymes also catalyzes the non-desired DHAP degradation (Suau et al. 2006). As presented in Fig. 6.5.5, DHAP remained stable in the presence of type I FruA (RAMA) at 4 °C, but its concentration decreased with time in the presence of class II FruA (from *E. coli*).

Thus, for an aldol addition reaction, the significance of non-synthetic DHAP decomposition has to be taken into account. In order to improve the yield and selectivity, different approaches have been employed: a) temperature reduction, exploiting the different temperature dependence of the reaction rates as a consequence of the different magnitudes of their energies of activation b) use of a high aldehyde excess to drive the process toward synthesis.

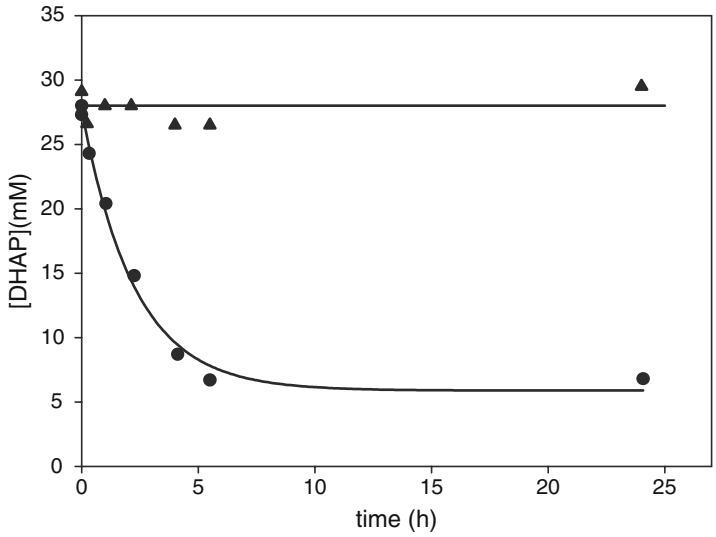
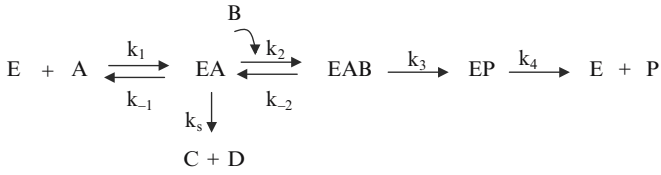


Fig. 6.5.5 Enzymatic DHAP consumption in the presence of type I (▲) and type II (•) FruA

Although the optimum temperature for aldolases ranges from 20 to 40 °C, a reduction to 4 °C eliminates the chemical DHAP decomposition and reduces the enzymatic DHAP degradation rate in a higher degree than the aldol addition, improving yields and selectivity. On the other hand, the operation of a reactor with discrete DHAP additions produces a significant improvement due to the combination of large aldehyde excess and low temperature. For instance, under optimized operation conditions, a final yield of 96% was reported for the FucA-catalyzed aldol addition between (*S*)-Cbz-alaninal and DHAP (Suau et al. 2006).

Concerning reaction kinetics, most of the reported aldolases have been biochemically characterized by determining the Michaelis constant and the maximum reaction rate over natural and non-natural substrates, but there are very few kinetic models describing the behavior of the aldol addition in synthetically interesting applications. For the case of class II fuculose-1-phosphate aldolase, a kinetic model has been developed, involving the desired synthesis reaction and the secondary DHAP enzymatic degradation, according to the following scheme:



where: E: aldolase enzyme, A: DHAP, B: aldehyde, C: methylglyoxal and D: phosphate.

Non-competitive inhibition by the acceptor aldehyde and competitive inhibition by methylglyoxal have been identified on both reactions, conducting to the following rate expressions:

– Rate of synthesis reaction (aldol addition):

$$v = \frac{V \cdot a \cdot b}{[K_M \cdot K_B\,(1 + K_{iC}) + K_B a + K_A b + ab] \cdot (1 + K_{iB})} \tag{6.5.1}$$

– Rate of secondary reaction of enzymatic DHAP degradation:

$$v_s = \frac{V_s a}{[K_M\,(1 + K'_{iC}) + a] \cdot (1 + K_{iB} b)} \tag{6.5.2}$$

where:

$$K_A = \frac{k_3 k_4}{k_1\,(k_3 + k_4)} \qquad K_B = \frac{k_4\,(k_{-2} + k_3)}{k_2\,(k_3 + k_4)}$$

K_{iC} and K'_{iC} are the methylglyoxal competitive inhibition constants for both reactions.

K_{iB} is the aldehyde non-competitive inhibition constant.

The Michaelis constant $K_M = (k_{-1} + k_2)/k_1$ is the same for both reactions, as they proceed via the same intermediate EA.

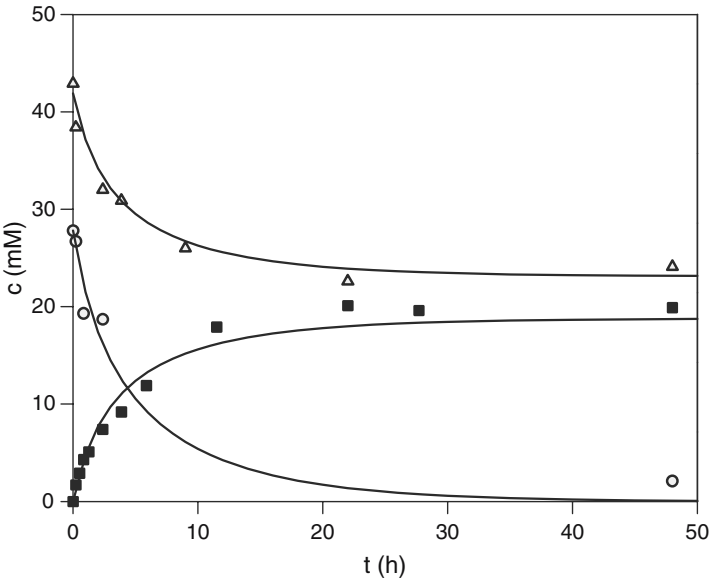


Fig. 6.5.6 Concentration profiles for an aldol addition catalyzed by 12 AU/mL of FucA enzyme. Substrates initial concentration: $[\text{DHAP}]_0 = 27.8\,\text{mM}$ and $[(S)\text{-Cbz-alaninal}]_0 = 42.9\,\text{mM}$. Experimental data: (○) DHAP, (Δ)(*S*)-Cbz-alaninal, (■) Product. Model: continuous line

This kind of kinetic model accurately describes the time course of the reactions, as is presented in Fig. 6.5.6 for the previously mentioned FucA-catalyzed aldol addition between (*S*)-Cbz-alaninal and DHAP in a DMF/water (20:80 v/v) reaction medium.

6.5.3.2 Medium Engineering

In many aldol additions catalyzed by aldolases the solubility characteristics of both donor and acceptor (aldehyde) substrates differ substantially. Whilst the donor is fully soluble in aqueous medium and insoluble in organic solvents, the solubility of the acceptor is generally the reverse. Aqueous-organic cosolvent mixtures, like dimethylformamide/water, are normally used to overcome this problem. Hence, 10–20% v/v cosolvent concentration is usually well tolerated by the enzyme but not enough for substrate solubility (Sobolov et al. 1994; Budde and Khmelnitsky 1999).

To overcome most of solubilization problems, colloidal surfactant systems (e.g. micelles, liquid crystals, microemulsions, vesicles, emulsions, etc.) are attracting a great deal of attention as alternative reaction media (Walde 1996; Holmberg 1997; Antonietti 2001). Their advantages are: they possess micro- and nanostructures consisting of well-defined hydrophilic and lipophilic domains separated by surfactant films with very large interfacial area, the exchange between chemical species

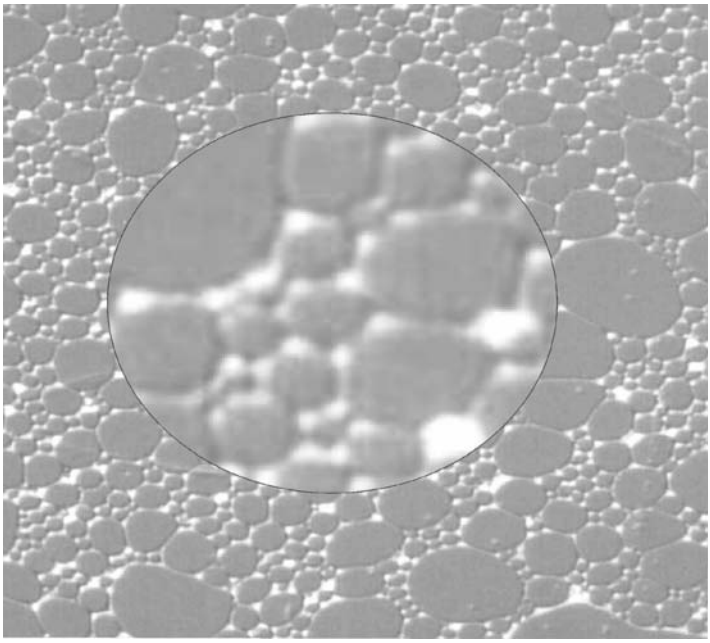


Fig. 6.5.7 Micrograph of a W/O gel emulsion under optical microscope

located in different domains is favored and chemical reactions with higher reaction rates and yields can be achieved, the self-assembled surfactant aggregates and the disperse phase of colloidal systems may act as micro- or nanoreactors where reactants are concentrated and consequently reaction yields can be considerably improved enabling total or partial replacement of organic solvents by aqueous media (Walde 1996; Holmberg 1997; Solans et al. 2001). Among the colloidal systems, highly concentrated emulsions also referred as high-internal-phase-ratio-emulsions (HIPRE) (Princen and Kiss 1986) or gel emulsions (Pons et al. 1993; Kunieda et al. 1994) have attracted much attention as novel reaction media. This type of emulsions are characterized by volume fractions of dispersed phase higher than 0.73, the critical value of close-packed monodispersed spheres (Princen 1979). Consequently, the droplets are deformed and/or polydisperse, separated by a thin film of continuous phase (Fig. 6.5.7). This foam-like structure confers them a viscoelastic rheological behavior, responsible for their gel appearance (Solans et al. 1998).

Water-in-oil (W/O) gel emulsions has been applied for the first time in α-chymotrypsin-catalyzed peptide synthesis (Clapés et al. 2001) and in aldolic condensation of DHAP with acceptor aldehydes such as phenylacetaldehyde and benzyloxyacetaldehyde, catalyzed by D-fructose-1,6-bisphosphate aldolase from rabbit muscle (RAMA). Gel emulsions of the ternary systems such as water/$C_{14}E_4$/oil, where $C_{14}E_4$ is a technical grade poly(oxyethylene) tetradecyl ether surfactant, with an average of four moles of ethylene oxide per surfactant molecule and oil can be

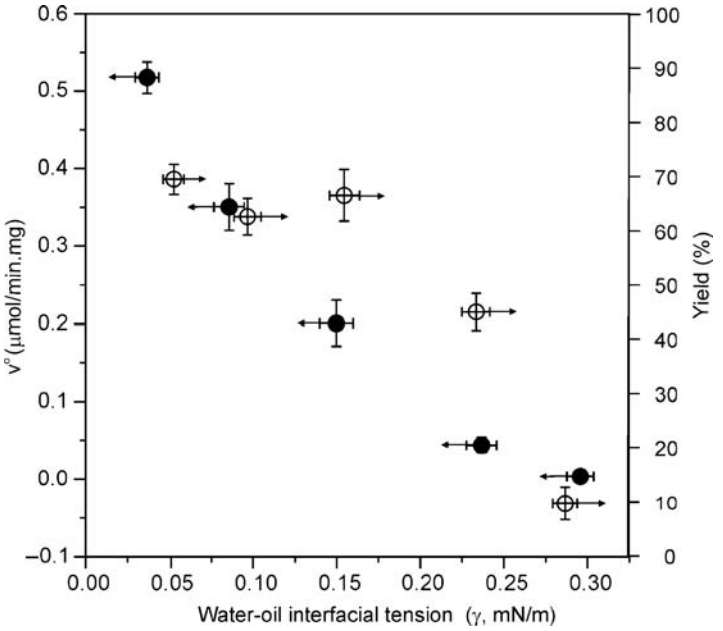


Fig. 6.5.8 Influence of the water–oil interfacial tension (γ) on the equilibrium product yield and initial reaction rate (v^o) for the RAMA-catalyzed aldol addition of DHAP (30 mM) to phenylacetaldehyde (50 mM) in water/$C_{14}E_4$/oil 90/4/6 w/w gel emulsion systems at 25 °C

octane, decane, dodecane, tetradecane, hexadecane or squalane, were typically chosen as reaction media (Clapés et al. 2001; Espelt et al. 2003a).

The first interesting observation was that the stability of RAMA in W/O gel emulsions was improved by 7- and 25-fold compared to that in aqueous medium or conventional dimethylformamide/water 1/4 v/v mixture, respectively. The reported experimental data allows concluding that the equilibrium yields and enzyme activity depends on both the oil/water partition coefficient and the water–oil interfacial tension. In general, the highest enzymatic activities were observed in W/O gel emulsion systems with the lowest water–oil interfacial tension (i.e. attained with the most hydrophobic oil component) (Fig. 6.5.8). Moreover, the highest equilibrium yields were achieved with the lowest values of both the oil/water partition coefficient of the acceptor aldehyde and the water–oil interfacial tension (Figs. 6.5.8 and 6.5.9). Thus, the best aliphatic hydrocarbons to formulate water/$C_{14}E_4$/oil gel emulsions systems for DHAP-dependent aldolases were tetradecane, hexadecane, and squalene where both the interfacial tension and oil/water partition coefficient of the acceptor aldehydes are among the lowest values.

On the light of the experimental data reported and although the precise mechanism of the reaction in W/O gel emulsion media is not known yet, it is likely that the reaction takes place either at the oil/water interface or into the dispersed phase that contains the enzyme and the donor DHAP (Fig. 6.5.10).

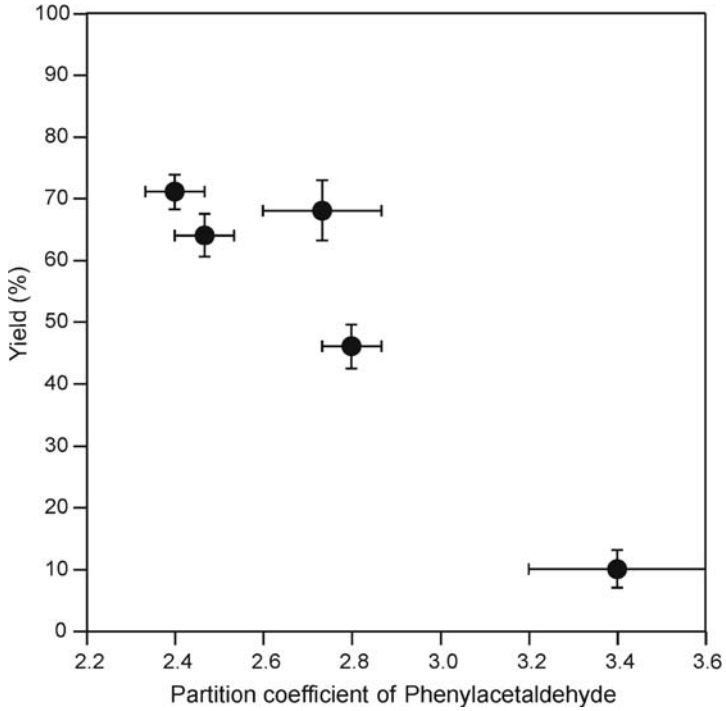


Fig. 6.5.9 Influence of the partition coefficient on the equilibrium product yield for the RAMA-catalyzed aldol addition of DHAP (30 mM) to phenylacetaldehyde (50 mM) in water/$C_{14}E_4$/oil 90/4/6 w/w gel emulsion systems at 25 °C

These systems were tested in the enzymatic aldolization of a variety of *N*-Cbz-aminoaldehydes catalyzed by D-fructose-1,6-bisphosphate aldolase from rabbit muscle (RAMA) and L-rhamnulose-1-phosphate aldolase and L-fuculose-1-phosphate aldolase from *E. coli* (Espelt et al. 2003 a,b, 2005). The largest differences between conventional DMF/water cosolvent systems and gel emulsions were observed with RAMA catalyst (Fig. 6.5.11).

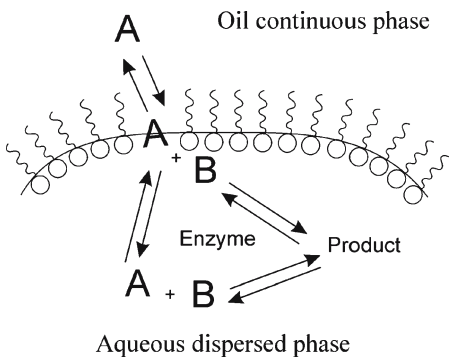


Fig. 6.5.10 Schematic representation of a possible reaction model for aldolase-catalyzed carbon–carbon bond formation in W/O gel emulsions. A: aldehyde acceptor; B: donor

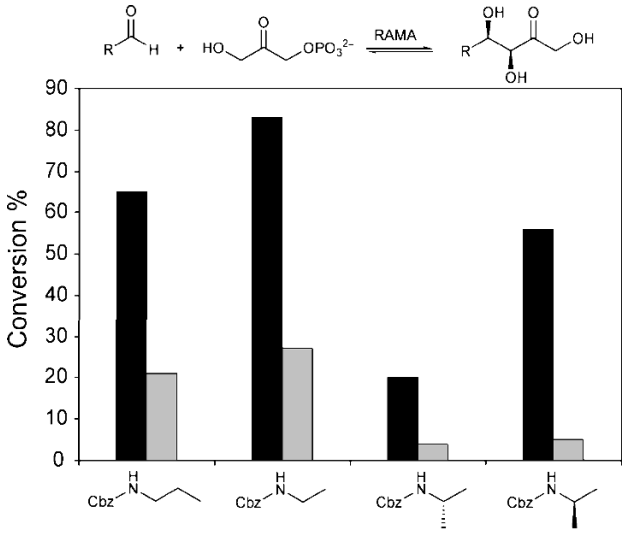


Fig. 6.5.11 Influence of the reaction medium on RAMA-catalyzed aldol additions of DHAP to *N*-Cbz-aminoaldehydes. Molar percentage conversion to product in water/$C_{14}E_4$/hexadecane (i.e. oil) 90/4/6 wt % (black bars) and dimethylformamide/water 1:4 mixture (grey bars)

6.5.4 Synthetic Applications

The main synthetic applications of aldolases are focused on the preparation of chiral polyhydroxylated building blocks in multistep syntheses (Fessner 1998, 2000; Machajewski and Wong 2000; Fessner and Helaine 2001; Whalen and Wong 2006). These intermediates are of paramount importance for the preparation of carbohydrates, complex carbohydrate mimetics and analogs as well as related bioactive compounds (Fessner and Helaine 2001). The configuration of their many stereogenic centers which define their precise biological activity and selectivity is of the utmost importance in all these compounds.

6.5.4.1 DHAP and DHA Aldolases

Dihydroxyacetone phosphate-dependent aldolases (DHAP-aldolases) have been used widely for preparative synthesis of monosaccharides and sugar analogs (Fessner and Walter 1997; Wymer and Toone 2000; Silvestri et al. 2003). Among them, RAMA RhuA and FucA from *E. coli* are the most available aldolases, especially the former which was one of the first to be commercialized (Fessner and Walter 1997; Takayama et al. 1997). In many of the chemo-enzymatic strategies they are involved, the biocatalytic aldol addition to the configuration of the newly stereogenic centers is fixed by the enzyme. However, pertinent examples have been reported in which

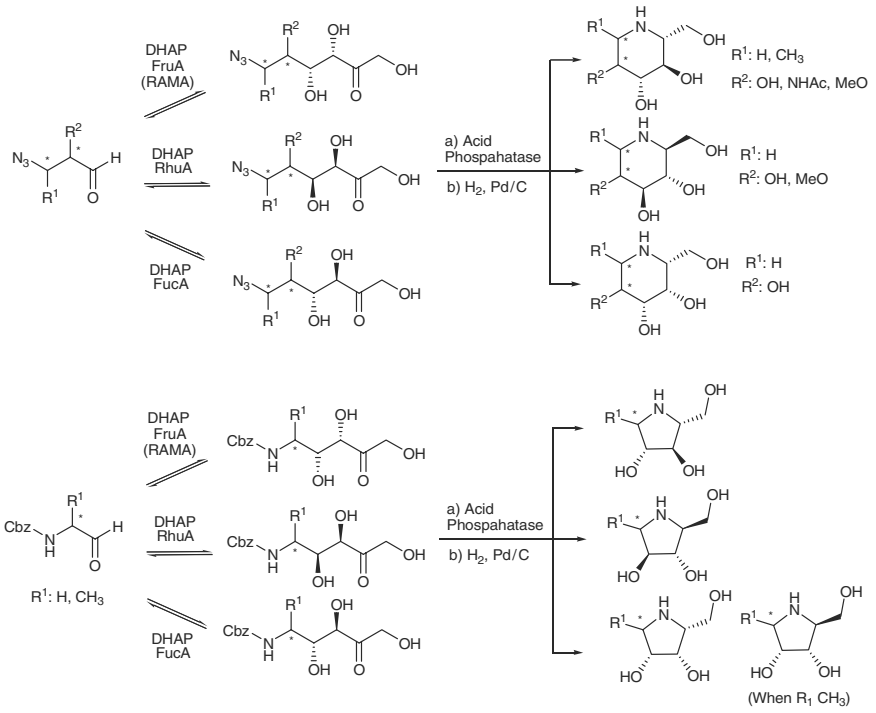


Fig. 6.5.12 DHAP-aldolases-assisted iminociclytol synthesis using azido and *N*-benzyloxycarbonyl (Cbz) aldehydes as key acceptors

the stereoselectivity of the aldolase depended upon the structure of the aldehyde acceptor (Fessner et al. 1991; Espelt et al. 2003b, 2005; Calveras et al. 2006).

DHAP-aldolases are powerful catalyst for the aldol addition of DHAP to azido aldehydes or *N*-protected amino aldehydes to furnish a wide structural, functional and stereochemical diversity of iminosugars (Fig. 6.5.12) (Espelt et al. 2003b, 2005; Whalen and Wong 2006). These compounds are carbohydrate mimics in which the endocyclic oxygen is substituted by nitrogen. Some aminosugars are among the most potent inhibitors of glycoprocessing enzymes (i.e. glycosidases and glycosyltransferases), with a great therapeutic potential for a wide range of diseases such as cancer, HIV, herpes, metabolic disorders and inflammation (Asano et al. 2000). The advantage of using *N*-protected aminoaldehydes over azido aldehydes is that the former can be readily obtained in a great variety of functional and stereochemical diversity.

Azido aldehydes were also utilized for the synthesis of 6-substituted D-fructopyranoside derivatives actives against *Trypanosoma brucei* parasite (Azema et al. 2000). Other interesting synthetic applications of DHAP aldolases are listed below:

– Preparation of 6-*C*-perfluoroalkylated-D-fructoses as surfactants and emulsifiers for biomedical applications (Zhu and Li 2000). RAMA was in this case the

selected aldolase and 6-perfluoroalkyldihydroxyaldehydes the acceptors which were obtained in a catalytic dihydroxylation reaction.
- Synthesis of sugar phosphonate analogs, which have attracted much attention for their potential bioactivity as inhibitors and regulators of metabolic processes (Guanti et al. 2000). These compounds are accessible by enzymatic aldolization of ω-diethylphosphonoylated-β-hydroxyaldehydes.
- Combination of FucA and fuculose isomerase for the synthesis of interesting L-fucose analogs having tails with increased hydrophobicity and reactivity (Fessner et al. 2000). Homologues and unsaturated analogs of L-lactaldehyde were well tolerated by FucA with high diasteromeric excess ($\geq 95\%$).
- Preparation of syringolides, which are molecular elicitors of bacterial plant pathogens that trigger a hypersensitive defense response in some plants. L-Fructose-1,6-phosphate aldolase, RAMA, was applied successfully in the multistep synthesis of the microbial elicitor (–)-syringolide (Chenevert and Dasser 2000). In the key step, *p*-methoxybenzyloxyacetaldehyde was subjected to enzymatic aldolization.

In the preceding examples, the phosphate group of the aldol adduct resulting from the enzymatic reaction must be removed to proceed with the synthetic scheme. This may represent a considerable effort since DHAP must be either chemically prepared or generated in situ by multienzymatic methods (Jung et al. 1994; Fessner and Walter 1997). This is one of the reasons why the DHAP-dependent aldolases have no wide industrial applicability. Efforts to overcome this problem were recently rewarded by the discovery of two novel D-fructose-6-phosphate aldolase isoenzymes (TALC and FSA) from the *E. coli* genome (Schürmann and Sprenger 2001; Schürmann et al. 2002). Both enzymes were found to catalyze reversibly the aldol addition of dihydroxyacetone (DHA) and glyceraldehyde-3-phosphate to furnish D-fructose-6-phosphate (Fig. 6.5.13). Thus, the tremendous advantage of

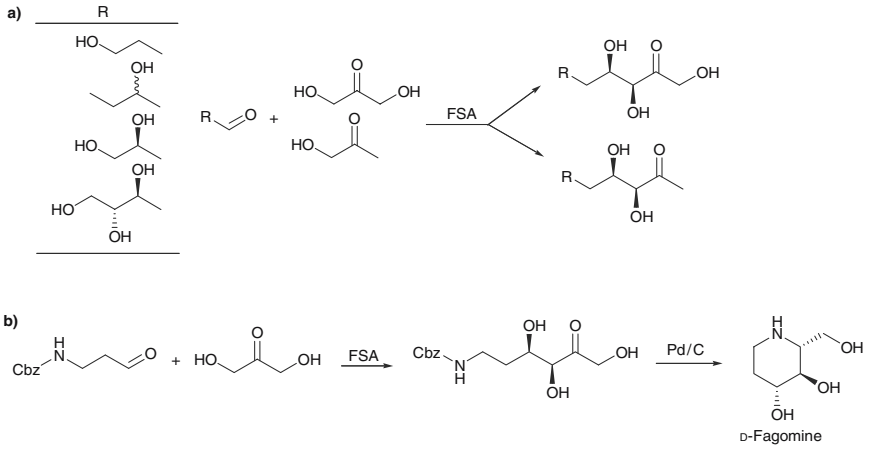


Fig. 6.5.13 d-Fructose-6-phosphate aldolase (FSA) catalyzed aldol additions of DHA to a number of aldehyde acceptors

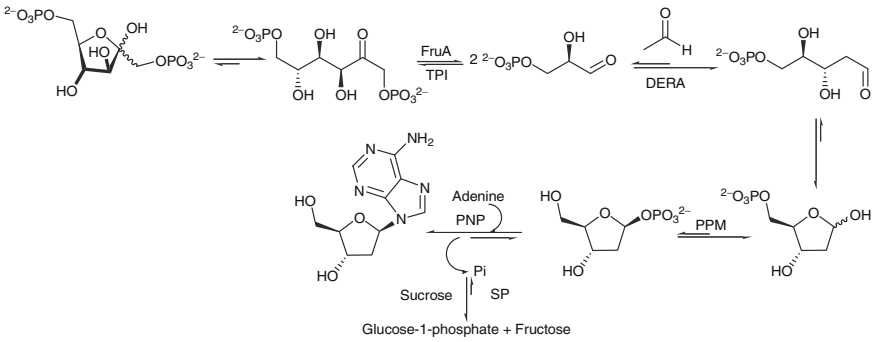


Fig. 6.5.14 Multienzymatic synthesis of deoxyribonucleosides

these aldolases is that they are able to use DHA as donor instead of the expensive and synthetically time consuming dihydroxyacetone phosphate that DHAP-aldolases need. Moreover, hydroxyacetone (acetol) can also be used as a donor with similar rates, expanding the synthetic potential of these aldolases towards the donor nucleophile. Recent examples of the synthetic capabilities of FSA are given in Fig. 6.5.13 (Castillo et al. 2006).

6.5.4.2 2-Deoxy-D-Ribose-5-Phosphate Aldolase (DERA)

An economically viable alternative to the synthesis of deoxyribonuclosides has been developed as a two stage process involving 2-deoxy-D-ribose 5-phosphate aldolase (DERA) (Fig. 6.5.14) (Tischer et al. 2001). The first step was the aldol addition of G3P to acetaldehyde catalyzed by DERA. G3P was generated in situ by a reverse action of FruA on L-fructose-1,6-diphosphate and triose phosphate isomerase which transformed the DHAP released into G3P. In a second stage, the action of pentose-phosphate mutase (PPM) and purine nucleoside phosphorylase (PNP), in the presence of adenine furnished the desired product. The released phosphate was consumed by sucrose phosphorylase (SP) that converts sucrose to fructose-1-phosphate, shifting the unfavorable equilibrium position of the later reaction.

Site-specific mutated 2-deoxyribose-5-phosphate aldolase (DERA) was used as catalyst for the synthesis of the key intermediate useful for the preparation of the cholesterol lowering drug atorvastatin (Lipitor) (De Santis et al. 2003).

6.5.4.3 Pyruvate Dependent Aldolases

N-acetyl neuraminic acid lyase (NAL) catalyzes the reversible aldol addition of *N*-acetylmannosamine to pyruvate to produce *N*-acetylneuraminic acid, a sialic acid. Sialic acid is an essential component of complex carbohydrates which are of paramount importance as recognition signals in a variety of biological process, such as parasite invasion, infectivity and survival of the invading organism in the host. Hence, sialic acids mimetics, such as the commercial Oseltamavir, Relenza and

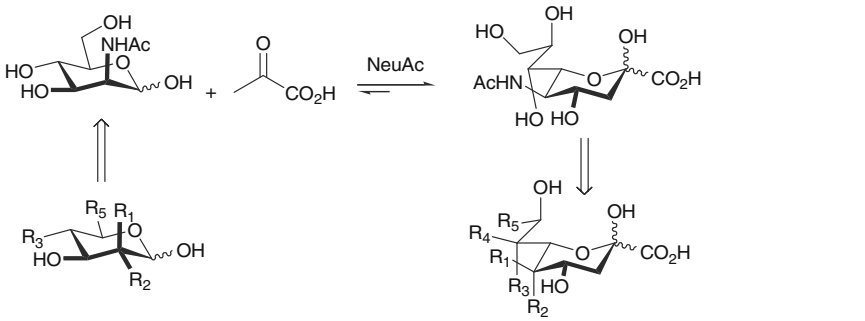


Fig. 6.5.15 Enzymatic synthesis of sialic acid mimetics

Tamiflu (Fig. 6.5.15), are behind potential chemotherapeutics against, for instance, influenza virus (Fessner and Walter 1997; Babu et al. 2000).

The fairly broad tolerance of NAL for stereochemically related *N*-acetylmannosamine allowed the synthesis of a number of natural and unnatural sialic acid derivatives (Fessner and Helaine 2001). Recently, a pair of stereocomplementary *R*- and *S*-stereoselective NAL variants for the synthesis of sialic acid mimetics has been described, using a combination of structure-guided mutagenesis and directed evolution (Williams et al. 2006).

6.5.4.4 Threonine Aldolases

Synthetic applications of threonine aldolase have been hampered due to the poor capacity for erythro/threo discrimination. *Erythro*-selective threonine aldolase from *Candida humicola* has been used for the preparation of a key chiral building block in the synthesis of the immunodepressive lipid mycestericin D (Fig. 6.5.16). The conversion was purposely low to ensure a kinetic control and therefore maximizing the yield of the *erythro* product.

A novel serine hydromethyl transferase (SHMT) with L-threonine aldolase activity has been cloned from *Streptococcus thermophilus* (Vidal et al. 2005b). It was observed that the K_M for L-*allo*-threonine was 38-fold higher than that for L-threonine, suggesting that this enzyme can be classified as *threo*-selective. The novel aldolase was capable of reacting with unnatural aldehydes for the production of β-hydroxy-α-amino acids with moderate stereoselectivity.

Threonine aldolases have been also successfully applied in the resolution of racemates of β-hydroxyamino acids. An interesting example is the use of a D-threonine aldolase from *Alcaligenes xylooxidans* to resolve DL-threo-β-(3,4-methylenedocyphenyl)serine, a synthetic intermediate for parkinsonism drug (Liu et al. 2000b).

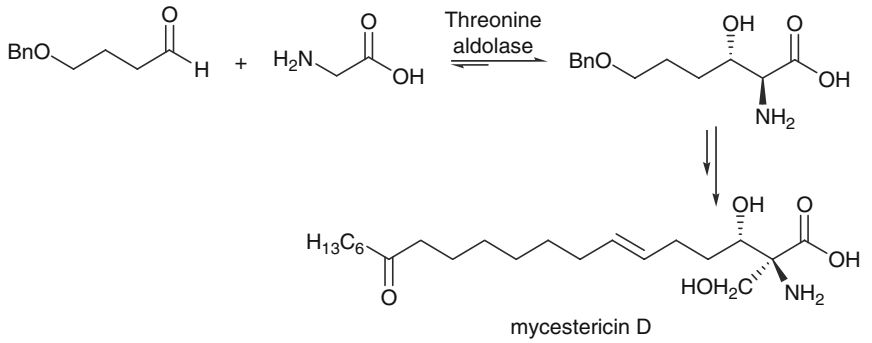


Fig. 6.5.16 Threonine aldolase-mediated chemo-enzymatic synthesis of immunodepressive lipid mycestericin D

The enzymatic aldolization of (*R*)-glyceraldehyde acetonide with glycine catalyzed by L-threonine aldolase from *Candida humicola* gave the key intermediates for the synthesis of 3,4-dihydroxyprolines in six steps (Fujii et al. 2000).

As mentioned before, one of the main drawbacks in the application of threonine aldolases is their lack of erithro/threo selectivity (kinetic limitation) and their equilibrium position (thermodynamic limitation). Recently, a tandem use of LD-threonine aldolases with low selectivity and L-amino acid decarboxylases with high selectivity has demonstrated to overcome the kinetic and thermodynamic limitations in the synthesis of phenyl serine (Steinreiber et al. 2007). Starting with benzaldehyde and glycine, *R*-phenyl ethanol was obtained in 58% isolated yield and R enantiomeric excess higher than 99% by the action of L-threonine aldolase (L-TA) from *Pseudomonas putida*, D-threonine aldolase (D-TA) from *Alcaligenes xylosoxidans* and L-tyrosine decarboxylase (L-TyrDC) from *Enterococcus faecalis* following the scheme depicted in Fig. 6.5.17.

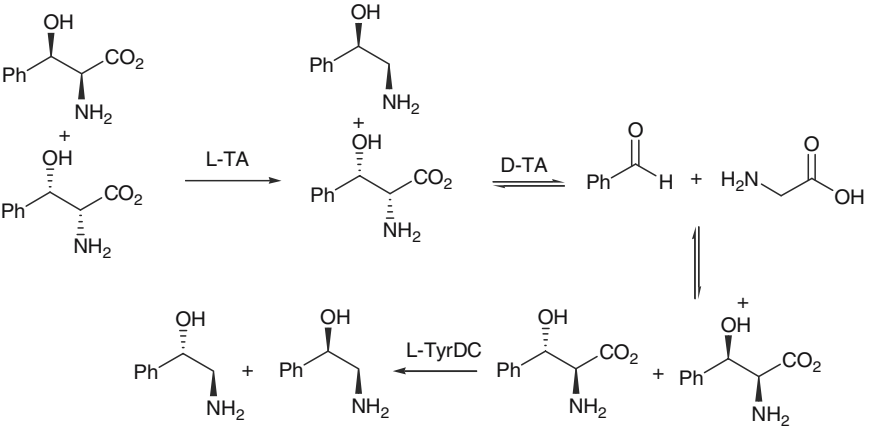


Fig. 6.5.17 Multienzymatic tandem synthesis of *R*-phenyl ethanol

6.5.5 Conclusions

Aldolases constitute a group of enzymes potentially important for the development of synthetic processes involving asymmetric aldol reactions. They can be employed for the synthesis of valuable products with unique stereochemistry, conducting to new synthetic approaches (new processes) or alternative routes to the chemical ways for the production of chiral intermediates. These facts are particularly important in the context of the production of enantiopure compounds to be tested for its biological activity. Nevertheless, major developments at both laboratory and process scale are still necessary for the full exploitation of their potential. Firstly, the insufficient supply and price of the enzymes has to be solved by developing efficient production processes. Secondly, the dependency of expensive donor compounds such as DHAP and PEP has to be overcome by using both molecular (new enzymes) and process (reaction engineering) strategies.

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6.6 Application of Enzymatic Reactors for the Degradation of Highly and Poorly Soluble Recalcitrant Compounds

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6.6.1 Potential Application of Oxidative Enzymes for Environmental Purposes

The discharge of recalcitrant compounds in the environment represents an important ecological concern since their complex structure and low bioavailability confer resistance for biodegradation and difficult their transformation in conventional sewage treatment plants (STP). Advanced oxidation processes such as ozonation, Fenton treatment or UV exposure, have arisen as possible alternatives to treat particular effluents before entering a STP (Chen et al. 2007; Lee et al. 2007; Suárez et al. 2007). Other possibility is based on biotechnological approaches, which consider the use of bacterial or fungal cultures to carry out their transformation. In particular, the utilization of white-rot fungi may be valuable, due to their ability to oxidize and decompose very complex and strong structures as lignin. The action of these fungi is attributed to extracellular oxidases and peroxidases, the most outstanding being lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Lac).

Degradation of organopollutants by fungal cultures has been carried out successfully at bench and pilot scale, preferentially in batch or semi-continuous operations (Alleman et al. 1995; Fujita et al. 2002; Mielgo et al. 2002). Continuous treatment systems are desirable alternatives to increase process efficiency. A modified conventional configuration (fixed-bed bioreactor) coupled with a pulsation system has

Table 6.6.1 Main Features of Fungal and Enzymatic Reactors for Environmental Purposes

Fungal Reactors	Enzymatic Reactors
Autocatalytic	Need of cofactors and mediators
Mineralization in most cases	Biotransformation to intermediates
Inhibition by pollutants	Higher tolerance to pollutants
Longer treatment time required (days)	Shorter treatment time (hours)
Difficult control	Easier and simpler control
Sterile conditions	Non-sterile conditions
Higher background experience	Cost of enzymes

proved to assure efficient and prolonged operation of the bioreactor (Mielgo et al. 2002). The feasibility of this approach was evaluated in the degradation of three different dyes: anthraquinone type (Poly R-478), azo type (Orange II) and phtalocyanine type (Reactive Blue 98). This configuration provided good oxygen transfer, controlled mycelia extension by pneumatic pulsation and prevented preferential

Table 6.6.2 In Vitro Degradation of Organopollutants by Oxidative Enzymes

Organopollutant	Enzyme	Source	References
Trinitrotoluene	MnP	*Nematoloma forwardii*	Scheibner and Hofrichter (1998)
	MnP	*Phlebia radiata*	van Aken et al. (1999)
Organochlorines	LiP, MnP	*Phanerochaete chrysosporium*	Valli et al. (1992)
Polychlorinated biphenyls	Lac	*Trametes versicolor*	Dec and Bollag (1995); Roper et al. (1995)
Bleach-plant effluent	MnP	*Penicillium chrysosporium*	Jaspers et al. (1994)
	Lac	*Trametes versicolor*	Archibald et al. (1990)
Synthetic dyes	LiP	*Penicillium chrysosporium*	Cripps et al. (1990); Ollikka et al. (1993)
	MnP	*Bjerkandera adusta Penicillium chrysosporium*	Heinfling et al. (1998)
	MnP	*Bjerkandera sp BOS55*	Mielgo et al. (2003); López et al. (2004)
	HRP[a]	Commercial	Bhunia et al. (2001)
Polycyclic aromatic hydrocarbons (PAHs)	LiP	*Penicillium chrysosporium*	Bumpus (1989); Hammel et al. (1986)
	LiP	*Nematoloma frowardii*	Günther et al. (1998)
	MnP	*Penicillium chrysosporium*	Bogan and Lamar (1995); Bogan et al. (1996)
	MnP	*Nematoloma frowardii*	Günther et al. (1998)
	Lac	*Trametes versicolor*	Johannes et al. (1996); Majcherczyk et al. (1998)
	Lac	*Coriolopsis gallica*	Pickard et al. (1999)

[a]Horseradish peroxidase

paths. By modifying the operating conditions of this system related to Mn^{+2} and hydrogen peroxide (H_2O_2) concentrations, temperature and oxygen supply, the decolorizing system was robust and flexible enough to maintain fairly high efficiency of removal (80–98%), even when working at conditions far apart from the optimal. However, the application of microbial reactors presented as drawbacks the operation at hydraulic retention times of about one day, the addition of nutrients and aeration and the necessity of maintaining sterile conditions.

Under a practical point of view, the potential use of enzymes for environmental purposes appears as a solution to overcome these drawbacks. Both strategies are compared in Table 6.6.1, being enzymatic treatment more advantageous than fungal degradation in many aspects. Several works report the ability of different enzymes for the oxidation of organopollutants (Table 6.6.2). However, the application of an enzymatic system for degradation of recalcitrant compounds is a scarcely explored alternative mainly due to the cost of the enzyme.

6.6.2 Requirements for an Efficient Catalytic Cycle

The first step to develop an enzymatic reactor requires a wide knowledge of enzyme behavior, regarding the substrates and cofactors involved in the catalytic cycle. In whole fungal cultures, these compounds are endogenously produced and their necessary replacement takes place naturally, whereas in enzymatic processes their addition is required to effectively complete the catalytic cycle. Let us consider MnP as a selected example. It has a similar catalytic cycle than other peroxidases,

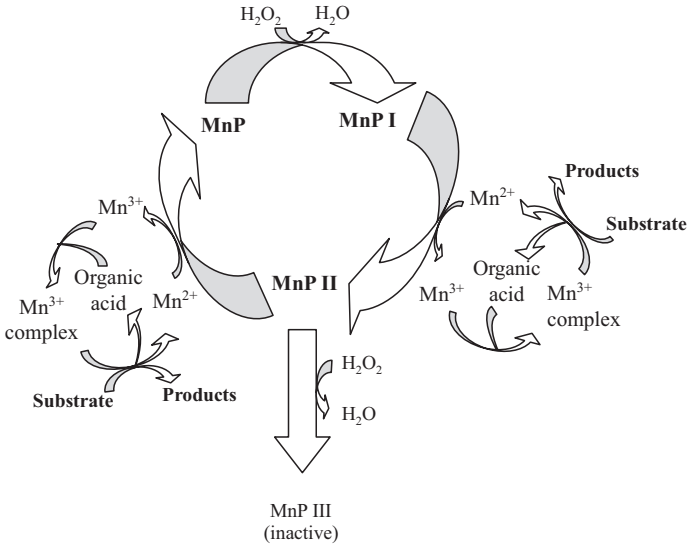

Fig. 6.6.1 Scheme of the catalytic cycle of MnP

involving a 2-electron oxidation (Fig. 6.6.1). The initial oxidation of MnP by H_2O_2 leads to an intermediate compound which promotes oxidation of Mn^{2+} to Mn^{3+}. Mn^{3+} is stabilized by organic acids and the Mn^{3+}-organic acid complex formed acts as an active diffusing oxidizer (1.54 V) that attacks organic molecules non-specifically at locations remote from the enzyme active site (Kuan and Tien 1993). It is important to highlight that an excess of H_2O_2 can cause irreversible enzyme inactivation.

In natural environments, fungi adapt their degradative capacity to the slowest reaction step. In wood decay, the microorganism uses Mn^{2+} present in wood and produces H_2O_2 endogenously by extracellular oxidases. In contrast, the in vitro application of MnP requires the addition of Mn^{2+}, H_2O_2 and organic acid not only to assure the completion of the catalytic cycle but also to enhance reaction kinetics and enzyme stability. Mn^{2+} affects positively the reaction rate, though this beneficial effect is greatly dependent on the H_2O_2 concentration (Palma et al. 1997). H_2O_2 has a dual effect, as it is directly involved both in the activation of the catalytic cycle at a balanced concentration and deactivation of the enzyme at a high concentration (Timofeevski et al. 1998). On the contrary, a low concentration of H_2O_2 implies kinetic or even stoichiometric limitations, while the enzyme activity is protected from inactivation (Mielgo et al. 2003). Organic acids provoke a similar cross effect: a high concentration favors the chelation of Mn^{3+} but it may affect the stability of the enzyme (Hofrichter et al. 1998). This effect could likely be due to the endogenous formation of H_2O_2 during the decarboxylation of sodium malonate (van Aken and Agathos 2002). Not only a high reaction rate is desirable, but also a minimum loss of enzymatic activity.

When the practical application of the enzyme for the transformation of a substrate is considered, an important goal is to attain the best efficiency, defined as the maximum amount of substrate degraded per minimum units of enzyme inactivated during the reaction.

6.6.3 Enzymatic Reactor Configurations

The choice of the enzyme reactor configuration depends on the properties of the reaction system. For each class of biotransformation, the medium in which conversion takes place is dependent on the properties of reactants and products. The reaction components may have high or low aqueous solubility, which will greatly affect the reactor selection. When the xenobiotic compound is highly soluble in water, the choice of the enzyme reactor is apparently simple, focusing the design on the retention of the enzyme. However, for poorly soluble compounds, bioavailability of the xenobiotic is the most restrictive step for degradation. Systems to improve the interaction between the enzyme or its related mediator and the substrate have to be applied. Among several possibilities, Table 6.6.3 shows different reactor configurations to treat highly and poorly soluble compounds, which are discussed below.

Table 6.6.3 Possible Configurations of Enzymatic Reactors Treating Highly or Poorly Soluble Compounds

Substrate	Key Factor	Alternative	Configuration	References
Highly soluble (dyes, phenols, nitroaromatic compounds, ...)	Retention of the enzyme	Enzyme recovery by means of a membrane	Monophasic reactor coupled to a membrane	Basheer et al. (1993); Pasta et al. (1999); López et al. (2002)
		Immobilization of the enzyme	Monophasic reactor with enzyme immobilized onto a support	Grabski et al. (1998); Hublik and Schinner (2000); Krastanov (2000); Sasaki et al. (2001)
Poorly soluble (PAHs, pesticides, polychlorinated byphenils, ...)	Availability of the pollutant	Addition of a surfactant	Emulsion reactor with free enzyme	Moen and Hammel (1994); Bogan and Lamar (1995); Steffen et al. (2003)
		Addition of a miscible solvent	Monophasic reactor with free enzyme	Field et al. (1996); Eibes et al. (2006); Rodakiewicz-Nowak and Jarosz-Wilkolazka (2007)
		Addition of an immiscible solvent	Biphasic reactor with free enzyme	Eibes et al. (2007)

6.6.3.1 Highly Soluble Compounds

The design of enzymatic reactors for the degradation of highly soluble compounds may consider the use of a semi permeable membrane for the separation of the enzyme and products or substrates (enzyme membrane reactors) or the immobilization of the enzyme in a support (immobilized enzyme reactors).

6.6.3.1.1 Membrane reactors

The system is provided with a membrane of suitable molecular cut-off, which acts as a selective barrier for the retention of the enzyme. Permeable substrates and products are taken out from the reaction mixture by the action of a gradient (chemical potential, pressure) through the membrane. Based on the combination of membranes and enzyme reactors two main configurations are considered, as shown in Fig. 6.6.2. In the first configuration, the enzyme may be immobilized by covalent binding between an activated group of the membrane and a functional group of the protein

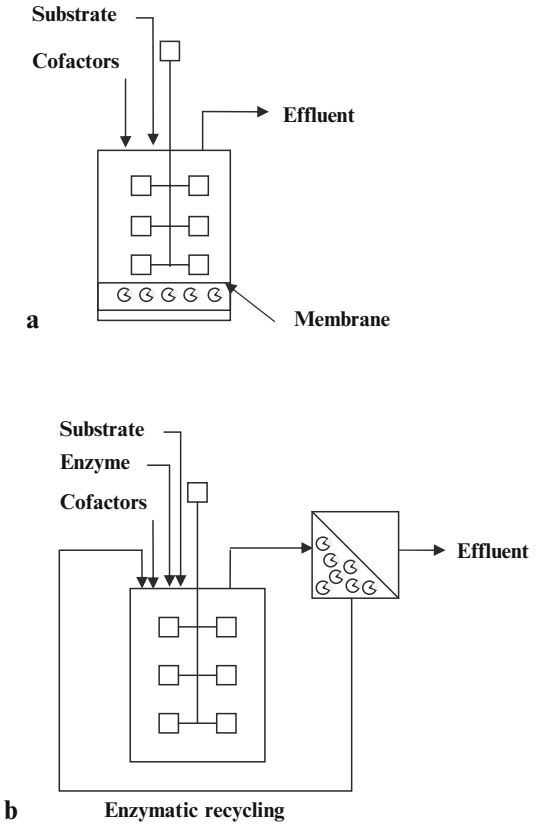

Fig. 6.6.2 Schematic diagrams of different configurations of enzymatic membrane reactors: (a) stirred tank reactor with enzyme immobilized or retained by a membrane; (b) stirred tank reactor coupled to ultrafiltration membrane

(Guisán et al. 1997), adsorbed by Van der Waals or electrostatic forces (Lante et al. 2000) or entrapped or encapsulated onto the matrix during the membrane preparation process (Isono and Nakajima 2000). The alternative configuration is based on the selective extraction of substrates from the influent by specific membranes. Once the substrate reacts with the enzyme, products diffuse back through the membrane to reach the effluent (Prazeres and Cabral 1994). The main drawbacks of this system are the requirement of low molecular weight substrates and the diffusion of compounds through the membrane, which usually slows down the kinetics of the process (Kelsey et al. 1990). In direct contact enzyme reactors, the substrate is fed directly to the compartment of the enzyme, enabling a direct contact between them. The most common system consists of a traditional stirred tank reactor combined with a membrane separation unit (Prazeres and Cabral 1994). The retained enzyme is recycled back to the reactor, whereas the degraded products are able to pass through the membrane. The main advantages of this configuration are: i) operation with free enzyme, avoiding mass transfer limitations; ii) retention of non-biodegradable molecules with high molecular weights; iii) ability of the degraded products to cross the membrane, being discharged in the effluent; and iv) easy operation.

6.6.3.1.2 Immobilized Enzyme Reactors

References about immobilized enzyme reactors for environmental purposes are scarce (Katchalski-Katzir and Kraemer 2000). The concept is based on the immobilization of the enzyme onto a support by covalent binding or ionic interaction. The feasibility of the immobilized enzyme reactors is determined by the following requirements: i) the specific activity of the derivative (units of enzyme per g of support) should be as high as possible; ii) the support or membrane should be applied with a secondary function, such as the separation of substrates or products; and iii) the support should have good mechanical resistance and minimum interaction with the substrates or products. Additionally, the immobilization process should be simple and inexpensive.

Different reactor configurations are proposed for the use of immobilized enzymes (Fig. 6.6.3): A) stirred tank reactors; B) fixed bed reactors; and C) fluidized bed reactors. The selection of the best option depends on the type of support and reaction kinetics. However, very often the activated supports do not present adequate characteristics for the performance of a fixed or fluidized bed reactor and, therefore, the development of stirred tank reactors based on immobilized enzyme or even, sequential stirred tank reactors appear as the more feasible options.

6.6.3.2 Poorly Soluble Compounds

6.6.3.2.1 Solvents and Surfactants

The in vitro degradation of poorly soluble compounds is limited by the extent to which they are available to the enzymes, which could lead to biocatalytic

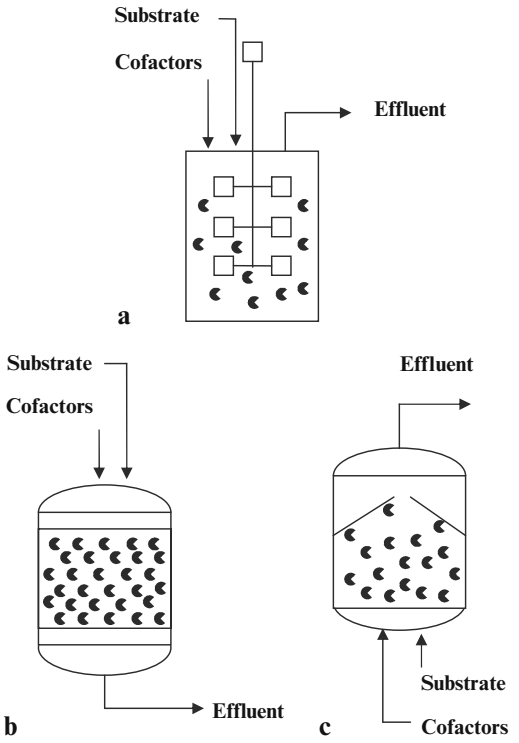

Fig. 6.6.3 Schematic diagrams of different configurations of immobilized enzyme reactors: (A) stirred tank reactors, (B) fixed bed reactor and (C) fluidized bed reactor

conversions at low rates. The addition of solubilizing agents, such as surfactants or solvents, might be considered to overcome this limitation. Although the use of surfactants may exert a deleterious effect on enzyme activity and the solubilization is rather limited, they have been frequently applied to increase the availability of certain substrates and in some cases they have been found to enhance the oxidation via formation of peroxyl radicals (Kotterman et al. 1998). Enzymatic catalysis in organic solvents has undergone rapid expansion in the last decades and, although believed to be a promising approach in decontamination, most of the work reported is related to hydrolytic enzymes. The potential of using more complex enzymes as ligninases, which require specific substrates and cofactors for the catalytic cycle, is almost untapped (Field et al. 1996). The addition of water miscible solvents (co-solvents) can enhance the solubility of compounds by several orders of magnitude, (Eibes et al. 2005; Eibes et al. 2006). However, the selection of the cosolvent requires special attention, because hydrophilic solvents have a great tendency to strip bound water away from enzyme molecules leading to a rapid inactivation (Klibanov 2001). Moreover, the solvent and enzyme recovery has to be considered to make this process economically feasible.

The use of a biphasic medium to carry out bioconversions at higher overall concentrations has clear advantages for those reactions where one or more

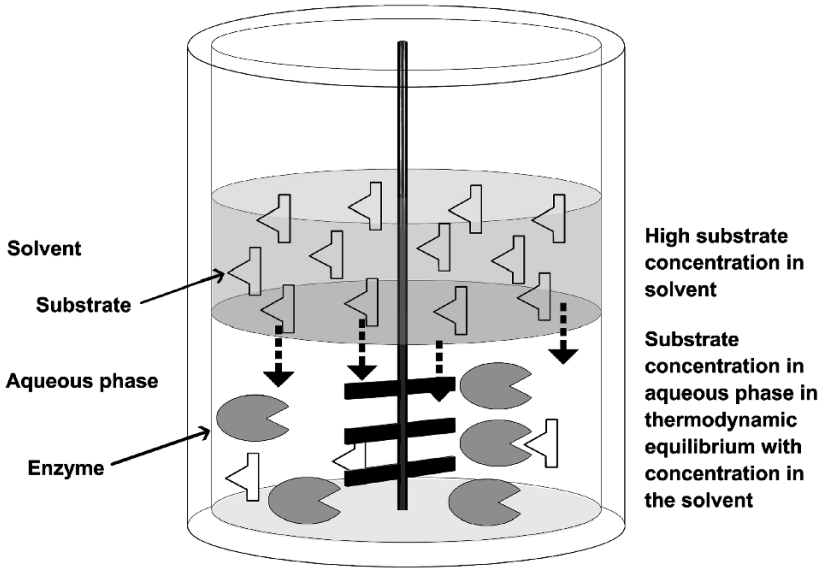

Fig. 6.6.4 Scheme of the enzymatic biphasic reactor for the degradation of poorly soluble compounds

reaction components are poorly water-soluble. The principle of a biphasic biocatalytic process is based on the addition of a virtually water-immiscible organic solvent to an aqueous phase (at concentrations well above the aqueous phase saturation limit) containing the biocatalyst to create a biphasic reaction medium (Fig. 6.6.4). When the substrate is hydrophobic, the organic phase will be initially rich in substrate which will then distribute into the aqueous phase at a concentration defined by its partition coefficient. Once in the aqueous phase, the enzyme will degrade the substrate, this being the driving force for the subsequent substrate spread out of the organic phase in order to maintain a thermodynamic equilibrium. Depending upon their solubility, degradation products may remain in the aqueous phase or partition into the organic phase.

The selected solvent should have suitable physical and chemical properties (be immiscible, non-volatile, etc.), and be inexpensive and readily available (Déziel et al. 1999; Marcoux et al. 2000; MacLeod and Daugulis 2003). Furthermore, the possible interaction between the solvent and the enzyme has to be considered. It is important that the presence of the solvent does not interfere with the degradation of the target substrate (MacLeod and Daugulis 2003) and its effect on the enzyme activity be as low as possible (Ross et al. 2000). The development of solvent-resistant enzymes will facilitate the application of two-liquid phase biocatalysis.

6.6.3.2.2 Factors Affecting Reactor Efficiency

The application of two phase partitioning bioreactors (TPPBs) presents some difficult biochemical engineering challenges including the selection of an appropriate

reaction medium, reactor design and operating parameters. Drawbacks arising from the use of biphasic reactors may be related to the non-direct contact between the enzyme and the substrate, which may imply diffusional resistance. The substrate transfer rate from the water-immiscible phase to the aqueous phase (Eq. 6.6.1) is another critical factor and it has to be enhanced so as not to limit the overall degradation rate.

$$J_S = k_L\, a \cdot (s_w^* - s_w) \tag{6.6.1}$$

where J_S is the flow of the substrate (M/h); k_L is the mass transfer coefficient (m/h); a is the specific interfacial area (m^2/m^3); and $(s_w{}^* - s_w)$ is the difference between the equilibrium and the actual substrate concentration in the aqueous phase (M). Mass transfer coefficient is dependent on the system geometry, the physical properties of medium (solvent viscosity, density, interfacial tension, substrate diffusivity, ...) and flow dynamics (Welty et al. 1984). Apart from these solvent properties, the partition coefficient of the substrate in the solvent is another factor which has to be taken into account. Solvents with high partition coefficient can sequester the target compound, thus reducing its biodegradation rate (Efroymson and Alexander 1995). Therefore, low values of partition coefficients are preferred in order to achieve high concentration of the substrate in the aqueous phase.

An increased surface area enables higher substrate transfer rate; therefore, the rate of biodegradation in a TPPB may be governed by the interfacial area (Köhler et al. 1994), as defined by Eq. 6.6.2:

$$a = \frac{6 \cdot \varphi}{d_{sm}} \tag{6.6.2}$$

where φ is the proportion of the organic phase in the reactor and d_{sm} is the Sauter mean diameter of the solvent drops, these parameters being very important when selecting the operating conditions.

6.6.4 Modeling of Enzymatic Reactors

Mathematical models, especially when coupled with computer techniques, are a very effective tool in searching for optimal operating conditions in the design, operation and control of enzyme reactors. The study of a reliable model for the enzyme reaction system is of significant importance for the industrial application of the biocatalyst. The model has to be effective in a wide range of values of the process variables.

The most important characteristics of an enzyme are the basic data about the kinetics of the catalyzed process and the knowledge about the factors that affect the kinetic properties. The following step for further developing the model is to find the relationship between the reaction rates and the reactor configuration. The above-mentioned formal kinetic model for the overall reaction rate and the balance equations for a reactor configuration are used to predict the process behavior and to

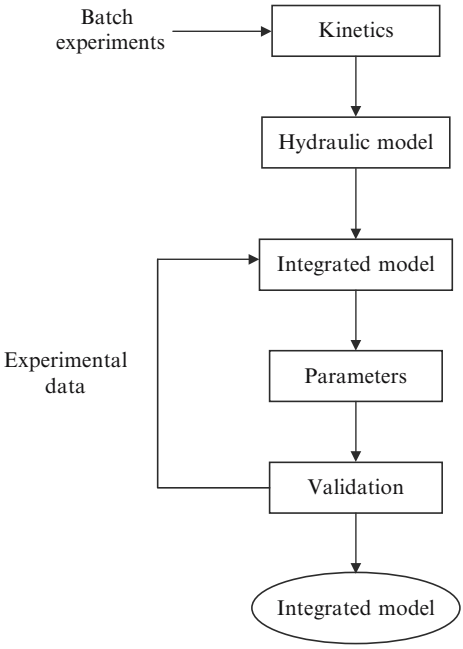

Fig. 6.6.5 Flow chart of the process to obtain the model of enzymatic reactors

give a rule for optimizing efficiency (see Chapter 5). To validate the kinetic model, the data from batch reactor experiments should be in good agreement with simulated data obtained by means of numerical integration. Figure 6.6.5 represents the scheme followed to obtain the model for an enzyme reactor operation.

Furthermore, the study of the reactor behavior in unsteady-state conditions will be indicative of the stability of the system as it shows its resistance against alterations, such as changes in the influent flow or pollutant concentration. The dynamic model of a particular process is an essential tool for developing an effective control strategy.

6.6.5 Case Studies

Two different reactor configurations were studied in order to evaluate the removal of a highly soluble compound, the azo dye Orange II and a poorly soluble compound, the polycyclic aromatic hydrocarbon (PAH), anthracene.

6.6.5.1 In Vitro Degradation of Orange II in an Enzyme Membrane Reactor

The degradation of the dye Orange II was carried out in an enzyme reactor consisting of a continuous stirred tank reactor (CSTR) coupled with a membrane to recover the enzyme (Fig. 6.6.2.B). From an economic point of view, a compromise

has to be made between the productivity, the conversion and the loss of enzyme activity. Before using a continuous enzymatic membrane reactor for the degradation of compounds, the optimization of both the parameters involved in the catalytic cycle of the enzyme and the operational conditions of the reactor must be considered; besides, the development of a control system is very useful for the simulation and optimization of the process.

6.6.5.1.1 Optimization of the Parameters Involved in the Catalytic Cycle

The effect of the variables involved in the catalytic cycle of the enzyme MnP was evaluated in batch experiments. Addition rate of H_2O_2, concentration of Mn^{2+}, pH

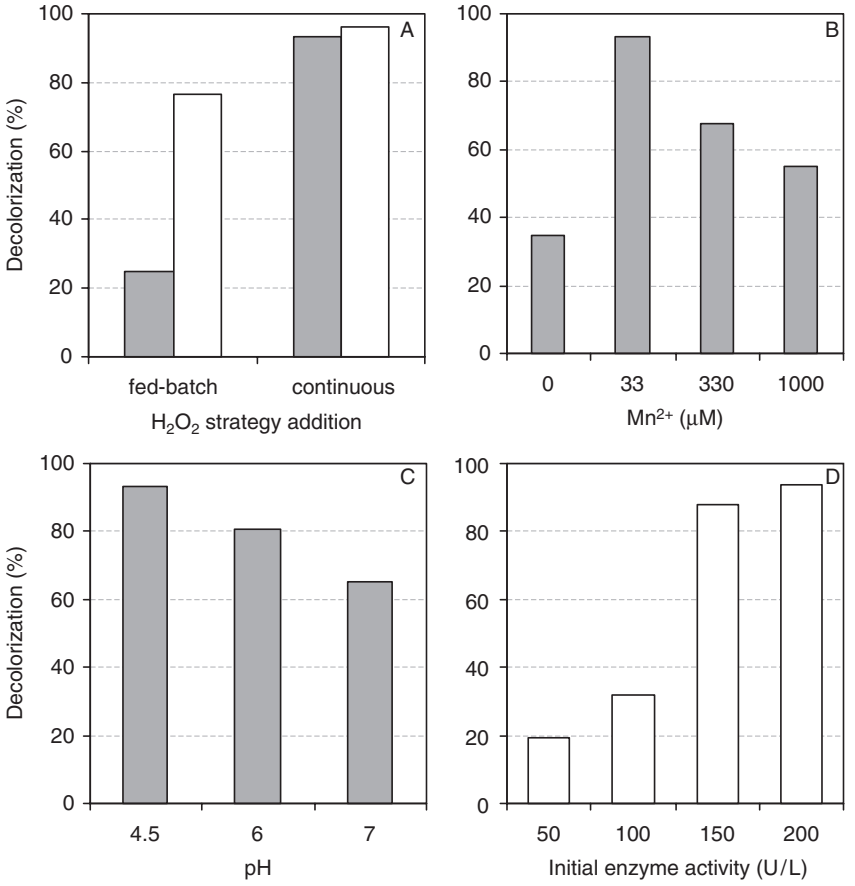

Fig. 6.6.6 Orange II decolorization in batch reactors after 10 min (grey bars) or 60 min (white bars) at different strategies of H_2O_2 addition (A), Mn^{2+} concentration (B), pH (C) and initial MnP activity (D). Basic conditions: MnP 200 U/L, H_2O_2 addition rate 1.36 μmol/min, Mn^{2+} 33 μM and pH 4.5

and MnP activity were investigated. Results are presented in Fig. 6.6.6 (Mielgo et al. 2003). The purpose was to obtain conditions to attain high decolorization rate of Orange II with low consumption of enzyme. Initial MnP activity for the experiments was 200 U/L unless indicated. When H_2O_2 was continuously pumped at an addition rate of 34 μmol $H_2O_2/L \cdot min$, almost complete degradation (93%) was obtained after 10 min of reaction, while in the case of fed-batch operation only 76% degradation was reached after 60 min (Fig. 6.6.6A). Therefore, continuous addition of H_2O_2 was considered for the following experiments. Only 33 μM of Mn^{2+} was required for Orange II decolorization and no further improvement was attained at higher concentrations (Fig. 6.6.6B). Values of pH around 4.5 were adequate for an efficient operation (Fig. 6.6.6C). Concerning the enzyme, 150 U/L of MnP was determined to be the minimum activity required to reach a good decolorization after 1 h (Fig. 6.6.6D). The optimization of all parameters permitted to reach 90% degradation of 100 mg/L of Orange II after 10 min with minimum inactivation of enzyme.

6.6.5.1.2 Optimization of the Operational Parameters of the Enzymatic Membrane Reactor

Based on the optimization of the conditions attained in discontinuous experiments (Mielgo et al. 2003), the degradation of the dye was performed in a continuous enzyme reactor (López et al. 2004). Different experiments were planned to maximize process efficiency, defined as the ratio between degradation rate and enzymatic consumption.

Three strategies for enzyme addition were considered (Fig. 6.6.7): i) single initial addition of MnP; ii) stepwise addition of enzyme every hour; iii) continuous pumping of the enzyme into the vessel in order to maintain a relatively constant level of activity (López et al. 2004). Continuous addition of enzyme implied high decolorization, low MnP activity loss and the highest efficiency of the process. Using this strategy of MnP addition, different flow rates of H_2O_2 were evaluated: 15, 25 and 50 $\mu mol/L \cdot min$, finding that a compromise solution should be selected depending on the particular objectives: maximizing decolorization or minimizing enzymatic deactivation.

6.6.5.1.3 Modeling

Deep knowledge of the enzymatic reaction is necessary for a proper selection of the variables that should be considered in the reaction model. In this case, two variables were selected: Orange II concentration, as the dye is the substrate to be oxidized, and H_2O_2 addition rate, as the primary substrate of the enzyme (López et al. 2007). The performance of some discontinuous experiments at different initial values of both variables resulted in the definition of a kinetic equation, defined using a Michaelis–Menten model with respect to the Orange II concentration and a first-order linear

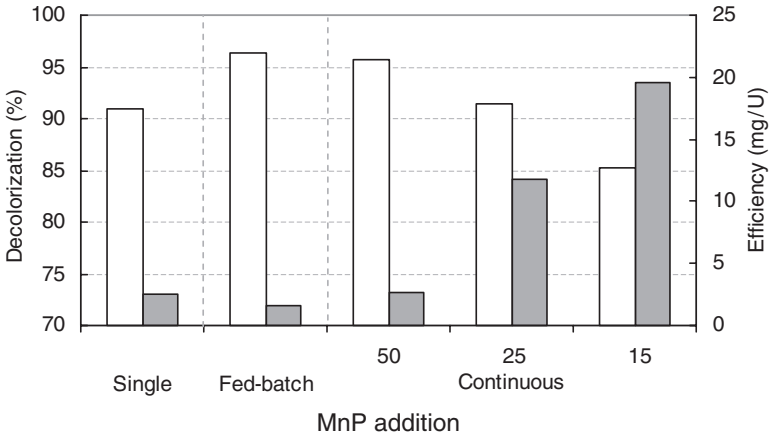

Fig. 6.6.7 Orange II decolorization (white bars) and efficiency (grey bars) in the enzymatic membrane reactor at different strategies of MnP addition. Continuous addition of MnP was performed at 50, 25 and 15 μmol $H_2O_2/L \cdot min$

dependence relative to H_2O_2 addition rate (Eq. 6.6.3).

$$r = \frac{r_m \cdot s}{K_M + s}\left(K_{H_2O_2} + Q_{H_2O_2}\right) = \frac{0.33 \cdot s}{58.2 + s}\left(2.4 + Q_{H_2O_2}\right) \qquad (6.6.3)$$

where r is the degradation rate $(\mu M/min)$; r_m is a constant dimensionless value; s is the Orange II concentration (μM); K_M is the Michaelis constant (μM); $K_{H_2O_2}$ is the constant for the H_2O_2 addition rate $(\mu M/min)$ and $Q_{H_2O_2}$ is the H_2O_2 addition rate $(\mu M/min)$.

A dynamic model was defined considering the kinetic equation and the hydraulics of the enzymatic membrane reactor. This model was validated comparing experimental data with model predictions at different experiments in steady-state conditions. Even when some modifications were performed, as changes in the Orange II concentration in the feed, the control system was able to predict the Orange II concentration in the reactor (Fig. 6.6.8).

The study of the behavior of the reactor in unsteady-state conditions was useful, not only to validate the dynamic model, but also to obtain information about the system. Figure 6.6.9 shows the results of an experiment where some perturbations of the steady state were performed at 1-h intervals. Orange II hydraulic retention time (HRT) and H_2O_2 addition rate were modified in order to cause a destabilization of the system, which recovered steady state in less than 3 h after the end of each alteration. As it can be observed, the dynamic model successfully simulated the behavior of the system.

The dynamic model was validated both in steady and unsteady-state conditions, which is quite interesting in case that a control based on feed-forward strategy is applied. The prediction of the final concentration of Orange II from the initial data would allow the system control to modify the flow rate of MnP, Orange II or H_2O_2, in order to adapt the conditions to the desired final value.

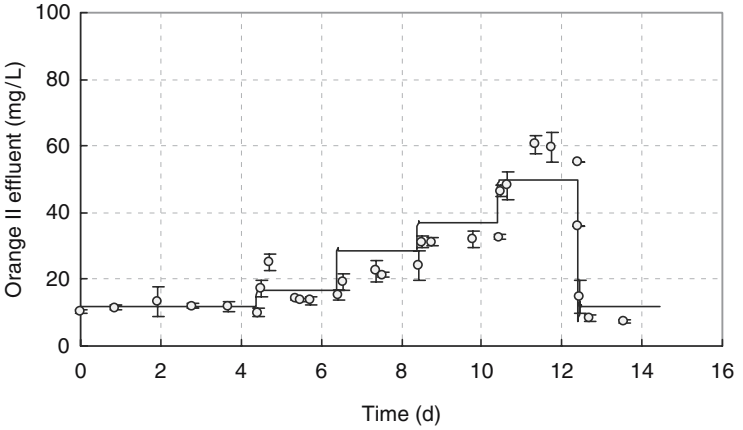

Fig. 6.6.8 Experimental (○) and simulated data (—) of Orange II concentration in a continuous experiment at different Orange II concentrations in the influent: days 0–4: 100 mg/L; days 4–6: 150 mg/L; days 6–8: 200 mg/L; days 8–10: 250 mg/L; days 10–12: 300 mg/L and days 12–14: 100 mg/L. Concentrations in the influent: malonate 1 mM, Mn^{2+} 33 μM.; HRT 60 min; H_2O_2 addition rate 15 μmol/L · min; pH 4.5

Dissolved oxygen (DO) concentration in steady state was observed to be dependent on the experimental conditions (López et al. 2004). DO decreases when there is an unbalance between H_2O_2 addition rate and organic loading rate (OLR) (Fig. 6.6.9). This suggests DO as a suitable parameter for monitoring the reaction behavior and a control variable to apply a feed-back control system (López et al. 2007).

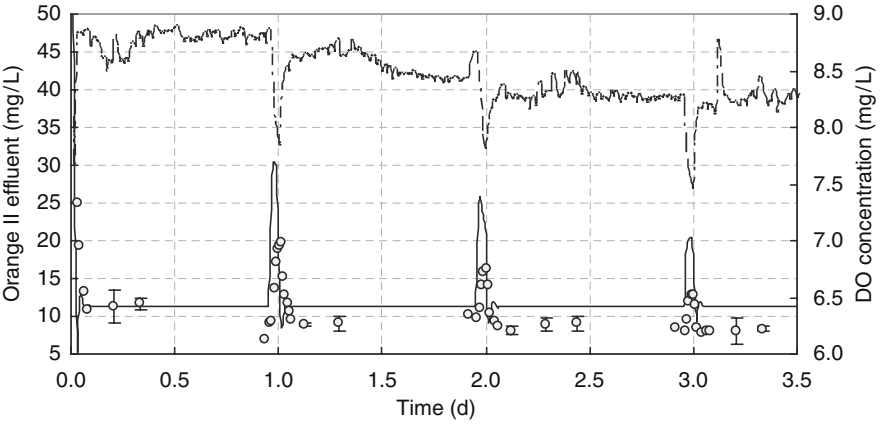

Fig. 6.6.9 Experimental (○) and simulated data (—) of Orange II concentration and dissolved oxygen (–·–) in a continuous process in unsteady state conditions. MnP activity 200 U/L; Concentrations in the influent: Orange II 100 mg/L, malonate 1 mM, Mn^{2+} 33 μM. Overload at 1 d: Orange II 200 mg/L; overload at 2 d: HRT 30 min; overload at 3 d: H_2O_2 7.5 μmol/L · min

6.6.5.2 In Vitro Degradation of Anthracene in TPPBs

The degradation of a poorly-soluble compound, anthracene, was carried out in a TPPB by the oxidative action of MnP. When dealing with biphasic reactors, the selection of the appropriate solvent is the first step in the optimization of the process. As well as with soluble compounds, the parameters involved in the catalytic cycle should be studied but also other operational parameters affecting mass transfer of the substrate. Finally, a model of the process will help to understand the whole process and to choose the most adequate operational parameters.

6.6.5.2.1 Selection of the solvent

Two factors must be considered to select an adequate solvent: partition coefficient of anthracene in solvent/water medium and stability of the enzyme in biphasic systems. A list of solvents presenting high boiling point, low water solubility, low cost, lack of toxicity and commercial availability was first considered to determine the partition coefficient of anthracene (K_{SW}) (Fig. 6.6.10). Solvents presenting high values of K_{SW} were avoided, since the degradation rate might be reduced due to the diminution of anthracene concentration in aqueous phase. Both silicone oil, with the minimum log K_{SW} 3.7, and dodecane, with an intermediate value of log K_{SW} 4, were selected for enzyme inactivation studies. Organic solvents can exert a deleterious effect on the biocatalyst, which may be due to the interaction with dissolved solvent molecules or with the interface between the aqueous and organic phases (Ross et al. 2000). Silicone oil and dodecane are nearly insoluble in water with high hydrophobicity values (log K_{OW} 6.6 and 11 for dodecane and silicone oil, respectively), being therefore the main mechanism of interfacial interaction. Different interfacial areas were produced by modifying agitation in the presence of silicone

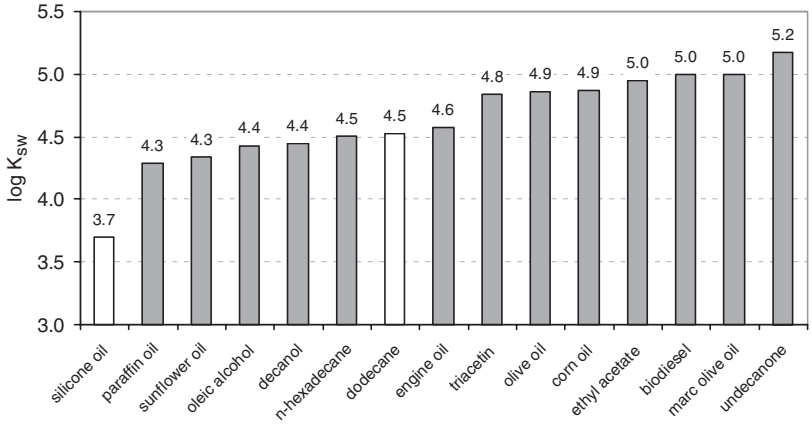

Fig. 6.6.10 Values of log K_{SW} obtained for 15 different solvents. Silicone oil and dodecane were selected as representative compounds for low and medium partition coefficients

oil and dodecane. Under the same agitation rate, silicone oil formed higher interfacial areas than dodecane due to its lower interfacial tension (20 and 53 mN/m for silicone oil and dodecane, respectively). The inactivation rates for silicone oil and dodecane were 6.7 and 11.8 $U/L \cdot h$ at 400 rpm; 61 and 81 $U/L \cdot h$ at 600 rpm; and 138 and 143 $U/L \cdot h$ at 800 rpm, respectively, dodecane causing higher enzymatic inactivation at all agitation rates. In consequence, even at higher interfacial areas, enzyme inactivation in silicone oil was lower. As silicone oil had the lowest partition coefficient and led to lower inactivation rates of the enzyme, it was selected for the following experiments.

6.6.5.2.2 Optimization of the Parameters Involved in the Catalytic Cycle

The effects of the main factors involved in the catalytic cycle of MnP on the degradation of anthracene were studied (Eibes et al. 2007). Figure 6.6.11 shows the degradation rate and efficiency, in terms of milligrams of anthracene degraded per unit of enzyme inactivated, for experiments at different H_2O_2 addition rate, malonate concentration and pH control.

Regarding H_2O_2 addition rate, similar efficiencies were obtained at 1 and 5 $\mu mol/L \cdot min$, but the highest degradation rate was obtained at 5 $\mu mol/L \cdot min$. Higher addition rates decreased the efficiency due to the inactivation caused by H_2O_2 excess. A similar effect was observed for the concentration of the organic acid, being necessary to reach a compromise solution between enzyme inactivation and degradation efficiency. Next, pH control was considered because a pH increase was observed along the reaction, reaching values closer to 8 after 70 h of operation. The addition of malonic acid to control the pH at 4.5 was evaluated, since

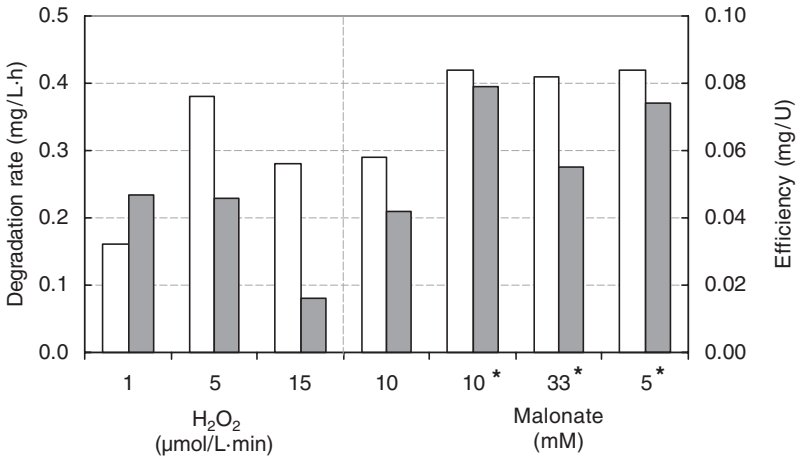

Fig. 6.6.11 Degradation rate of anthracene (white bars) and efficiency (grey bars) at different hydrogen peroxide addition rate, malonate concentration and control of pH (*) by addition of malonic acid (250 mM)

the concentration of sodium malonate was observed to decrease in the reactor. Efficiency was then increased 1.9-fold compared to the experiment with no pH control.

6.6.5.2.3 Optimization of the Mass Transfer Coefficients

Two factors were considered to affect substrate transfer rate: fraction of silicone oil and agitation rate. Diffusion from the organic phase was favored by increasing both factors because they increase interfacial area (Eq. 6.6.2), but this may also affect enzymatic activity. Since both variables, solvent fraction and agitation rate, are likely to be co-dependent, a 2^2 experiment design was considered to optimize the efficiency of the system (Eibes et al. 2007). The ranges considered were 200–300 rpm and 10–30% of silicone oil (v:v) and the response surface corresponding to efficiency (η) is represented by Eq. 6.6.4:

$$\eta = 0.152 + 0.026 \cdot \upsilon + 0.054 \cdot \sigma + 0.026 \cdot \upsilon \cdot \sigma \tag{6.6.4}$$

being η the efficiency of the system, υ the agitation rate and σ the fraction of silicone oil (all factors normalized).

The equation shows that efficiency was mainly dependent on the ratio of the organic and aqueous phases and higher fractions of silicone oil led to higher efficiency values. Both agitation and the combined effect had similar relative weights in the equation, both being positive. The highest efficiency was obtained at 300 rpm and 30% silicone oil, where a nearly complete oxidation was achieved after 56 h. Although different experiments were performed following the pathway of the steepest ascent, considering a golden section optimization protocol, none of them improved efficiency.

6.6.5.2.4 Modeling of the Process

Process modeling has to consider the two major aspects involved: i) mass transfer of anthracene and ii) enzymatic kinetics; thus the coefficients for each mechanism of the proposed model were evaluated. In order to quantify mass transfer coefficients, anthracene balance in the aqueous phase in the absence of enzymatic reaction has to be considered, being the substrate concentration in the aqueous phase given by Eq. 6.6.5:

$$\ln\left(s^* - s_w\right) = \ln s^* - k_L a \cdot t \tag{6.6.5}$$

The substrate concentration was measured in the aqueous phase for a period of time, thus enabling to find the mass transfer coefficient ($k_L a$) for each condition of agitation rate and fraction of solvent. Figure 6.6.12 shows the experimental $k_L a$ values for the conditions evaluated, presenting a great increase in a short range of agitation rates (200–250 rpm), being more pronounced when low fractions of silicone oil were present. Although mass transfer coefficients were maximized at 250 rpm for all the evaluated fractions of silicone oil, the experimental results of anthracene degradation indicated that 300 rpm and 30% silicone oil are the optimal conditions.

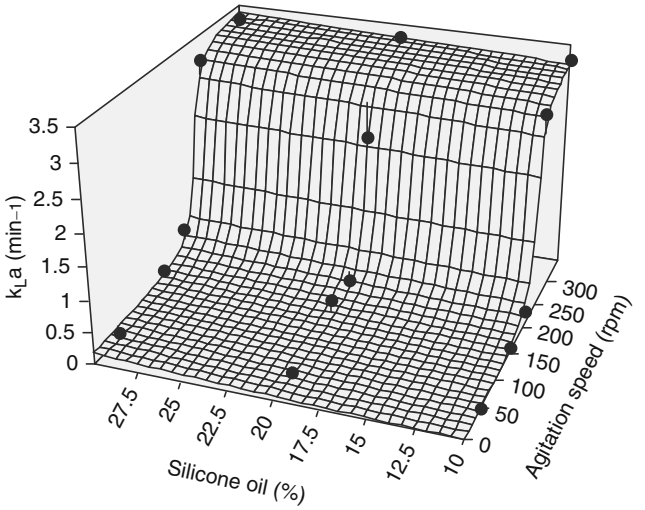

Fig. 6.6.12 Values of k_La obtained for experiments at different agitation rates and volume of silicone oil

The values of k_La were fitted to a surface ($r^2 = 0.986$) represented in Fig. 6.6.12 and thus related to the agitation rate (υ) and the fraction of silicone oil (σ) through an empiric correlation with five parameters (Eq. 6.6.6).

$$k_La = b + c \cdot \sigma + d \cdot \left(0.5 + \frac{\arctan\left(\frac{(\upsilon - e)}{f}\right)}{\pi} \right) \tag{6.6.6}$$

In order to obtain the catalytic coefficient, both balances in organic and in aqueous phase were considered. The enzymatic degradation of anthracene by MnP was considered as pseudo-first order kinetics with an autocatalytic effect due to the presence of the degradation products (Eq. 6.6.7). Quinones, which are the main degradation products of PAHs, can act as electron carriers as described by Méndez-Paz et al. (2005), thus accelerating the overall degradation.

$$r_S = (\alpha + \beta \cdot p) \cdot s \tag{6.6.7}$$

Including the kinetics onto the mass balance of anthracene in the aqueous phase and substituting in the organic phase balance, Eq. 6.6.8 is finally obtained which describes the behavior of anthracene in the organic phase (s_S) for each hydrodynamic condition:

$$\frac{ds_S}{dt} = -\frac{k_La}{k_{sw}} \cdot \frac{V_w}{V_s} \cdot \left(s_S - \frac{k_La \cdot s_S}{k_La + \alpha + \beta \cdot \frac{s_{S0} - s_S}{\frac{V_w}{V_s} + k'_{sw}}} \right) \tag{6.6.8}$$

The partition coefficient of anthracene in silicone oil was previously determined ($k_{sw} = 5012$, Fig. 6.6.10), the mass transfer coefficient is given by the empiric

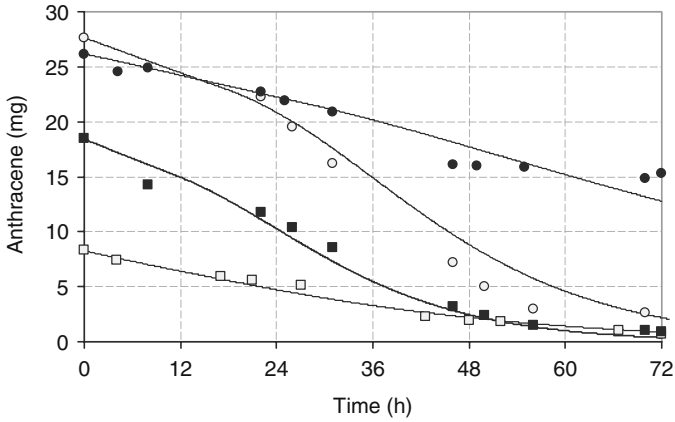

Fig. 6.6.13 Experimental data and fitted model (—) of anthracene degradation in batch experiments at different conditions of agitation speed and silicone oil volume: □ 200 rpm – 10%; ■ 250 rpm – 20%; • 200 rpm – 30%; ○ 300 rpm – 30%

correlation previously obtained (Eq. 6.6.6) and the kinetic constants α and β were estimated by using the method of least squares from the experiments at different agitation rates and fractions of silicone oil. The experimental data of anthracene degraded along the reactor operation and the fitted model for four different hydrodynamic conditions are plotted in Fig. 6.6.13. The highest degradation rate was obtained at 300 rpm and 30% silicone oil (v/v), oxidizing 90% of anthracene present in the organic phase after 56 h. Taking into account the efficiency function (η), the path of the steepest ascent for agitation was $0.55 \cdot s + 1$, and for silicone oil volume $0.84 \cdot s + 1$. The final experiment was carried out considering a step $s = 2.4$, which meant 365 rpm and 50% (v/v) silicone oil. The degradation rate obtained, $1.29\,mg/L \cdot h$, was 1.36-fold lower than that of the optimal conditions. The activity loss was slightly higher, $7.8\,U/L \cdot h$ and the efficiency, 0.166 mg/U, represented 73% of that obtained at 300 rpm and 30% (v/v) silicone oil.

6.6.6 Conclusions and Perspectives

Ligninolytic fungi are able to oxidize dyes and other soluble compounds, achieving high degrees of degradation. The main problem of fungal treatments is the requirement of sterile conditions and high hydraulic retention time (HRT) (12–24 h). On the contrary, enzyme reactors present several advantages, such as the possibility to work at much lower HRT, due to the fast kinetics of the enzymatic reaction. Thus, high conversions and degradation efficiencies are expected.

The application of enzymes in continuous processes can be performed in non sterile conditions, and allows working with high loads of enzymes in the reactor,

to maintain a stable enzyme activity, to reduce the risk of product inhibition and to minimize costs, energy and waste products. Enzyme membrane reactors are proposed as a promising technology, as they are very easy to operate and control, and the degradation process can be carried out continuously during more than 20 days with no cleaning or membrane replacement. Furthermore, the enzymatic reactors are quite versatile, since there is a wide variety of membrane shapes, materials and modules commercially available. The selection process must mainly consider the characteristics of the effluent and the enzyme molecular weight.

The addition of a second immiscible phase for the enzymatic degradation of poorly-soluble compounds provides several advantages, such as a simpler operation, mainly due to the easy recovery of the solvent depleted of substrate and its reuse in subsequent operations. Mass transfer could be considered a priori as a limitation for this system and to be the determinant of lower efficiencies. However, the selection of the appropriate solvent, as well as the determination of the adequate conditions which lead to the maximum efficiency allowed us to obtain unprecedented degradation rates in enzyme reactors.

Enzyme reactors appear as an attractive technology for the degradation of hardly biodegradable compounds, which has to be further optimized and tested at higher scale. Increasing the enzyme efficiency (mass of pollutants/units of enzyme deactivated), improving the reliability in continuous systems and implementing control strategies for a more stable operation, are some of the goals to be attained in the near future.

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