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Preface

The use of fermentation in food processing was originally developed as a method for preserving food and has probably been practised since mankind has been living in settlements. Nowadays food biotechnology involves a lot more than simply preventing spoilage and ranges from improving salubriousness, improving texture to enriching foodstuffs with substances that are favourable to health and well-being and the prevention of illness. The basic principle of fermentation is mostly to exploit the metabolism of a specific microorganism. The activity of these microorganisms takes place in the foodstuff itself or in the intestine (probiotica), whereby they produce primary or secondary metabolites via the fermentation process. Naturally, probiotica or metabolites in particular can be produced as pure cultures or as pure substances and can then be administered as pharma products or food additives.

The food biotechnology field is extremely broad and is developing rapidly. An important factor is that the average age of humans is increasing, but the age of well-being, that is how long one feels fit and healthy, is stagnating. It has been shown that this so-called age of well-being can be influenced by individual nutrition habits and, complemented by genetic predisposition, can even be increased. In addition, common diseases and illnesses such as the prevalence of being overweight or diabetes can even be prevented through food, pro- and prebiotica and food additives. One emphasis of this volume is on this area.

Apart from looking at pro-, pre- and synbiotica as regards health, the volume also focuses on the genetic optimisation of plant raw materials and fermentation organisms used in the processing of food. Although currently molecular genetic methods are generally spurned in Western Europe, in the long term there will be no choice but to use such methods to optimise the utilisation of food resources. In addition, it should not be underestimated that plant cells, in contrast to microorganisms, hold great potential as regards the production of secondary metabolites and food additives, particularly in the areas of "wellness" ingredients. Even though plants have little to do with filamentous fungi, they are an interesting alternative for producing food additives. Now that genetic methods have become more accessible for fungi, they can be used as a powerful biological catalyst that has good secretion abilities. Even though

much seems possible in the field of food biotechnology, one should not lose sight of ethical considerations. The last chapter examines this aspect.

In conclusion, I want to sincerely thank the co-editors and authors who have contributed to this volume for their dedicated effort and their excellent contribution. I hope that you as reader will enjoy the volume.

Berlin, July 2008 Ulf Stahl

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Probiotics, Prebiotics, and Synbiotics

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Abstract According to the German definition, probiotics are *defined viable microorganisms, sufficient amounts of which reach the intestine in an active state and thus exert positive health effects*. Numerous probiotic microorganisms (e.g. *Lactobacillus rhamnosus* GG, *L. reuteri*, bifidobacteria and certain strains of *L. casei* or the *L. acidophilus*-group) are used in probiotic food, particularly fermented milk products, or have been investigated—as well as *Escherichia coli* strain Nissle 1917, certain enterococci (Enterococcus faecium SF68) and the probiotic yeast *Saccharomyces boulardii*—with regard to their medicinal use. Among the numerous purported health benefits attributed to probiotic bacteria, the (transient) modulation of the intestinal microflora of the host and the capacity to interact with the immune system directly or mediated by the autochthonous microflora, are basic mechanisms. They are supported by an increasing number of in vitro and in vivo experiments using conventional and molecular biologic methods. In addition to these, a limited number of randomized, well-controlled human intervention trials have been reported.

Well-established probiotic effects are:

1. Prevention and/or reduction of duration and complaints of rotavirus-induced or antibiotic-associated diarrhea as well as alleviation of complaints due to lactose intolerance.
2. Reduction of the concentration of cancer-promoting enzymes and/or putrefactive (bacterial) metabolites in the gut.
3. Prevention and alleviation of unspecific and irregular complaints of the gastrointestinal tracts in healthy people.
4. Beneficial effects on microbial aberrancies, inflammation and other complaints in connection with: inflammatory diseases of the gastrointestinal tract, Helicobacter pylori infection or bacterial overgrowth.
5. Normalization of passing stool and stool consistency in subjects suffering from obstipation or an irritable colon.
6. Prevention or alleviation of allergies and atopic diseases in infants.
7. Prevention of respiratory tract infections (common cold, influenza) and other infectious diseases as well as treatment of urogenital infections.
   Insufficient or at most preliminary evidence exists with respect to cancer prevention, a so-called hypocholesterolemic effect, improvement of the mouth flora and caries prevention or prevention or therapy of ischemic heart diseases or amelioration of autoimmune diseases (e.g. arthritis).

A prebiotic is "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well being and health", whereas synergistic combinations of pro- and prebiotics are called synbiotics. Today, only bifidogenic, non-digestible oligosaccharides (particularly inulin, its hydrolysis product oligofructose, and (trans)galactooligosaccharides), fulfill all the criteria for prebiotic classification. They are dietary fibers with a well-established positive impact on the intestinal microflora. Other health effects of prebiotics (prevention of diarrhoea or obstipation, modulation of the metabolism of the intestinal flora, cancer prevention, positive effects on lipid metabolism, stimulation of mineral adsorption and immunomodulatory properties) are indirect, i.e. mediated by the intestinal microflora, and therefore less-well proven. In the last years, successful attempts have been reported to make infant formula more breast milk-like by the addition of fructo- and (primarily) galactooligosaccharides.

Keywords Health effects · Host immunity · Intestinal flora · Prebiotics · Probiotics · Synbiotics

Probiotics, prebiotics, and synbiotics are based on the same idea: to create foodstuffs which after ingestion multiply "healthy" bacteria in the intestine. This can be performed by adding either health-promoting "probiotic" bacteria or undigestible but fermentable "prebiotic"[1] carbohydrates. Such an enhancement of health-promoting qualities beyond the basic function of a food as a supplier of relevant nutrients, complies to a large extent with common definitions of functional foods.[2]

Indeed, pro- and prebiotics are food components fulfilling nearly ideally those definitions and particularly the term "beyond nutrition", since bacteria and undigestable carbohydrates have no nutrient character. Furthermore, fermented milk with health-promoting "probiotic" properties is one of the oldest functional foods. Fermented milk has not only been consumed throughout the world for thousands of years, as evidenced by their depiction in Sumerian wall paintings dating back to 2500B.C., but in a Persian version of the Old Testament (Genesis 18:8) it can be read that Abraham owed his longevity to the consumption of sour milk. And in 76B.C. the Roman historian Plinius recommended the administration of fermented milk products for treating gastroenteritis (reference cited in Bottazzi [1]).

The function of the probiotic bacteria added to foods includes the reduction of potential pathogenic bacteria and/or harmful metabolites in the intestine, normalization of gastrointestinal motility and modulation of the immune response, whereas so-called prebiotic food components should promote favorable bacteria of the indigenous intestinal flora of humans, or also improve survival of probiotic bacteria which have been ingested at the same time.

1 Probiotics

1.1 Introduction

According to a recent definition used in Germany, probiotics[3] are

> *defined viable microorganisms, sufficient amounts of which reach the intestine in an active state and thus exert positive health effects* [3].

Although often used synonymously, probiotics are not the same as probiotic foods:

---

[1] (Almost) all established prebiotics are undigestible but fermentable carbohydrates like inulin or galacto- and fructooligosaccharides (oligofructose) [2].

[2] E.g. "a functional food is similar in appearance to conventional foods, is consumed as part of a usual diet, and has demonstrated physiological benefits and/or reduces the risk of chronic disease beyond basic nutritional functions" (Bureau of Nutrition Science, Canada).

[3] According to this definition the terms "probiotics" and "probiotic microorganisms" (often limited to "probiotic bacteria") can be used synonymously.

> *"Probiotic foods contain living probiotic microorganisms in an adequate matrix and in sufficient concentration, so that after their ingestion, the postulated effect is obtained, and is beyond that of usual nutrient suppliers."* [3].

Probiotics are not only ingested as a food component. The term "probiotics" was created in the 1950s by W. Kollath [4], whereas Lilly and Stillwell in 1965 used this term for live bacteria and spores as animal feed supplements that should help limiting the use of antibiotics in animal husbandry [5]. The first generally accepted definition was given by Fuller in 1989 [6]: [a probiotic is] "a live microbial feed supplement which beneficially affects the host animal[4] by improving its intestinal microbial balance".

Pharmaceutical products with live bacteria have also been on the market for a long time, although not labeled as "probiotic", and for years without a sufficient proof of efficiency.

The idea, to suppress and displace harmful bacteria in the intestine by orally administered "beneficial" ones and by this improve microbial balance, health and longevity, was born nearly a century ago by Carre [7], Tissier [8], and Metchnikoff [9]. Tissier recommended the administration of bifidobacteria to infants suffering from diarrhea, claiming that bifidobacteria supersede the putrefactive bacteria causing the disease. He showed that bifidobacteria were predominant in the gut of breast-fed infants. And Nobel Prize winner (1908) Elie Metchnikoff from the Pasteur Institute in Paris claimed in his famous book "The prolongation of life" that the intake of lactobacilli-containing yogurt, results in a reduction of toxin-producing bacteria in the gut and this is associated with increased longevity of the host. It may be of interest, that the first industrially produced yogurt was developed according to the ideas of Metchnikoff to help children suffering from diarrhea and was sold in pharmacies.

Probiotic microorganisms do not act exclusively in the large intestine via affecting the intestinal flora. They also affect other organs, either by modulating immunological parameters, intestinal permeability and bacterial translocation, or by providing bioactive or otherwise regulatory metabolites. Therefore, broader definitions have been suggested, i.e. by Schrezenmeir and de Vrese [10], by the International Life Sciences Institute (ILSI) Europe, according to which "[a probiotic is] a viable microbial food supplement which beneficially influences the health of the host" (cited according to [11]) or by the FAO/WHO 2001 [12], according to which probiotics are "live microorganisms which when administered in adequate amounts confer a health benefit on the host".

Irrespective of some differences, all definitions have in common that probiotic microorganisms must (1) be living and (2) exert scientifically proven health effects.

---

[4] This definition was restricted to probiotics in animal nutrition.

Although neither "viability" nor "survivability of the gastrointestinal transit" are indispensable qualities of health-promoting microorganisms, since dead cells and cell components may also exert some health-promoting physiological effects, it is consumers' and scientific understanding that a probiotic food must contain living microorganisms [13].

1.2 Health Claims for Probiotics

Regardless of the results of legal discussions, composition and effects of probiotic foods and their detection methods need to be clearly defined.

1. A primary prerequisite is that such foods be healthy and safe, and free of pathogenic and toxic effects.
2. Postulated health effects have to be proven by clinical studies in humans. In vitro studies and animal experimental analyses only give indications to possible health relevant effects. They may be useful for identifying mechanisms of action, or for the search for new probiotics.
3. Clinical studies should follow clearly defined study goals and a randomized, double-blind and placebo controlled design. Their results should be confirmed by independent research teams, and documented in peer-reviewed scientific journals and be documented according to the rules of "good clinical practice" (GCP).
4. As even closely related bacteria strains of the same species may have different physiological effects, proofs for health effects are only valid for the (probiotic) bacteria strain with which the study had been performed. A prerequisite for unambiguous study results are bacteria strains, clearly defined with modern molecular biological detection methods. A strain allocation based on phenotypical characteristics only is generally not sufficient.
5. The extent to which the intake of probiotic microorganisms leads to the desired health effect does not only depend on their absolute numbers in the ingested product, but also on its composition and physical state. This also means that, using a probiotic bacteria strain in different matrices or together with different probiotic bacteria, the postulated effect should be identified for each combination. As this claim is not realistic it has been eased to such an extent that study results can be transferred to similar foods, for which, in the present state of knowledge, no different matrix effects are expected.
6. The effectiveness of a probiotic and therefore the lowest concentration of probiotic microorganisms in the product from which a health effect may still be expected, depends on the kind of probiotic microorganism, the claimed effect, the duration of application, the food matrix, and, last but not least, the target group. Often 10^8–10^9 probiotic bacteria per day are

mentioned as the minimum amount for probiotic effects. This value, however, is rather a makeshift than scientifically proven, because in clinical studies health effects by certain strains have been demonstrated at lower dosages: e.g. ingestion of $\sim 5 \times 10^7$ cfu/d LA5 plus Bb12 decreased gastric *Helicobacte pylori* activity before and frequency and severity of side effects during Helicobacter eradication therapy [14]. Anyway, a probiotic product should guarantee the ingestion of that number of probiotic microorganisms at the end of its shelf life, which was used in the studies substantiating its health effect.

7. Probiotic effects are target specific. The effect of probiotic microorganisms on study participants may vary with age, health and gender, diet, residence and environment, e.g. rural or urban etc. There are differences with respect to maturity or efficiency of the immune system to the predominant microflora and/or to hygiene standards. This has the consequence that results from studies in children/aged subjects, in diseased people or from the Third World cannot be transferred without further examination to adults, healthy people or people from industrialized countries, respectively. On the other hand this means—particularly in the case of small experimental groups and/or a small number of studies—that inconsistent results do not necessarily cast doubt on the investigated probiotic effect, but more likely on its transferability from the participants of a successful study to the general population. The often-stated phrase: "more well-planned studies are necessary to corroborate an effect" should be reformulated to "more studies are necessary to find out which section of the population may profit from a probiotic and under which conditions".

1.3 Probiotic Microorganisms

The majority of probiotic microorganisms belong to the genera *Lactobacillus* and *Bifidobacterium*. However, other bacteria and some yeasts may have probiotic properties as well (Table 1). *Lactobacilli* and *Bifidobacteria* are Gram-positive lactic acid-producing bacteria that constitute a major part of the normal intestinal microflora in animals and humans.

Lactobacilli are non-spore forming rod-shaped bacteria. They have complex nutritional requirements and are strictly fermentative, aerotolerant or anaerobic, aciduric or acidophilic. Lactobacilli are found in a variety of habitats where rich, carbohydrate-containing substrates are available, such as human and animal mucosal membranes, on plants or material of plant origin, sewage and fermented milk products fermenting or spoiling food.

Bifidobacteria constitute a major part of the normal intestinal microflora in humans throughout life. They appear in the stools a few days after birth and increase in number thereafter. The number of bifidobacteria in the colon of adults is 10^{10}–10^{11} cfu/gram, but this number decreases with age. Bi-

Table 1 Microorganisms used as probiotics [17, 18]

Lactobacilli [a]	Bifidobacteria	Others
L. acidophilus-group	*B. longum* (BB536) *B. longum* (SP 07/3)	*Enterococcus faecalis* [b]
L.acidophilus (LA-5)	*B. bifidum* (MF 20/5)	*Enterococcus faecium* [c]
L. crispatus (*L. acidophilus* "Gilliland")	*B. infantis*	*Lactococcus lactis*
L. johnsonii (LA1)	*B. animalis* (*B. animalis* ssp. *lactis* BB-12)	*Streptococcus thermophilus*
L. gasseri(PA 16/8)	*B. adolescentis*	*Propionibacteria*
L. casei- group	*B. breve*	*E. coli* [c] (*E. coli* "Nissle 1917")
L. (para)casei (*L. casei*) "shirota" *L. casei* "defensis")		*Sporolactobac. Inulinus* [c]
L. rhamnosus (LGG)		*Spores of Bacillus cereus* "toyoi"
L. reuteri		
L. plantarum (*299 and 299v*)		*Saccharomyces boulardii* [d]

[a] Commercial names of specific strains are given in brackets
[b] Mainly used in pharmaceutical preparations
[c] Mainly used in animal husbandry
[d] Re-classified as a strain of *S. cerevisiae*

fidobacteria are nonmotile, nonsporulating rods with varying appearance. Most strains are strictly anaerobic.

While conventional starter cultures, above all, have been optimized in respect to technological and tasting properties as well as culture stability in acidified milk, probiotic microorganism strains have been selected from the broad spectrum of lactic acid bacteria and other microorganisms for their health-promoting qualities.

For this purpose a number of selection criteria were established.

- Safe for humans, i.e. free of pathogenic and toxic effects.
- Origin from the intestinal tract of healthy persons,[5] as such microorganisms are regarded safe for humans and best adapted to the ecosystem of the gut.
- Tolerance to gastric and bile acid as well as sufficient resistance against digestive enzymes enable the survival during the passage through stomach and upper intestinal tract,[6] and have health-promoting effects in the gut. As the decrease in pH of the ingested food in the stomach is low due to the buffer capacity of the gastric acid, resistance against gastric acid is less critical than tolerance of the bacteria to bile acid and digestive enzymes in the small bowel.

[5] No essential criterion; some successful strains had been isolated from animals or vegetables.
[6] Survival is no longer strictly required by some definitions of probiotic [15].

- Detection of parameters enabling a (positive) influence on the intestinal flora[7] like adhesion to intestinal epithelial cells, survival and reproducing capacity in the human large intestine, or production of antimicrobial substances. A permanent colonization of bacteria in the large bowel has not been proven. It is not requested for attaining probiotic effects, as far as a daily or at least regular bacteria supply occurs via regular intake of probiotics.

The yeast *Saccharomyces boulardii*, used in pharmaceutical products, was shown to exert beneficial effects against diarrhea, and *Enterococcus*-containing pharmaceuticals are used in pediatrics.

Probiotic bacteria must also comply with the technological requirements,[8] and a certain probiotic content must be guaranteed until the expiry of shelf life.

1.4 Health Relevant Effects of Probiotics

Most health effects attributed to probiotic microorganisms are related, directly or indirectly, i.e. mediated by the immune system, to the gastrointestinal tract (Table 2). This is not only due to the fact that probiotics in food or therapeutically used microorganisms are applied normally via the oral route.[9] The mechanisms and the efficacy of a probiotic effect often depend on interactions with the specific microflora of the host or immunocompetent cells of the intestinal mucosa. The gut (or the gut-associated lymphoid system (GALT), respectively), is the largest immunologically competent organ in the body, and maturation and optimal development of the immune system since birth depends on the deve lopment and composition of the indigenous microflora [19].

Many strains of probiotic bacteria have been shown (1) to modulate (temporarily) the intestinal microflora and/or (2) to inhibit colonization of the gut by (potential) pathogens, as well as (3) translocation of pathogenic bacteria through the intestinal wall and the infection of other organs. Suggested, but unconfirmed mechanisms for these effects include:

> Reduced intestinal pH, production of bactericidal substances (e.g. organic acids, H_2O_2 and bacteriocines), agglutination of pathogenic microorganisms, strengthening barrier function of the intestinal mucosa [21–23],

[7] An impact on the intestinal flora is no longer required by some definitions of probiotic [15, 16].

[8] Food probiotics must be able to grow or at least survive in the food matrix before and after fermentation and taste and consistency of probiotic food should not be inferior to that of conventional products.

[9] Modulation of the microflora of the mouth or the urogenital tract and attempts to destroy tumors in mice can be done by direct local application or injection, respectively, of the probiotic microorganisms. Furthermore, the entrance of probiotic bacteria into the body via the mucus layer of the respiratory tract has also been demonstrated in mice.

Table 2 Established and proposed probiotic health effects

Probiotic effect	Validity of scientific proofs
- Prevention and/or reduction of duration and complaints of rotavirus-induced diarrhea - Prevention or alleviation of antibiotic-associated diarrhea - Alleviation of complaints due to lactose intolerance	Effect well-established by clinical studies and accepted by the scientific community
- Modulation of the autochthonous (usually intestinal) microflora - Immunomodulation and/or -regulation - Reduction of the concentration of cancer promoting enzymes and/or putrefactive (bacterial) metabolites in the gut	Well-established effect. However, due to methodological difficulties and complex interdependencies between regulatory mechanisms, the correlation with true health effects is unclear
- Prevention or alleviation of allergies and atopic diseases in infants - Beneficial effects on microbial aberrancies, inflammation and other complaints in connection with: inflammatory diseases of the gastrointestinal tract, Helicobacter pylori infection, bacterial overgrowth - Treatment of urogenital infections - Prevention and alleviation of unspecific and irregular complaints of the gastrointestinal tracts in healthy people - Prevention of respiratory tract infections (common cold, influenza) and other infectious diseases	Effects observed in certain target groups. However, more studies are necessary to find out which section of the population may profit from a probiotic and under which conditions
- Cancer prevention - Normalization of passing stool and stool consistency in subjects suffering from obstipation or an irritable colon - Prevention or therapy of ischemic heart diseases - Amelioration of autoimmune diseases (e.g. arthritis)	Due to insufficient clinical and/or epidemiological data, effects cannot be considered as well established and scientifically proven
- Hypocholesterolemic effect - Improvement of mineral absorption - Improvement of the mouth flora, caries prevention	In the light of existing data (long term) reliable effects are not proven at all

competition for fermentable substrates or receptors on the cellular surface of the mucosa, release of gut-protective (arginine, glutamine, short-chain fatty acids, CLA) and absorption and metabolization of potentially pathogenic, toxic, or cancerogenic metabolites and enzymes [24–26], modulation of immunologic mechanisms [27], or stimulation of the intestinal motility and mucus production [28].

Because of these effects it is understandable that beside the immunomodulatory properties particulary the potential use of probiotics for prevention or therapy of diarrhea or inflammatory bowel disease have been studied [29, 30]. A recently published meta analysis of 34 randomized placebo-controlled human studies concluded that probiotics do significantly reduce diarrhea, amongst others antibiotic-associated diarrhea incidences by 35 to 65%, travelers diarrhea incidences by 6 to 21%, and diarrhea incidences due to other reasons by 8 to 53% [31]. Overall the risk of acute diarrhea was reduced by 57% in children and by 26% in adults.

1.4.1 Infectious Diarrhea Caused by Viruses or Bacteria

Rotavirus-induced diarrhea is still a major problem and frequent cause of death, especially in hospitalized children and in developing countries. Protection by probiotic bacteria and yeasts with immunostimulatory properties or the alleviation of symptoms and shortening of acute infections is perhaps the best-documented probiotic effect. It has been demonstrated many times in the past in clinical studies fulfilling scientific requirements. Beneficial effects such as decreased frequency of infections, shortening of the duration of episodes by 1–1.5 days [32, 33], less shedding of rotaviruses or an increase in the production of rotavirus-specific antibodies have been demonstrated for *Lactobacillus rhamnosus* GG (LGG), *L. casei* Shirota, *L. reuteri*, *Bifidobacterium animalis* ssp. *lactis* Bb-12 and a number of other probiotic strains [34–44]. Beneficial effects were frequently less pronounced with stronger infections.

Further demonstration of the effectiveness of *L. rhamnosus* GG failed in two recent studies in infants where LGG was ineffective in nosocomial rotavirus infections [45] and in severe dehydrating diarrhea [46].

There are three studies in young healthy children from day-care centers, where, however, the nature of the causative pathogens (probably mainly viral) was not examined. In a French study, 287 children (aged 18.9 ± 6.0 months) in day-care nurseries were administered daily either unfermented jellied milk, conventional yogurt, or a probiotic yogurt product containing 10^8 cfu/ml *L. casei* spec. Products were given for one month each, interrupted by one month without supplementation. The conventional yogurt shortened the mean duration of diarrhea from 8.0 days down to 5 days, the probiotic product even down to 4.3 days ($p < 0.01$), while the incidence of diarrhea was not different between groups [47]. This study was expanded to a randomized, controlled multicenter clinical trial with a total of 928 children (aged 6–24 months). During daily administration of *L. casei*-containing fermented milk for two months a lower frequency of diarrhea was observed compared with the administration of conventional yogurt (15.9 vs. 22%, $p < 0.05$; [48]). And Finnish children from day-care centers, who consumed milk contain-

ing a probiotic *Lactobacillus rhamnosus* strain during the winter, had 16% less days of absence from day care due to diarrhea and gastrointestinal and respiratory tract infections then controls [49].

The addition of Bb-12 or *L. reuteri* ssp. to infant formulas did prevent infectious diseases in Israeli child-care centers [50], and in 204 undernourished Peruvian children (6–24 months) rLGG compared with a placebo did reduce frequency of diarrhea from 6.0 to 5.2 episodes per child and year ($p < 0.05$; [51]).

On the other hand analogous studies were performed more seldom in adults, and overall the beneficial effects were less pronounced. For example, when 529 Israeli soldiers consumed yogurt with or without probiotic *L. casei* cultures, diarrhea frequency and duration were 12 as compared to 16% and 2.6 versus 3 days. These differences were not significant [52].

Investigations on the effect of probiotic bacteria on traveler's diarrhea showed inconsistent results, possibly due to differences between probiotic strains, the traveled countries, the local microflora, specific (eating) habits of the travelers, or the method of administration of the probiotic (before or during travel, as a capsule or a fermented milk product). Whereas some studies revealed less or shortened episodes of diarrhea in subjects consuming the probiotic [53–55], others found no such effect [56].

Although in vitro and animal studies provided good evidence that some probiotic strains inhibit growth and metabolic activity as well as the adhesion to intestinal cells of enteropathogenic bacteria like *Salmonella, Shigella* or *Vibrio cholerae*, few studies have been published demonstrating positive effects in humans.

1.4.2 Antibiotic Associated Diarrhea

Administration of certain probiotic strains before and during antibiotic treatment did in most studies reduce the frequency and/or duration of episodes of antibiotic-associated diarrhea and the severity of symptoms [57–65], although there are reports of lacking effects [66]. Administration of a fermented milk product (200 g/d) containing 10^5–10^7 cfu/g *Bifidobacterium animalis* ssp. *lactis* and *Lactobacillus acidophilus* four weeks before and during a *Helicobacter pylori* eradication therapy led to significantly less episodes of diarrhea (7% versus 22% of the subjects) compared with the placebo group (Fig. 1, [14]).

In some cases antibiotic treatment may result in life-threatening pseudomembranous colitis, which is associated with abundance of anaerobic toxigenic bacteria (e.g. strains of *Clostridium difficile*). Application of probiotics did also significantly decrease the number of relapses in successfully treated *Clostridium difficile* infections [67].

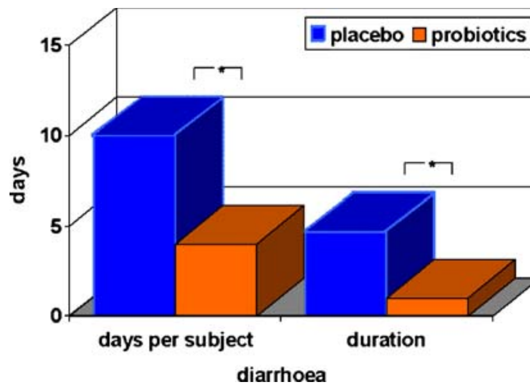

Fig. 1 Effects of probiotic lactobacilli and bifidobacteria on frequency and duration of antibiotic-associated diarrhea during H. pylori eradication [14]

1.4.3
Diarrhea in Immunocompromised Subjects

Chemo- and radiotherapy frequently cause severe disturbances of the immune system and the indigenous intestinal microflora, accompanied by diarrhea and/or increased cell counts of fungi (*Candida albicans*) in the gastrointestinal tract and other organs. Both side effects were ameliorated by the administration of probiotic bacteria before and during chemo- [68] or (in a mouse model) radiotherapy [69–71].

Whether regular consumption of probiotics exert beneficial effects in HIV patients has not been studied up to now, but it has been shown that probiotic products are well tolerated by these patients [72].

1.4.4
Lactose Intolerance

Without any doubt, the most thoroughly investigated health-relevant effect of fermented milk products is the enhancement of lactose digestion and the avoidance of intolerance symptoms in lactose malabsorbers, namely in persons with an insufficient activity of the lactose-cleaving enzyme β-galactosidase in the small intestine. This effect is based mainly on the fact that fermented milk products with live bacteria contain microbial β-galactosidase that survives the passage through the stomach, to be finally liberated in the small intestine to support lactose hydrolysis (Fig. 2, [73]). Moreover, it has been recently demonstrated in mice that during its transit living *Streptococcus thermophilus* or *Lactobacillus casei defensis* [74] are also able to perform the lactose hydrolysis.

However, depending on the definition of "probiotic" this is not a specific probiotic effect, because it doesn't depend on survival of the bacte-

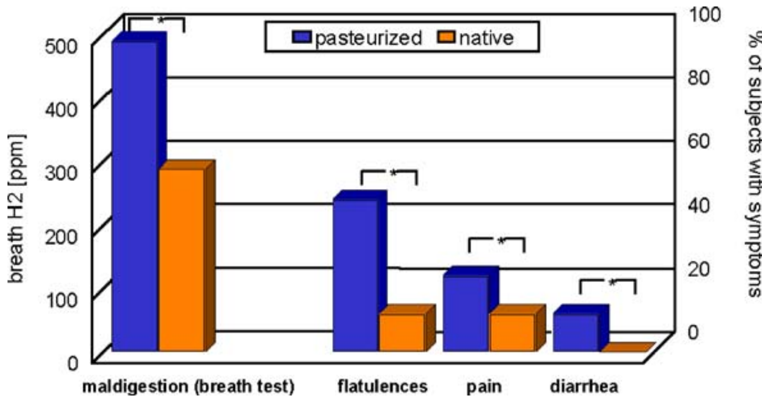

Fig. 2 Effect of fermented milk with live or heat-killed lactobacilli on lactose malabsorption (breath H2) and clinical symptoms in ten healthy African and South-East Asian nurses consuming pasteurized or native fermented milk [73, 75]

ria in the small intestine, yogurt is mostly more effective [75, 76] and, last but not least, primary or adult-type hypolactasia (the reason for lactose malabsorption) is not a disease, but rather the normal physiological situation. Many probiotic bacteria show either a lower β-galactosidase activity, or, due to their high resistance against acid and bile salts, do not yet release most of their β-galactosidase in the small intestine, opposite to yogurt bacteria [77].

Independent from such effects on lactose maldigestion, probiotics seem to reduce gastrointestinal complaints like flatulence or diarrhea, possibly by their impact on the intestinal microflora [75, 78].

1.4.5
Inflammatory Intestinal Diseases

Although the exact causes are not yet fully understood, there is evidence that disturbances of the autochthonous intestinal microflora and the stimulation of pro-inflammatory immunological mechanisms play a role in a number of inflammatory diseases of the intestine. Therefore, numerous efforts have been undertaken to improve health and well-being of affected patients by the administration of probiotics with anti-inflammatory properties and a demonstrated positive impact on the intestinal flora. Studies in experimental animals give a clue about the potential application of lactobacilli, bifidobacteria, *Lactococcus lactis* or non-food probiotics, particularly nonpathogenic strains of *Escherichia coli* (e.g. strain Nissle 1917) to prevent or treat colitis [79–82].

Likewise, patients with inflammatory bowel diseases (Crohn's disease and ulcerative colitis [83–88], necrotizing enterocolitis [79], diverticulitis [89] or

inflammation of an ileal pouch [90, 91]) responded positively too. Longer remissions due to the administration of probiotics were associated with a decreased expression of inflammatory markers ex vivo [79] and increased IgA secretion, lower drug consumption and all in all a higher quality of life of the patients [92]. In recent times more positive study outcomes have been reported [93–97] and review papers concerning the potential mechanisms like regulation of intestinal flora [98–101] or immunological mechanisms [102–105] were published. However, other studies showed no positive effects, and no case of complete recovery has been reported [106–110].

1.4.6
Gastrointestinal Motility Disorders

In the past normalization of the intestinal motility of obstipated subjects by administering probiotic bacteria has been demonstrated, however, more frequently by anecdotal reports than by controlled clinical trials [111, 112]. Many studies suffered from an unclear definition of obstipation, a lack of appropriate end-point markers, insufficiently detailed symptoms questionnaires, an unsatisfactory recording of health and well-being of the subjects before the study. This resulted in numerous confusing and contradictory results. Recent controlled clinical studies showed that administration of certain probiotic strains belonging to *L. casei* [113] and *B. animalis* [114] reduced gastrointestinal transit time, and very recently a probiotic fermented milk product was introduced in the market with the claim to fight obstipation. Nevertheless, more controlled clinical studies with clearly defined end-point markers and sufficient numbers of participants are necessary.

Beneficial effects of probiotics in subjects suffering from an irritable bowel syndrome[10] are still contradictory [115]. Whereas some studies showed a positive modulation of the intestinal flora and the alleviation of symptoms [116–123], other studies failed to do so [124, 125], and further investigations are required to move from hopeful findings to conclusive results.

1.4.7
Miscellaneous Diseases due to Microbial Imbalances

The use of probiotics in diarrheal diseases due to virus or bacterial infections or disturbances of the intestinal microflora have been investigated over a long period, and beneficial effects in rotavirus- and antibiotic-induced diarrhea or in lactose intolerance belong to the best documented and established health effects of probiotic microorganisms. In certain other diseases, which are associated with imbalance of the local microflora and bacterial infection and/or

[10] Functional disorder of the colon without provable biochemical or structural irregularity. Symptoms include intermittent abdominal pain and a succession of diarrhea and obstipation.

overgrowth as well, beneficial effects of probiotics are less established, the number of controlled studies or study participants is small and study results are inconsistent (Table 3).

Table 3 Miscellaneous non-diarrheal diseases and complaints due to bacterial infections and imbalances of the local microflora and benefits resulting from probiotics

Locus	Disease	Health effects
Mouth and teeth	Caries, gingivitis	Reduction of gingivitis by *L. reuteri* [126]; effects on *Streptococcus mutans* [127, 128]; colonization of the teeth' surface by lactobacilli from a "bio-yogurt" [129], less caries after ingestion of living [130] or oral vaccination with heat-killed lactobacilli [131]; all in all very few positive controlled studies
Stomach, (duodenum)	Helicobacter pylori infection	Inhibition of growth and adhesion to mucosal cells [29, 57, 132], decrease in gastric *H. pylori* concentration [133], less side effects during antibiotic therapy [14]; no effects [134, 135]
Small bowel	Bacterial overgrowth	Few successful studies: normalization of the small bowel microflora [136], decreased frequency of diarrhea [137], decreased release of toxic N-metabolites [138]
Intestinal microflora plus host metabolism (liver, kidney)	Decreased detoxification/ excretion of toxic microbial metabolites due to liver/renal failure; hepatic encephalopathy	Increased bifidobacterial cell counts and shift from a preferably protein- to a carbohydrate-metabolizing microflora, less toxic and/or putrefactive metabolites, improvement of hepatic encephalopathy after administration of bifidobacteria and lactulose [139, 140]
Urogenital tract	Irritation or inflammation of the vagina, urethra, bladder, ureter, kidney, or cervix due to infections by endogenous (from the gut) or exogenous bacteria and imbalances of the local microflora	Restoration of an imbalanced microflora by selected lactobacilli [141–143], decreased incidence and increased curing-rates in bacterial vaginosis and vaginitis (mostly candiasis) due to the local [144] or oral [145, 146] application of lactobacilli; decreased incidence or recurrence of urinary tract infections [147–150]; no effects [151]

1.4.8 Immunemodulation

Probiotic microorganisms and their cell-wall components (peptidoglycanes, lipopolysaccharides), DNA and metabolites were shown to have immunomodulatory properties.

Modulation of the systemic and secretory immune response [38] is well-established in mice and other experimental animals: inhibition of bacterial translocation [152]; increased proliferation in organs of the immune system (Peyer's patches, spleen); stimulation of phagocytes/macrophages and natural killer cells [153–157]; increased release of cytokines (IFN_{α}, IFN_{γ}, INF_{α}) and defensines[11] [153, 158], shifts in the Th1/Th2[12]-balance (Fig. 3) towards less allergy/atopy [159–161], increased production of specific antibodies [162–165] and increased resistance and prolonged survival during co-administration of viruses, toxines, and bacteria (rotavirus, *Klebsiella pneumoniae*, *Salmonella thyphimurium, Shigella, Vibrio cholerae*, *Listeria monocytogenes*). Similar effects on parameters of the cellular and humoral immunity have also been proven in human studies.

But because of the complexity of the immune system and the numerous interactions with the indigenous gut flora and administered probiotic bacteria, interpretation of animal and particularly in vitro data is often difficult. Stimulation of the immune system by itself does not necessarily imply a positive health effect. Controlled clinical studies showing therapeutic effects of probiotics, protection against infections or reduction of allergic reactions, and the investigation of the mechanisms are required.

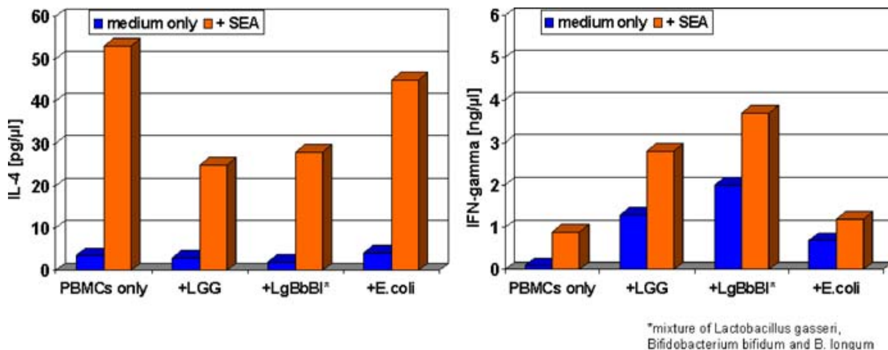

Fig. 3 Probiotic bacteria decrease production of "proallergic" Th2-cytokines [interleukin-4 (IL-4)] und increase production of "antiallergic" Th1-cytokines [$interferon_{\gamma}$ (IFN_{γ})] in stimulated Peripheral Blood Mononuclear Cells (PBMCs) of house dust mite allergic subjects [161]

[11] Defensines = protein molecules released from cells within the body and involved in defense against bacteria.

[12] Th1, Th2: T-helper cells.

1.4.9
Common Virus and Respiratory Tract Infections

Probiotics with proven immune stimulatory properties may be appropriate candidates for the prevention or treatment of some common viral infections including those of the respiratory tract. This has been thoroughly investigated in rotavirus infections, but enteroviruses have also been investigated, of which the target organ is not exclusively the intestine.

A randomized, double-blind, placebo-controlled clinical study [162], where strains of *Lactobacillus rhamnosus* and *paracasei* were applied orally to young adults before and during oral vaccination with attenuated polio viruses, showed that probiotics induce an immunologic response (IgA, IgG) and provide protection from polioviruses by increasing production of virus-neutralizing antibodies.

A few studies gave evidence, that certain strains of probiotic bacteria may prevent viral respiratory tract infections (common cold and influenza), alleviate complaints and/or shorten the duration of the disease. In a double-blind, placebo-controlled Finnish study, children from day-care centers (1–6 years), consuming milk with a probiotic *L. rhamnosus* strain for 7 months, were 0.7 days less absent from the centers because of illness of the gastrointestinal and respiratory tract and had a lower risk of respiratory tract infections than controls. No differentiation, however, was made between viral and microbial infections [49]. A probiotic *Enterococcus faecalis* preparation did reduce the incidence of respiratory tract infections in well- and malnourished children [166], whereas a *L. casei* strain was effective on winter-infections in elderly subjects in a pilot study [167].

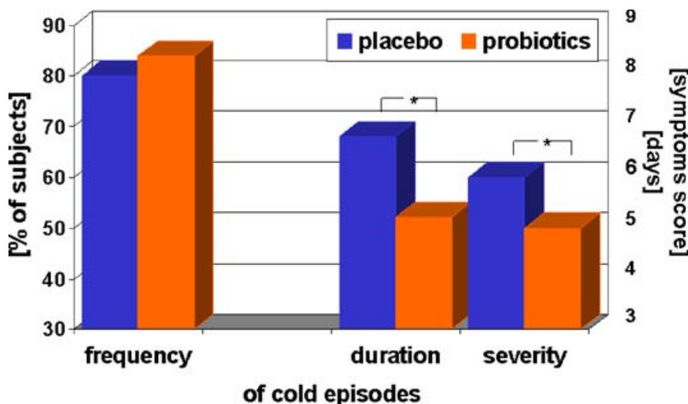

Fig. 4 Effect of the regular consumption of three strains of probiotic lactobacilli and bifidobacteria (5×10^7 cfu/day) on frequency, duration, and severity of common cold episodes in 244 healthy subjects during a winter/spring period [168, 170]

In a double-blind, controlled clinical trial in healthy adults, film-coated tablets containing a vitamin-mineral-mixture plus *L. gasseri*, *B. longum* and *B. bifidum* (verum), or without probiotic bacteria were applied to a total of 500 study participants over two winter-spring periods (3 and 5.5 months). In the verum group, almost two days shorter cold episodes ($p < 0.05$), less severe complaints ($p = 0.056$) and less days with fever ($p = 0.03$) were recorded (Fig. 4), accompanied by modulations of cellular immunity [168–170].

1.4.10 Probiotics in Allergy and Atopic Diseases of Children

One of the most interesting study results of the last years was the finding that probiotic bacteria do not exclusively stimulate immunity, but may modulate immune reactions in persons with allergies and atopic diseases or in at-risk infants [171, 172].

In a Finnish study [173] children who manifested atopic eczema during exclusive breast-feeding (nine children per group, on average 4.6 months old) received a hypoallergenic, extensively hydrolyzed formula on a whey basis without (control) or with 3×10^9 cfu/g *L. rhamnosus* or *B. animalis* ssp. *lactis*. After 2 months a significant improvement in skin condition occurred in patients given probiotic-supplemented formulas, but not in the controls. This was recorded both subjectively and objectively by physicians by means of a valuation scale (SCORAD).

Similar curative results were obtained with *L. rhamnosus* plus *L. reuteri* preparations [174], whereas *L. rhamnosus* did not show an effect in adults allergic to birch-pollen [175].

The incidence of atopic eczema in at-risk infants at two and four years of age was reduced to 50% through administration of *L. rhamnosus* to their mothers, one month before through six months after delivery, or to the infants themselves. This provided for the first time the option of a causal, preventive and/or therapeutic treatment of this disease [176–178].

However, studies in recent years yielded in part contradictory results. On the one side did probiotic single- and multi-strain cultures, in part combined with prebiotics, reduce the risk of atopic dermatitis in mice [179], children [180], high-risk children [181], school children [182], and children with food allergies [183]. This is also true for hey fever and for house dust and other allergies in children and mice [184, 185]. It was therefore suggested to stop the so-called "atopic march"[13] by an early application of probiotics. In other studies, however, probiotics did not ameliorate the complaints of children with neurodermatitis [187] and did not decrease the risk of atopic dermatitis or asthma in at-risk children [187, 188] and in long-distance run-

[13] The theorized "atopic march", in which atopic dermatitis (AD) precedes the development of asthma, is less well established than an association between AD and other allergic conditions.

ners [189]. More studies are needed in order to ascertain the findings and to find out the conditions under which probiotics may exert beneficial effects in the case of allergic illnesses.

Many mechanisms have been proposed for this beneficial effect, ranging from improved mucosal barrier function to a direct influence on the immune system, for example by the suppression of pro-inflammatory cytokines, by affecting regulatory T-cells and by improving the Th1/Th2-balance. Only living bacteria were effective in this way [190]. The modulation of the indigenous microflora during early life may be crucial, since it has been demonstrated that allergic infants have an aberrant intestinal microflora, containing more clostridia and less but more adult-type bifidobacteria [191]. However, the exact mode of action is not yet known. More in vitro and in vivo investigations and clinical trials are necessary for the future to elucidate the mechanisms of these effects and optimal conditions for application.

1.4.11 Inflammatory Autoimmune Diseases

Preliminary positive results from a rat study warrant further studies, especially in humans, to investigate whether probiotics with anti-inflammatory and immune regulatory properties may ameliorate arthritis and other inflammatory autoimmune diseases [192].

1.4.12 Cancer Prevention

Cancer-preventing properties are ascribed to probiotic bacteria in fermented milk products, but also in genuine yogurt cultures. Most studies dealt with probiotic effects on the colon carcinoma [193] being for decades the most frequent cancer of the intestinal tract in the Western industrial nations. Nevertheless, positive effects were also described for other types of cancer.

In mice, the growth of implanted or chemically induced tumors could be inhibited by injecting yogurt cultures or certain probiotic bacteria strains [194, 195].

A *L. casei* shirota preparation had a preventive effect on the recurrence rate of superficial bladder cancer after surgery in a controlled, double-blind study [196]. A large Japanese case control study [197] suggests that the habitual intake of lactobacilli and especially *L. casei* Shirota may reduce the risk for bladder cancer in the Japanese population. Besides this, only few epidemiological investigations have been performed concerning other probiotic strains and other types of cancer [198].

Several mechanisms have been suggested as a cause of these effects and have been investigated in vitro and in animal experiments:

- Inhibition of tumor-growth and proliferation of tumor cells by glycopeptides and cytotoxic metabolites of lactobacilli [38].
- Reduction of (pro)carcinogenic, mutagenic, and genotoxic substances (aflatoxines, nitrosamines; [15, 199]) and cancer-promoting enzymes (nitro-, azoreductase, β-glucuronidase) in the colon due to modifications of the gut flora, a decrease in pH, chemical modification, and ad- and absorption by the bacteria [23, 112, 200–202].
- Antimutagenic properties of probiotics and probiotic milk products [203, 204].
- Strengthening of the immune system and stimulation of the production of the tumor-necrosis-factor (TNF_α) by macrophages [196].

The factual relevance of these mechanisms of action on cancer risk is not known. Because of the long duration of carcinogenesis, it is difficult to investigate them in clinical human studies. More epidemiological data and more and longer lasting studies in humans using internationally recognized markers for cancer are necessary.

1.4.13
Hypocholesterolaemic and Cardioprotective Effects

The ability of different probiotic bacteria, above all members of the *L. acidophilus* group, to deconjugate bile acids in bile acidic and cholesterol-containing media, and to reduce their solubility, has been investigated in vitro. By coprecipitation with deconjugated bile acids, and by adsorption on/in the bacteria cell the cholesterol concentration in the medium is lowered by 50% [205–207]. Investigations in vivo on the mechanism of action are lacking. Therefore, a conclusive statement whether a thinning of the sterol pool can be obtained via these mechanisms in vivo, and finally a lowering of the concentration of serum and lipoprotein-cholesterol by ingestion of appropriate "probiotic" lactic acid bacteria is hardly possible. A placebo-controlled short-term study showed a transient decrease of the LDL cholesterol in serum of healthy adults by approx. 10% after intake of a probiotic milk product, fermented with *Enterococcus faecium* and *S. thermophilus* [208]. However, this effect disappeared with a longer observation period (> 6 months) and was no longer different from effects in the control group [209].

Apart from studies showing beneficial effects of *Enterococcus faecium* [210] and *L. plantarum* [211] on cardiovascular risk factors, a slightly increased HDL-concentration in sera of subjects consuming fermented dairy products for several months [212], or a direct cardioprotective effect of orally administered lactobacilli [213] most other studies found only transient [214, 215] or no effects at all [154, 216–219] of probiotics on serum lipids and more evidence and particularly clinical studies are required before improvement or even prevention of various ischemic heart diseases can be ascertained.

1.4.14 Probiotics for the Healthy Population?

Healthy people who regard their intestinal flora as balanced and their immune system as effective do often ask the question about the benefit of probiotics for the healthy consumer. The frequently given answer, that probiotics may prevent complaints due to occasional imbalances of an otherwise balanced system, is likely but still speculative, as long as one doesn't know enough about the composition of a balanced "healthy" microflora and its role on the host, especially on its immune system. On the other hand, proven or supposed health benefits like prevention or alleviation of occasional gastrointestinal complaints, common infectious diseases (e.g. cold) or atopic diseases of otherwise healthy people, as well as normalization of a decreased intestinal motility or reduction of certain long-term risks (cancer, ischemic heart disease) is surely of interest for the common population. In no case, however, consumption of probiotics should substitute a healthy lifestyle and a balanced nutrition.

1.5 Safety of Probiotics

The best evidence for the general safety of lactic acid bacteria and bifidobacteria is their long tradition of use without any harmful effects on human health [220, 221]. With the exception of one strain belonging to the *L. rhamnosus* species, lactobacilli and bifidobacteria used for food production are "generally recognized as safe" (GRAS) by the Food and Drug Administration of the USA. In Germany, all but two strains of lactobacilli and bifidobacteria are classified as "1" (absolutely safe) by the "Berufsgenossenschaft der chemischen Industrie" [222]. Moreover, certain strains of probiotic bacteria have been proven to be free of risk factors like: transferable antibiotic resistances, cancer-promoting and/or putrefactive enzymes and metabolites, hemolysis, activation of thrombocyte-aggregation or mucus degradation in the mucus layer of the gastrointestinal tract.

Despite the absence of a pathogenic potential, lactic acid bacteria were found in $< 0.1\%$ (enterococci 1%) of clinical samples from severe infections (endocarditis, meningitis, or bacteremia [223]). Most probably these bacteria originated from the indigenous microflora, whereby in many cases the translocation was facilitated by underlying disease, lesions or inflammations in the oral cavity and in the gastrointestinal tract, or by an impaired immune system.

Two cases have been published concerning food probiotics: in 1999 a *Lactobacillus* strain was isolated from a liver abscess which was undistinguishable from the food probiotic *L. rhamnosus* GG [224]. In a second case a man accidentally put the contents of a probiotic capsule (*L. rhamnosus*, *L. aci-*

dophilus and *Streptococcus faecalis*) into the mouth after a tooth extraction instead of swallowing the capsule without chewing. When an endocarditis occurred a short time later, the probiotic bacteria were recovered from the clinical sample [225]. And the probiotic yeast, *Saccharomyces boulardii*, was found in several cases of fungaemia, mostly in immunocompromised subjects or due to catheter infections, when suspensions of *S. boulardii* were prepared at the bedside [226, 227].

However, there is no evidence for a higher risk due to the ingestion of probiotic products in comparison with conventional products. This conclusion is supported by a study from Finland, where the consumption of *L. rhamnosus* GG has increased considerably during the last two decades without an increase in the incidence of infections by lactobacilli [228]. Moreover, studies in immune-compromised persons (HIV-positive subjects, patients with leukemia) did not show undesired effects [72], but rather positive effects as, e.g. lower incidence of Candida during a chemotherapy [68]. Health risks due to overdosage or long-term ingestion have also not been observed.

1.6 Probiotic Food

Apart from the health-promoting properties, probiotic microorganisms in foods have to fulfill a lot of other conditions. These include a sufficient stability during production and storage, so that the probiotic content of the food during the whole shelf life does not drop below the bacterial concentration required for a probiotic effect [229]. Survival and bacterial counts of probiotic microorganisms in the food, and the maintenance of its probiotic activity depend on the production process, on the properties of the product matrix, and on the physiological state of the bacteria. These include chemical composition, water activity, oxygen concentration, and redox potential, pH value, acid concentration, and synergetic or antagonistic interactions between conventional starter cultures and added probiotics.

Additionally, the quality of probiotic foods should not be less than that of corresponding conventional products. To avoid that metabolically active probiotic cultures adulterate taste, flavor, consistency, and shelf-life of the food through post-acidification, lypolysis and/or proteolysis probiotic milk products should be stored at $\leq 8\,^{\circ}$C [230]. Furthermore, probiotics producing bacteriocines may inhibit the activity of the conventional starter cultures and vice versa [231].

1.6.1 Fermented Milk Products with Probiotic Properties

Yogurt-like, solid or liquid milk products containing living probiotic bacteria are the most popular probiotic foodstuffs at the moment, whereas other

dairy and non-dairy probiotic products are seen far less on the supermarket shelves. One reason may be that consumers associate yogurt not only with palatability but also with health promotion. Even the idea that yogurt contains living bacteria does not scare the consumers. The large variety of fermented milk products allows a diversified and thus regular consumption. With the technically realizable probiotic concentrations in the product (10^6–10^9 cfu/g food), the current portions of 125 and 250 ml allow an intake of a relevant quantity of probiotic microorganisms.

The appropriate production process depends on fermentability and acid tolerance of the added probiotic microorganisms. Only a few can be used as sole starter cultures. In most of the cases fermentation occurs exclusively or predominantly through conventional starter cultures (*Streptococcus thermophilus* and others). The probiotic culture starter and the starter are added to the milk. In case of sensitive probiotics this occurs after fermentation. Hereby, the survival of oxygen-sensitive bacteria (e.g. bifidobacteria) in the product is favored by oxygen-consuming conventional starter cultures (*S. thermophilus*), and by the lowering of the redox potential. In Germany the prevailing consumer's desire for mildly acidified products favors the use of acid-sensitive probiotics.

Most of the marketable products have a consistency and appearance similar to that of set style yogurt or liquid yogurt. Probiotic variants of other fermented milk products like sour milk, sour whey, sour cream, buttermilk, or kefir are not very popular. In Europe, unfermented milk with added probiotics (sweet acidophilus milk, bifidus milk) are much less popular than yogurt. Positive human studies with these products have not been published yet.

1.6.2 Probiotic Cheese

Probiotic fresh or ripened cheeses are far-less popular foodstuffs than yogurt-like probiotic dairy products and seen far less on the supermarket shelves, although they can be an alternative for persons that do not like yogurt, for lactose-intolerant subjects, and in countries where yogurt is less popular than in Europe (e.g. USA, Canada).

One reason for this may be that consumers buy cheese mainly for its palatability. Furthermore, the relatively small serving sizes of cheese are believed to be a disadvantage, which requires increased concentrations of probiotic bacteria in the cheese. And last but not least, the cheese market is characterized by a great number of small manufacturers and established brand names of well-known manufacturers are rather the exception. This makes it difficult for the producers of probiotic cheese to establish a branded product and to amortize the high costs of research, legal provisions, and marketing.

In principle, probiotic bacteria may simply be added to the cheese together with the starter culture before renneting or clotting, respectively, or may oth-

erwise be mixed into the already cut curd. If probiotics are added to the cheese after fermentation, the physiological state of the probiotics is an important determinant of survival during ripening and storage [232, 233]. This state depends on (1) the nutritional composition of the growth medium of the probiotics in relation to the cheese, (2) harvesting of the culture (whether in logarithmic or stationary phase), (3) conditions leading to transition to stationary phase and (4) treatment of the probiotics during and after harvesting.

However, draining off the whey and—in the case of ripened cheese or cottage cheese—a scalding temperature of up to 55 °C may cause uncontrollable losses of probiotic bacteria. The long ripening time—several days in the case of certain surface-ripened soft cheeses, up to two years in the case of some extra hard cheeses—may prove negative for the survival of probiotic bacteria as well. Adverse effects on product or production quality can result from interactions between product and probiotic bacteria due to factors like pH, O_2, redox potential, water activity [234], proteolysis [235], and lipolysis [236], whereas reasons of antagonisms between starter culture and probiotic bacteria may be H_2O_2, benzoic acid, lactic acid, bacteriocines, and biogenic amines [231, 237–239].

On the other hand, cheese, perhaps with the exception of fresh cheese, might protect probiotic bacteria and particularly acid-susceptible bifidobacteria of human origin against acid due to its buffering capacity. The inclusion into the fat-protein-matrix of the cheese might protect probiotic bacteria against gastric juice, bile salts, and digestive enzymes during gastrointestinal passage.

1.6.2.1
Fresh Cheese

At first glance fresh cheese (quark, cottage cheese) appears to be particularly suited to serve as a carrier for probiotic bacteria, because it is produced without (prolonged) ripening, must be stored at refrigeration temperatures, and has a rather limited shelf-life.

Although there is one report on the manufacture of Argentinean Fresco cheese with added *Lactobacillus acidophilus, L. casei, Bifidobacterium bifidum*, and *B. longum* [240] reporting acceptable viable probiotics counts after 16 days of storage at ~5 °C, most published data show poor survival rates of potential probiotic bacteria in fresh cheese. This was explained above all by the low pH value in this type of cheese (~4.5). Viable bacteria counts in fresh cheese typically decreased by 1–2 log per week, falling below a minimum value of 10^6 to 10^7 cfu/g cheese after 15 days of storage at 4 °C [241].

Another problem, especially in the course of the manufacture of cottage cheese or "Hüttenkäse" is the rather high scalding temperature of up to 55 °C, which, however, may be circumvented by the admixture of the probiotics to cream and salt, which were added to the curd after heat treatment.

1.6.2.2 Ripened Cheese

Salting, the long period of ripening, or the scalding temperature proved not to be insurmountable obstacles for the production of probiotic ripened cheese. Although some studies showed a poor survival of the probiotics or unsatisfactory organoleptic properties of the cheese after the ripening period [242], most investigators successfully produced ripened cheese containing sufficient numbers of viable *Lactobacillus acidophilus, L. rhamnosus, L. paracasei, Bifidobacterium infantis, B. lactis*, or *Enterococcus faecium* [243–247]. The probiotics were added to the cheese milk or, more typically, as adjuncts together with or immediately after the starter.

In all these studies more than 5×10^6 cfu/g probiotic bacteria survived ripening periods between 5 and 39 weeks. Sometimes the cheese matrix improved survival of probiotic bacteria more than yogurt. After feeding *Lactobacillus paracasei* NFBC 348 or *Enterococcus faecalis* Fargo® 688 to minipigs, more probiotic bacteria were found in the small intestinal chyme or in the faeces, respectively, when they were administered in cheddar cheese instead of yogurt [245]. Furthermore, *E. faecium* in cheddar survived a 2 h incubation in gastric juice in vitro better then *E. faecium* in yogurt [245, 246].

1.6.2.3 Outcome of in Vitro Experiments and Animal Studies

Up to now there have been no clinical studies showing beneficial health effects of so-called "probiotic cheese". Most of the published investigations were confined to provide proof of survival and sufficient numbers of probiotic bacteria in cheese, and the term "probiotic cheese" was used as a synonym for cheese containing *Lactobacillus acidophilus*, bifidobacteria, bacteria of human origin or bacterial strains, for which probiotic properties have been reported in other matrices, e.g. in yogurt or in pharmaceutical preparations (Table 4). The term "sufficient number" was used when a regular daily serving contained 10^8 probiotic bacteria.[14] Accordingly hard cheese (daily consumption 1–3 slices à 30 g) should contain $\geq 3 \times 10^6$ cfu/g.

Several investigators tested the idea that the embedding of probiotic bacteria in the fat-protein-matrix of cheese may improve their survival. Vinderola et al. [240] demonstrated pH tolerance of strains of *B. longum*, *B. infantis*, *L. acidophilus*, and *L. casei* in homogenates of Argentinean Fresco cheese in HCl of pH 3. *Propionibacterium freudenreichii* and a *cidopropionici* from Emmental-like cheeses in artificial gastric and intestinal fluid showed improved survival and acid- and bile-tolerance in vitro, when Emmental cheese

[14] More precisely: the concentration should be so high, that a daily ration provides that amount of bacteria which exerted the respective probiotic effects in the corresponding scientific study.

Table 4 Probiotic bacteria strains used in cheese making and postulated health-related effects

Strain	Cheese	Survival in cheese cfu/g cheese (time of storage)	Effects (tested in cheese)	Postulated strain specific beneficial effects (on)[d] (not tested in cheese)	Refs.
L. rhamnosus GG	Gefilus cheese: Emmentaler Edam	3–4 slices of Edamer are equivalent to 150 mL yogurt		Survival of gastrointestinal passage, colonization of the – Rotavirus-induced diarrhea – Traveler's diarrhea – Antibiotic-induced diarrhea – M. Crohn – Constipation – Premature infants – Immune modulation – Allergy, atopic diseases – Prophylaxis of respiratory and gastrointestinal infections – Decrease of cancer promoting enzymes – Reduction of caries risk	Valio, Finland 2000 (company communication)
L. acidophilus LA 5 + B.*animalis* BB12	Soft cheese			Survival of gastrointestinal passage – Rotaviral diarrhea – Travelers diarrhea – Antibiotic-induced diarrhea	[248]
B. animalis BB12	Cheddar	6×10^7 (2 months)		– Infants – Modulation of the immune system –	[249]
B. animalis BB12	Cheddar	$\geq 10^8$ (6 months)		Allergy – Cancer – Serum cholesterol	[250]
B. longum BB536	Cheddar	$\sim 10^5$ (6 months)		Survival of gastrointestinal passage, modulation of the fecal flora – Less candida in immunecompromised subjects – Increased gastrointestinal well-being, reduction of antibiotic-induced diarrhea	[250]
L. paracasei NFBC 338	Cheddar	Survival and growth in cheese $> 10^8$ (6 months)	Better fecal recovery in cheddar than in yogurt[a]	Bile tolerance	[244]
E. faecium PR 68	Cheddar	Survival and growth in cheese $> 10^8$ (15 months)	Better survival of GI passage of *E. faecium* PR 68 in cheddar than in yogurt[c]	Bile and acid tolerance. Alleviation of symptoms of Irritable Bowel Syndrome[b]	[245, 246]

Table 4 (continued)

Strain	Cheese	Survival in cheese cfu/g cheese (time of storage)	Effects (tested in cheese)	Postulated strain specific beneficial effects (on)[d] (not tested in cheese)	Refs.
B.lactis Bo plus *L.acidophilus* Ki	Gouda; Goat ch.	Bo: $> 10^8$ (9 weeks) Ki: $> 10^6$ (9 weeks)		Bile tolerant. Establishment in intestinal ecology – Bactericidal effects on *S. typhimurium* and *C. difficile* – Cholesterol control	[242]
B.longum, *B.bifidus*, *L.acidophilus*, *L.casei*	Argent. Fresco	– 1 Log_{10}/2 months or less	3 h survival in a cheese-HCl-homogenate of pH 3		[240]
Propionibact. freudenreichii/ acidipropionici isolated from cheese	Emmen-taler-like		Survival in cheese juice of bacteria exposed to artificial gastric and intestinal fluid. Bile and acid tolerant		[251]

[a] Feeding 10^9 or 10^{11} cfu/d NFBC 338 in cheddar or yogurt, respectively, to three pigs led to a recovery of 10^5 or $10^{4.5}$ cfu/mL small intestinal chyme
[b] After an initial load by gastric intubation 17 patients with otherwise incurable IBS received for 4–30 months lyophilized E. faecium. Weekly examination of fecal samples; assessment of condition scores before and after treatment
[c] Feeding 1.3×10^{10} or 3.7×10^9 cfu/d PR 68 in cheddar or yogurt, respectively, to eight pigs led to a fecal recovery of 2×10^6 or 5.2×10^5 cfu/g feces
[d] Not tested in cheese

juice was added [251]. And in an Estonian smear-ripened, semi-soft cheese, to which 10^9 cfu/mL of *Lactobacillus fermentum* strain ME-3 had been added together with the starter culture, approximately 5×10^7 cfu/g ME-3 cells survived a ripening and storage period of about 54 days, sustaining moderate antimicrobial and high antioxidative activity [252].

Other investigators applied an inverse strategy: they isolated microorganism strains from cheese and tested their potential as candidate probiotics. Strains of *Lactobacillus plantarum* and *casei/paracasei*, isolated from unpasteurized Camembert [253] and yeast strains from blue cheese and kefir [254] were sufficiently acid, bile, and protease-resistant and adhered to CACO-2 cells. Yeast strains from Feta cheese [255] and some bacteriocin-producing, antimicrobial-active strains of *Enterococcus faecium* from Argentinean Tafi cheese [256] showed (limited) bile and acid resistance and in vitro cholesterol reduction.

The number of in vivo experiments is rather limited. In two animal studies it was found, that feeding three or eight pigs per group, respectively, with cheddar cheese containing *L. paracasei* NFBC 338 [244] or *E. faecium* PR 68 [245, 246] led to significantly higher mean fecal counts of the respective probiotic bacteria than feeding yogurt produced with the same bacteria. There was a positive serum IgG response in the probiotic group, but no effect on fecal coliforms or on pig growth, food efficiency, and animal health. Medici et al. [257] prepared a probiotic fresh cheese, which showed adequate survival through 60 days after manufacture of the starter (*Streptococcus thermophilus*, *Lactococcus lactis* A6) and added (potential) probiotic bacteria (*Bifidobacterium bifidus* A12, *Lactobacillus acidophilus* A9 and *L. paracasei* A13). Feeding the probiotic fresh cheese to mice was associated with an increased mucosal immune response in the small, but not in the large intestine. There was a significant increase in the phagocytic activity, the number of IgA-producing cells, and the $CD4^+/CD8^+$ T-cell ratio compared with a non-probiotic fresh cheese or no cheese.

Some health-related effects of cheese produced with probiotic bacteria are, according to conventional definition, not probiotic. The high microbial β-galactosidase-activity of cheeses (Canestrato, Cheddar [247]) supports lactose digestion in lactose-intolerant people, but this may be caused by non-probiotic lactic acid bacteria as well, and is not confined to viable microorganisms. Blood pressure-reducing (ACE-inhibitory) bioactive peptides are released by microbial proteolysis in Festivo cheese during ripening. In rat feeding studies this cheese did reduce blood pressure [258]. However, this health effect, too, does neither require living bacteria in the cheese after ripening nor survival of these microorganisms during gastrointestinal transit.

When established probiotic strains were used for cheese production, their health effects were not proven in clinical trials, where the bacteria were provided to subjects in a cheese matrix. Table 4 lists some of those strains which have already been in use for the production of probiotic cheese. Despite all

efforts almost no marketable probiotic cheeses exist so far. In 1999 a patent for production of probiotic cheese was granted, and in 2000 probiotic cheese containing Lactobacillus GG was introduced into the Finnish market. In Germany, the first cottage cheese called probiotic contained *L. acidophilus* La5 and *B. animalis* BB12 and appeared on the market in 1998. However, although Lactobacillus GG or LA5 plus BB12 are some of the best-characterized probiotic bacterial strains with well-established health-related properties, so far no data exists on their probiotic properties when supplied in a cheese matrix.

1.6.3 Other Probiotic Food and Food Ingredients

Apart from fermented milk products, including cheese and fermented whey-based drinks [259], other probiotic dairy and non-dairy probiotic food can be manufactured as well, using either metabolically active probiotic cultures or inactive, freeze- or spray-dried cultures or powdered probiotic dairy products. All these products have in common that their production has been described in the scientific or patent literature, but that they have not been tested in clinical trials and that they did not stay on the market for long.

1.6.3.1 Ice Cream

Ice cream with *acidophilus*- and bifidobacteria has been known since the 1960s. It is made without further fermentation by adding high-concentrated probiotic bacterial cultures, fermented milk products, or probiotic yogurt powder to the ice cream mixture, or by fermentation of a pasteurized ice cream mixture with selected non-probiotic and/or probiotic starter cultures. Appropriate strains of *L. acidophilus* and *Bifidobacterium* easily grow in the ice cream mixture, and produce acidity. Even if the final freezing of the ice cream mixture is accompanied by a considerable loss in the bacterial count, bacterial concentrations of $\geq 10^7$ cfu/g can be easily obtained in probiotic ice creams. These products have a good storability. In one study [260] probiotic ice cream was made by fermenting a standard ice cream mix with strains of *L. acidophilus* and *B. bifidum* and then freezing the mix in a batch freezer. During 17 weeks of storage at −29 °C *L. acidophilus* and *B. bifidum* counts as well as β-galactosidase activity in the product decreased from 1.5×10^8 cfu/ml, 2.5×10^8 cfu/ml or 1800 units/ml, respectively, to 4×10^6 cfu/ml, 1×10^7 cfu/ml or 1300 units/ml, respectively. Potentially probiotic frozen yogurt products were made in a similar manner using a standard [261] or acerola [262] ice cream mix, yogurt starters (*Streptococcus thermophilus* and *L. delbrückii* ssp. *bulgaricus*) and potentially probiotic bacteria (strains of *L. acidophilus* and *B. longum* in the first and *B. longum* plus *B. lactis* in the second study). The products could be stored at

– 20 °C for 11 or 15 weeks, respectively, without decrease in culture bacteria and sensory characteristics. No human studies have been performed to test health effects of the product.

1.6.3.2 Sweets

In other sweets, e.g. chocolate, bacterial counts similar to those in ice cream, are much more difficult to achieve. This and the small portion size and storage at ambient temperature are the reasons that (non-refrigerated) sweets are less appropriate vehicles for probiotic bacteria.

In the Anglosaxon countries frozen desserts, "cookies", and sweets with probiotic bacteria are being sold. In Japan, where bifidobacteria-containing functional foods are highly popular, seven brands of sweets with bifidobacteria were on the market already in 1993, besides 30 varieties of fermented (20), fresh (8), or powdered (2) milk products and 16 types of so-called "health food" (cited according to [263]).

1.6.3.3 Vegetable Food

Cereals ("flakes"), to which sugar and lyophilized probiotic cultures were added, were used as a simple, direct delivery vehicle for dried probiotics. Fermented cereals and other fermented vegetable products (e.g. "Sauerkraut", Kimchi, or "pickles"), although containing live lactobacilli, have up to now not been tested for probiotic health effects, nor have such effects been claimed.

1.6.3.4 Meat Products

Raw sausages are made from raw processed meat, i.e., the meat is not boiled or otherwise heated even during the further course of processing. Raw sausages are subdivided into spreadable types (German Mett- and Teewurst) and firm types, which are either cold-smoked (German "Landjaeger") or air-dried (Salami, Cervelat sausage). They are reddened and preserved by drying, smoking, and/or acidification by adding glucono-δ-lactone or by microbial fermentation. Fermentation takes between 3 to 4 days (e.g. German "frische Mettwurst" or "Teewurst") and about 6 months, as in the case of Italian salami [264]. Whereas in Southern Europe the spontaneous and accidental inoculation with the natural "local" microflora predominates, in Northern and Central Europe about 80–100% of industrially manufactured raw sausages are fermented by adding commercial starter cultures. These starters directly affect shelf life, nitrate reduction and flavor, texture and color of the final

product. Starter microorganisms used in the meat industry include the genera *Lactobacillus*, *Pediococcus*, *Staphylococcus*, and *Kocuria*, as well as certain yeasts and molds.

Typical viable lactobacilli cell counts in the sausage mixture and in the final product go beyond 10^8 cfu/g. Therefore, it should be possible to add probiotic lactobacilli in sufficient concentrations by mixing them with the starter culture [265]. Indeed, certain probiotic strains (*L. rhamnosus* GG, *Bifidobacterium animalis* Bb12) have been shown to be applicable for raw sausage manufacture, but probiotic health effects of the "ready-to-eat" sausages, however, have not been proven in human studies [154, 266].

1.6.3.5
Dried Probiotic Products

Most bacteria require a water activity of about 0.98 in the product matrix for survival and growth. In order that (probiotic) bacteria do survive in foods, pharmaceutical products and other delivery systems for an extended period of time, the water activity needs to be either high enough that the bacteria can maintain a normal metabolic activity or otherwise low enough that the bacteria can survive in an inactive state. The latter approach requires drying of the bacteria cultures, which can be carried out by freeze-drying (lyophilization) or by spray-drying.

Drying means a considerable stress for the bacteria, associated with cell damage and decreased viability, not only due to mechanical stress and enhanced temperature [267], but also to the process of drying per se. The (nearly complete) water-loss causes protein denaturation, protein destabilization and (partial) removal of proteins from the cell surface [268], and transformation of the liquid-crystalline structures of the phospholipid bilayer of the bacterial cell membrane into a gel phase [269]. If this phase separation is not completely reversed after rehydration, leaks in the membrane and disturbed molecular transport may remain [270]. Therefore, cells should be stabilized before drying by the addition of protective substances, for example hydrophilic polyhydroxy compounds like sugars [269, 271] or skimmed milk powder [272], which partly can replace the missing water molecules.

Spray-dried probiotic bacteria can be applied directly in the manufacture of probiotic infant food as well as of sweets and confectionary pastries. Alternatively milk powder (skimmed milk, whey, buttermilk, or yogurt powder) can be used as a delivery system. Probiotic milk powder is obtained by spray or freeze drying of the respective fermented or unfermented, probiotics containing milk product, or by adding the spray- or freeze-dried probiotic culture to the respective milk powder [263]. Survival of the bacteria in the dried products will be improved by increasing the dry matter of milk, whey, buttermilk, or yogurt through evaporation or sugar addition before processing, and by spray drying the partially neutralized, cooled milk product

concentrate after the addition of starch, lactose, and stabilizers (sodium citrate, dihydrogen phosphate) at 70 °C. To avoid too early germination, the microorganisms have to be integrated into a low-water matrix, or kept frozen until consumption.

Further improvement in spray-drying techniques is necessary to avoid cell damage and loss of viability of the probiotic bacteria [273–275]. On the other hand, certain manufacturers of starter cultures, although unpublished in the scientific literature, have the technology to produce stabilized lyophilisates of probiotic bacteria, that retain a high level of viability during storage [12]. Therefore, despite the higher price compared with spray-dried products, incorporation of lyophilized probiotic bacteria into powdered milk products may be the procedure of choice, at least for premium products.

All things considered, the manufacture of probiotic milk powder containing more than 10^8 cfu/g probiotic bacteria is possible and has been published. However, there is little information available on the stability of probiotic bacteria in powdered milk products and on the persistency of probiotic efficacy.

1.6.3.6 Microencapsulated Probiotics

During the last two decades numerous efforts have been made to embed metabolically active bacteria as well as lyophilized or spray-dried cultures in microcapsules or microparticles, in order to enhance their stability in, to extend the shelf-life of the corresponding probiotic food products manufactured with them and to improve viability of the probiotics after ingestion. Embedding them in polymers like alginate is a promising procedure to stabilize metabolically active bacteria [276]. For that purpose a bacteria-containing aqueous solution of the respective polymer is emulsified in oil and hardened by the addition of polyvalent metal ions (mainly Ca^{2+}) or dropped into a solidification solution of polyvalent ions using a vibration nozzle, a piezoelectric nozzle, or a coaxial air-jet. The most commonly reported encapsulation method for probiotic bacteria is embedding them in calcium-alginate gel microcapsules, other potentially suitable polymers are κ-carrageenan, guar gum, gelatin or starch. Other procedures have been reported, including spray-drying and coating, extrusion, emulsion and phase-separation techniques.

In some [277] but not all [278] studies alginate-encapsulated, potentially probiotic bacteria (e.g. strains of *L. acidophilus*) showed increased survival in frozen milk products and enhanced gastric and bile acid tolerance. However, in recent studies in pigs and humans alginate capsules after administration were not disintegrated in the intestine and did not release their contents. Therefore, this type of microcapsule, although frequently used, seems to be unsuitable as a carrier for probiotic bacteria [272].

To protect dried cultures of probiotic bacteria against rehydration and unintended germination in a humid or aqueous food matrix, they can be encapsulated in food-grade hard fat particles. Such particles could be produced from suspensions of the spray- or freeze-dried bacteria in melted fat by two techniques: by dropping the fluid suspension into a cooled solidifying bath or by grinding down the bacteria-fat suspension after congealing. When fat microparticles with melting points near body temperature were administered to pigs or men, they disintegrated in the gastrointestinal tract due to fat-softening, the gastrointestinal peristaltic and/or lipase activity [272].

However, until now, neither polymer encapsulation nor hard fat techniques had resulted in sufficiently small, impermeable, and protective microcapsules or -particles to provide the large numbers of shelf-stable, viable probiotic bacteria necessary for use in industrial processing, and no human in vivo studies have been published showing beneficial health effects of encapsulated probiotic bacteria in a food matrix. Therefore, the number of reports and patents concerned with small-scale microencapsulation of probiotic bacteria for use in the food industry, and the number of food items containing encapsulated probiotic bacteria are inversely related.

2 Prebiotics and Synbiotics

2.1 Prebiotics—The Definition Revisited

A prebiotic was first defined in 1995 by Gibson and Roberfroid [2] as

> "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health."

Especially the third criterion for prebiotic properties—improvement of health by selective stimulation of the growth and activity of a limited number of colonic bacteria—which is implied in this definition, is difficult to verify. An answer to the question, how many strains of "positive" bacteria are "a limited number" can hardly be given. It is also difficult to test the selective stimulation of individual bacterial strains between the more than 400 cultivable and non-cultivable bacterial strains in the human gut. The demonstration, that a potential prebiotic increases the cell counts of individual bacterial strains is not a sufficient test of prebiotic properties, but at most a screening parameter.

Therefore, the authors revisited their concept and proposed a new definition [279, 280]:

> "[A prebiotic is] a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well being and health."

This new definition, after all, results in an equalization of "prebiotic" and "bifidogenic". This shows also in the fact that Roberfroid defined a so-called prebiotic index. This index gives the absolute increase of the fecal bifidobacteria concentration per gram of daily consumed probiotics. Prime criterion is the effect on the intestinal flora, not a (potential) health effect derived from this change. As the prebiotic or rather bifidogenic effects depend on the type and concentration of the prebiotic and on the bifidobacteria concentration in the intestine of the host, no simple dose-effect relationship exists. According to the opinion of the author only carbohydrates like inulin and oligofructose (OF) [281], (*trans*-)galactooligosaccharides (TOS or GOS) or lactulose, which are non-digestible but can be fermented by the intestinal flora, fulfill the criteria (see Table 5; [282]).

According to this definition, candidate prebiotics must fulfill the following criteria which are to be proven by in vitro and—finally—in vivo tests:

- Non-digestibility
  Resistance to gastric acid, enzymatic digestion, and intestinal absorption was demonstrated in vitro [283] or in vivo using germ-free or antibiotic-treated rats [281], proctocolectomized individuals (ileostomy patients [284, 285]) and other models measuring recovery of undigested prebiotics in feces, in the distal ileum or in small intestinal effluent, respectively.
- Fermentation by the intestinal microbiota
  is often measured in vitro by adding the respective carbohydrates to fecal slurry, suspensions of colon contents, or pure or mixed bacteria cultures in an anaerobic batch or continuous culture fermentation system [286]. In vivo experiments are often performed in rats or heteroxenic rats harboring a human fecal flora [287]. The prebiotic can be admixed to food or drinking water, and the animals will be sacrificed in pre-defined time intervals to collect and analyze gastrointestinal contents and feces. Intestinal fermentation in humans can be investigated by measuring breath hydrogen or fecal recovery of the administered carbohydrate after a single prebiotic meal.
- Selective stimulation of growth and activity of intestinal bacteria
  The selectivity of the promotion of microbial growth and fermentation activity by prebiotic oligosaccharides is difficult to be proven by in vitro experiments, because the complexity and temporal variations of the intestinal microflora and differences between the segments of the gastrointestinal tract can hardly be simulated. The best in vitro model for that purpose is to measure bacterial counts in fecal samples (or intestinal content) before and during exposure to the test material in batch or multichamber fermentation systems [286].

Table 5 Prebiotic oligosaccharides, candidate prebiotics, and "colonic food" [282]

Prebiotic	Structure	Source	Proven effect
Prebiotic oligosaccharides			
Fructooligosaccharides (FOS)			
Fructooligosaccharides	α-D-Glu[-(1 $\rightarrow$ 2)-β-D-Fru]$_n$, $n = 2$–4	Transfructosylation of Sac by β-Fru	B↑, P↓
Oligofructose	[α-D-Glu-]$_m\beta$-D-Fru[-(1 $\rightarrow$ 2)-β-D-Fru]$_n$, $m = 0$–1, $n = 1$–9	Enzymatic hydrolysis of inulin	B↑, P↓
Inulin	α-D-Glu[-(1 $\rightarrow$ 2)-β-D-Fru]$_n$, $n = 10$–60	Chicoree (hot water-extraction)	B↑, P↓
Galactooligosaccharides, Trans-galactosyloligosaccharides (TOS)	α-D-Glu-(1 $\rightarrow$ 4)-β-D-Gal[-(1 $\rightarrow$ 6)-β-D-Gal]$_n$, $n = 1$–4	Transgalactosylation of lac by β-Gal)	B↑, P↓
Soy bean oligosaccharides: raffinose ($n = 1$) + stachyose ($n = 2$)	[α-D-Gal-(1 $\rightarrow$ 6)-]$_n\alpha$-D-Glu-(1 $\rightarrow$ 2)-β-D-Fru, mit $n = 1$–2	Soy beans	B↑
Oligosaccharides, undigestible but fermentable in the colon ("colonic food")			
Lactulose	β-D-Gal-(1 $\rightarrow$ 4)-β-D-Fru	Lac (alkaline isomerization of Glu)	B↑, P↓, PM↓
Lactosucrose	β-D-Gal-(1 $\rightarrow$ 4)-α-D-Glu-(1 $\rightarrow$ 2)-β-D-Fru	Lac + Sac (transfructosylation by β-Fru)	B↑
Glucooligosaccharides (GOS)		Sac + Mal (transglucosylation by GlT)	B↑, nnE↓
Xylooligosaccharides (XO)	β-Xyl[-(1 $\rightarrow$ 4)-β-Xyl]$_n$, $n = 1$–8	Extraction of corn cobs → xylan → hydrolysis	B↑
Gentiooligosaccharides	β-D-Glu[-(1 $\rightarrow$ 6)-β-D-Glu]$_n$, $n = 1$–4	Glucose syrup (enzymatic transglucosylation)	B↑
Isomaltooligosaccharides (IMO)	α-D-Glu[-(1 $\rightarrow$ 6)-α-D-Glu]$_n$, $n = 1$–4	Hydrolysis of starch (α-Amy → β-Amy + α-Glase)	B↑
Maltooligosaccharides	α-D-Glu[-(1 $\rightarrow$ 4)-α-D-Glu]$_n$, $n = 1$–6	Hydrolysis of starch (Iso-Amy + α-Amy)	pB↓
Cyclodextrines	[-α-D-Glu-(1 $\rightarrow$ 4)-]$_n$, cyclic, $n = 6$–12	Hydrolysis of starch (CmGt)	B?, L↓, nF
Chito-oligosaccharides	[β-GluNAc-(1 $\rightarrow$ 4)-]$_n$	Chitin (shrimps)	Antimicrobial, nF
Polysaccharides like starch, hemicelluloses, pectines, and gums are undigestible but fermentable "colonic foods"			

Abbreviations: Glu = Glucose, Fru = Fructose, Gal = Galactose, Xyl = Xylose, Sac = Saccharose, β-Gal = β-Galactosidase (EC 3.2.1.23), β-Fru = β-Fructofuranosidase (EC 3.2.1.26), GlT = Glucosyltransferase, α/β/Iso-Amy = α/β/Iso-Amylase, + α-Glase = α-Glucosidase, CmGt = Cyclomaltodextrin-Gluconotransferase (EC 2.4.1.19), eX = Endo-1,4-β-Xylanase (EC 3.2.1.8), B↑= bifidogen, P = putrefactive/pathogenic bacteria, PM = putrefactive metabolites, NH_3, nnE = neonatal necrotising Enterocolitis

Modern molecular biological methods which are used for strain detection and identification or for the analysis of a whole bacterial community bypass some of the difficulties associated with culture-based technologies, especially the necessity of strictly anaerobic sampling or the impossibility to detect unculturable bacteria. Fluorescence in situ-hybridization (FISH) allows detection of cultivable and non-cultivable bacteria by incorporating specific fluorescence labels into bacteria cells in situ. For that purpose short sequences of single-stranded DNA, which are complementary to DNA sequences of the bacteria, are prepared. After binding to bacterial DNA (hybridization), the probes, which have been labeled with fluorescent tags, allow visualization of the respective bacteria by microscopy.

The polymerase chain reaction (PCR) and techniques based on this reaction do not detect the bacteria themselves, but characteristic sequences of bacterial DNA or RNA, respectively (16S rDNA, 16S rRNA). Examples of molecular genetic techniques, which can be used to study microbial communities are: total DNA isolation and characterization, G+C composition, PCR amplification of rDNA linked with denaturing gradient gel electrophoresis (DGGE), PCR amplification of functional genes, rRNA sequences and in situ hybridization of rRNA oligonucleotide probes [288].

2.2 Composition and Technological Properties of Prebiotic Oligosaccharides

With the exception of inulin, which is a mixture of fructooligo- and -polysaccharides, the known prebiotics are mixtures of undigestible oligosaccharides, i.e. chains consisting of 3 to 10 carbohydrate monomers (Table 5). Since 1980, oligosaccharides have been increasingly used by the food industry (beverages, sweets) for modifying viscosity, emulsification capacity, gel formation, freezing point, and color of foods. They show nutrition- and health-relevant properties like moderate sweetness (typically 30–60% of the sucrose value), low cariogenicity, a low calorimetric value, and a low glycemic index.

They exhibit properties typical of dietary fibers. That means, that they are not, or only to a small extent, hydrolized by the digestive enzymes of the human intestinal tract but serve as a fermentable substrate in the colon, above all for bifidobacteria. In this process, they are metabolized to short-chain fatty acids (acetic, propionic, and butyric acid), lactic acid, hydrogen, methane, and CO_2. For example, the $(1 \rightarrow 2)$-bond between the fructose- and glucose unit of fructooligosaccharides resists the human digestive enzymes, whereas most bifidobacteria possess the respective β-fructosidase [289].

In the Anglo-Saxon language, indigestible carbohydrates, which are fermented in the large intestine, are sometimes called "colonic food", as they support indirectly the host organism through a supply of energy, metabolizable substrates, and essential nutrients. Table 5 gives a survey of commercially

used bifidogenic oligosaccharides. From these, only the natural and synthetic fructooligosaccharides, galactooligosaccharides, and oligosaccharides from soybeans are counted as prebiotics [290–292]. The remaining carbohydrates of the table represent "colonic food" as they are metabolized in the large intestine by more than a limited number of "beneficial" bacteria.

2.3
Health Effects of Prebiotics

As a consequence of the modified definition of prebiotic [280], the question has to be answered: whether prebiotic health effects must be demonstrated in human studies for each carbohydrate, each effect and for each target group, or if the demonstration of increased bifidobacteria or lactobacilli cell counts or a decrease in potential harmful bacteria is a sufficient criterion for health promotion. Because of methodical difficulties and insufficient knowledge of the composition of a "healthy" intestinal microbiota and the complex interactions between its members, it is hard to deduce concrete preventive or curative health effects from changes in bacterial cell counts, even if those changes, such as the bifidogenic effect, are generally regarded as positive. Therefore, a final proof of health relevant effects by controlled human intervention studies should be performed.

The association between the strength of the bifidogenic effect and the amount of prebiotics administered is only weak [280], because the increase in bifidobacteria cell counts after prebiotic ingestion depends mainly on the actual number of bifidobacteria in the host.[15] Although in some human studies 4 g inulin or its hydrolysis product oligofructose were administered [293] or even less [294], health-relevant effects [38] of inulin and oligofructose were demonstrated only with daily intakes of > 8 g/day.

2.3.1
Prebiotics are Dietary Fibers

Prebiotic carbohydrates are dietary fibers, as they are not digested by human enzymes but fermented by the flora of the large intestine. Thus, they increase biomass, feces weight, and feces frequency, have a positive effect on constipation and on the health of the mucosa of the large intestine [295, 296].

[15] However, the reverse conclusion, that a very low dose of inulin or oligofructose might be as effective as a significantly higher one, is wrong as well. Otherwise an increase in the daily amount of prebiotics ingested, e.g. from 2 to 10 g/d, would provide no additional positive effect. This would mean that prebiotic effects are impossible, because the mean dietary intake of inulin and other bifidogenic oligosaccharides in industrialized countries is already about 4 g/d (Europe 3–9 g/d, USA 1–4 g/d).

2.3.2
Impact on the Intestinal Flora

Positive effects of pre- and synbiotics on the intestinal flora [297, 298], i.e. growth-promotion of potentially protective bacteria (bifidobacteria and in part also lactobacilli) and/or the inhibition of potentially pathogenic microorganisms, as well as stabilization of the intestinal environment by lowering the pH and release of short-chain organic acids, have been investigated and confirmed frequently in in vitro and in vivo trials. Inulin, oligofructose, or TOS as well as their synbiotic combination with probiotic bacteria (strains of *L. plantarum*, *L.paracasei*, or *B. bifidum*) increased bifidobacteria and lactobacilli or inhibited various human- and animal pathogenic bacteria strains (*Clostridium* spec., *E. coli, Campylobacter jejuni, Enterobacterium spec., Salmonella enteritidis*, or *S. typhimurium*) in vitro [299] or in mice [300], piglets [301], or humans [302, 303].

Only relatively few studies observed or examined at all preventive or therapeutic health effects resulting from this. At least there are some experimental indications as to the beneficial effects of inulin, oligofructose, or galactooligosaccharides, given alone or as a synbioticum, in the case of experimental colitis in rats [304], of rotavirus-induced, *C. difficile*-associated and other diarrheas [303, 305], and of refractory enterocolitis [306]. The administration of 12 g/day oligofructose for prevention of traveler's diarrhoea showed moderate success [307], while the frequency of antibiotic-associated diarrheas in the elderly [308] or children [309], infectious diarrheas in children [310] as well as diarrheas associated with an irritable colon [311] could not be reduced significantly. There are no recent findings concerning the potential application of prebiotics in the case of obstipation.

2.3.3
Cancer Prevention

In different animal models (rats, mice), feeding inulin and/or oligofructose did reduce the genotoxicity of fecal water [312],[16] decreased the number of chemically induced[17] pre-cancerogenic lesions (aberrant krypt foci [313, 314]) and stimulated defense functions, amongst others, an increased level of IL-10 and of NK-cell activity [315]. On a longer-term basis, the tumor incidence in the large intestine [316] and in other organs (breast cancer in rats and mice, metastases in the lung [317]) was lowered by adding 5 to 15% inulin or oligofructose to the diet. This effect was even more pronounced when a combination of prebiotics and probiotics was given [318].

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[16] Risk factor for colon carcinoma.

[17] By azoxymethane or dimethyl hydrazine.

The following mechanisms are discussed:

1. Production of short-chain fatty acids (lactic, acetic, propionic, and butyric acid) during fermentation of prebiotic carbohydrates. A more acidic pH and modulations of the intestinal flora, especially growth stimulation of carbohydrate-fermenting bacteria, decreased the concentration of putrefactive[18], toxic, mutagenic, or genotoxic substances and bacterial metabolites, as well as of secondary bile acids and cancer-promoting enzymes;
2. Butyric acid supports the regeneration of the intestinal epithelium;
3. Immune modulation.

However, the question whether these mechanisms are relevant for human health and cancer prevention cannot be answered from clinical intervention studies due to experimental difficulties and ethical limitations.

2.3.4 Effects on Lipid Metabolism

Inulin and oligofructose modulate hepatic lipid metabolism in rats and hamsters fed a so-called "western-style" diet, which is rich in fat and energy, and low in dietary fiber. Postprandial cholesterol and triglyceride levels in serum were reduced by 15% and up to 50%, respectively [319, 320], and the triglyceride accumulation in the liver was inhibited [321], mainly through a decreased lipogenic enzyme activity and a reduced VLDL particle concentration. In LDL-receptor-knockout ($LDLR^{-/-}$)-mice with spontaneous hypercholesterolemia (elevated LDL + IDL[19]) and atherosclerosis the daily administration of a diet rich in carbohydrates and fat plus 10% inulin over a period of 16 weeks lowered the total LDL and VLDL cholesterol concentration but not the HDL cholesterol concentration, and reduced not significantly atherosclerosis in the aorta (measured as intima:media ratio) by 15% [322].

In humans the findings are more controversial, possibly as the fatty acid synthesis in the liver plays a lesser role in man than in rodents. Three out of nine clinical studies with inulin and oligofructose showed no effect, whereas in four investigations the triacylglycerol and total cholesterol concentration and/or the total and LDL-cholesterol concentration in serum were significantly lowered [323–325]. In a more recent review the authors came to the conclusion that probiotics, prebiotics, or synbiotics only lowered the cholesterol level in hypercholesterinemic, whereas a reduction of the plasma triglyceride level was observed in normolipidemic persons [326].

There is less evidence of beneficial effects of prebiotics on other symptoms and diseases associated with the metabolic syndrome (overweight/obesity,

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[18] Putrefactive: causing the (typically) anaerobic decomposition of organic material, especially of proteins, with the formation of foul-smelling incompletely oxidized products.

[19] IDL: intermediate density lipoproteins.

disorders of lipid metabolism, atherosclerosis, hypertension, insulin resistance/diabetes). In rats oligofructose or inulin reduced energy intake with food and the proportion of body fat [327], and in humans inulin reduced the fasting insulin levels [328]. Whether and to what extent prebiotics may be able to decrease the risk of atherosclerosis and heart attack is not clear.

2.3.5
Stimulation of Mineral Adsorption and Bone Stability

Lowering the pH in the gut improves the absorption of calcium,[20] iron, and magnesium in the large intestine, probably due to an increased mineral solubility. It was demonstrated in ovariectomized rats, an established osteoporosis model, that lowering the pH increases bone mineralization, inhibits bone degradation induced by estrogen deficit, and preserves the bone structure [329, 330].

Beneficial effects on calcium absorption and bone mineralization were also demonstrated in pigs [331] and humans [332–334]. To the contrary there was no significant effect of fructooligosaccharides plus CPP[21] on the absorption of calcium phosphate in young adults [335]. A decrease of the risk of osteoporosis has not been shown to date.

2.3.6
Immunomodulatory Properties

Although inulin and oligofructose have no direct immunogenic effect, they can, by influencing the intestinal flora, indirectly modulate various parameters of the immune system, like the NK-cell activity, the secretion of IL-10 and interferon, and the lymphocyte proliferation [316, 336–338]. Mice, which were fed inulin or oligofructose for six weeks showed an increased T-cell activity, higher resistance against microbial infections and lower mortality when afflicted with enteral (*Candida albicans*) and systemic (*Listeria monocytogenes*, *Salmonella typhimurium*) infections [339]. The administration of inulin to rats with chemically induced colitis had an anti-inflammatory effect and reduced lesions of the intestinal mucosa [304]. In a small group of elderly people oligofructose had no immunostimulating effect [340], whereas a synbioticum from galactooligosaccharides, *Bifidobacterium breve*, and *Lactobacillus casei* had an immunotrophic effect in heavily diseased infants with laryngotracheo-esophageal cleft [341].[22] Potential benefits from applications of prebiotics in the case of allergic diseases were not examined [342, 343].

[20] That under these conditions contributes also to calcium supply in humans.

[21] Caseino-phosphopeptides.

[22] Rare, hereditary deformity in infants in the area of the larynx, trachea, and oesophagus, being easily inflamed and then life-threatening.

2.3.7 Infant Formulae

In recent years efforts have been made to promote softer and more acid (pH 5–6) "infant feces" also in bottle-fed babies and to induce an intestinal flora with high bifidus content similar to that of breast-fed babies in the first 2 to 3 months [344, 345]. This was done by feeding infant formulae on a milk basis, to which either probiotic bifidobacteria and lactobacilli [344–347] or bifidogenic fructo- and galacto-oligosaccharides were added.

The measurable success, i.e. a beneficial health effect for the infant, is the only decisive factor for the choice of the applied fructo- and galacto-oligosaccharides. An attempt to copy the conditions in human milk has not been made because the industrially prepared prebiotics do not (actually) by far reach the complexity of the more than 130 different oligosaccharides and glycoconjugates in human milk. The so-called mother's milk oligosaccharides are present in human milk in very high concentrations (12–14 g/L) compared to cow's milk (< 1 g/L), they can be short- or longer-chain, linear or branched chain, neutral or acidic, and apart from simple sugars like galactose, glucose, and fructose they also contain sugar derivatives like amino sugars or uronic acids. They play a major role in the bifidogenic, protective, and immunomodulating properties of human milk [344, 345]. At least at the present time, a further property of human milk can (still) not be simulated with commercially available prebiotics, namely the inhibition of the adhesion of (pathogenic) bacteria on endothelic cells. This inhibition is due to the fact that certain, more complex oligosaccharides in human milk are receptor-analogues to the adhesion molecules of the intestinal mucosa [344, 345].

Although the results have to be corroborated by further studies, and above all the added prebiotics need to be further optimized as to quantity, structure, and composition, several studies with beneficial effects have already been published. Above all the addition of oligofructose or (more frequently) galacto-oligosaccharides or both to conventional infant formulae in quantities from 0.4 to 1 g/100 mL for feeding periods of 3 to 12 weeks led to a significant increase in bifidobacteria in the fecal flora from 20% to approx. 60% (breast-fed babies ∼80%), and to feces characteristics similar to that of breast-fed babies [348–352].

Additionally, experiments in animals as well as studies in infants and children (1 and 14 years), show other possible advantages of supplementing infant food with prebiotics, probiotics, or synbiotics like, for example, less necrotizing enterocolitides [353, 354] or less rotavirus- and otherwise-induced diarrhea in children [355, 356]. In children in Thailand, Brazil, Mexico, Spain, and Portugal suffering partly from malnutrition, administration of prebiotics led to an increase in calcium adsorption and improved growth and health as well as immunostimulation and relief of atopic and allergic problems [334, 357–359].

2.3.8 Adverse Effects of Prebiotic Carbohydrates

Because of fermentation in the large intestine, the ingestion of higher quantities of prebiotics may lead to flatulence, abdominal disorders, and diarrhea. In general, 10–20 g oligofructose or inulin, regardless of whether ingested in a liquid or solid meal, are considered to be without side-effects. In a trial with 80 healthy probands the ingested quantity, after which at least one of the tested symptoms (headache, belching, flatulence, bowel contractions, or liquid stools) had been observed, was between 31 and 41 g oligofructose, corresponding to 0.04–0.06 g/kg body weight [360]. Nevertheless, some investigations and personal communications revealed that some of the probands felt they had gastrointestinal disorders after the ingestion of significantly smaller quantities of prebiotics (down to < 2 g). Whether this is due to the composition of the subjects' intestinal flora, or to a higher sensitivity against gases and other products of the prebiotic fermentation, is not known.

2.3.9 Prebiotic and Synbiotic Food

Every year a remarkable number of new food and drink items are launched onto the market, to which fructooligosaccharides and other prebiotic carbohydrates, mostly inulin and oligofructose, have been added. In contrast to probiotic food, however, only a minority of them, if any at all, is seen by consumers as a food of its own kind. The consumers see prebiotics more as a health-promoting additive to normal food—analogous e.g. to vitamins. Furthermore, often only small amounts, less than 4 g per daily serving, are added, perhaps to avoid the risk of gastrointestinal complaints and indisposition in sensitive individuals.

Very often prebiotics are added to probiotic foods, whereby their concentration in the product is typically below 10%. For this combination, the term "synbiotic" has been coined. For example, two European companies from France and The Netherlands, respectively, launched combinations of *L. acidophilus* strains with fructooligosaccharides (FOS) or inulin, respectively, in the market, claiming to lower blood cholesterol.

Andersson et al. [361] defined synbiotics as mixtures of probiotics and prebiotics that beneficially affect the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract of the host. This expression, however, should only be used in the case of a true "synergistic" mutual reinforcement. Most food items containing both probiotic bacteria and prebiotic carbohydrates do not fulfill this criterion. Either the amount of the prebiotic per serving is too low to ascertain an effect, as is the case in various fermented products on the German market, which contain approximately 2.5 g inulin or oligofructose, respectively, in order to avoid gas-

Table 6 Recent investigations of potential synbiotics

Subjects	Test groups	Duration	Results	Refs.
129 AOM-treated rats	S: rat diet + 100 g (I + OF)/kg + (5×10^{11} cfu LGG + 5×10^{11} cfu Bb12)/kg C: rat diet P_1: rat diet + 100 g (I + OF)/kg diet P_2: (5×10^{11}LGG + 5×10^{11}Bb12) cfu/kg	32 weeks	Adenomas/rat $S = P_1^* < P_2 = C$ Cancers/rat: $S < P_1^* \leq P_2 = C$ Apoptose Index: $S \leq P_1 < P_2^* > C$ Cecal SCFAs: $S = P_1^* > P_2 = C$	[318]
80 Rats	S: rat diet + 100 g (I + OF)/kg + (5×10^{11} cfu LGG + 5×10^{11} cfu Bb12)/kg P_1: rat diet + 100 g (I + OF)/kg diet P_2: (5×10^{11}LGG + 5×10^{11} Bb12) cfu/kg C: rat diet	4 weeks	Ileal sIgA: $S^* \geq P_1 \geq P_2 > C$ Cecal sIgA: $P_1^* \geq P_2 \geq S > C$ IL-10 prod. in PP: $P_1^* \geq P_2 \geq S > C$ IFN prod. in PP: $P_1 \geq S \approx P_2 = C$	[315]
Weaning piglets	S: *L paracasei* + (FOS) P: *L. paracasei* C: placebo		Fecal anaerobes$_{total}$: $S^* > P = C$ Fecal aerobes$_{total}$: $S^* > P = C$ Fecal bifidobact$_{total}$: $S^* > P = C$	[362]
129 Children, 1–6 y, AB treated	S: Supplement + 3.5 g/LFOS + 10^9 cfu/g (*L. acidophilus* + *B.* spec.) C_1: Supplement C_2: Fruit flavored drink	14 days post AB	% Subj. without illness: S(94), C_1(88), C_2(81) Weight gain: $S > C_2^*$ Fecal Lactobacilli: $S > C_2$	[363]
90 Critically ill patients	S: 15 g/d OF + 10^{10} cfu/d prob.mix C: placebo	8 days	Incidence of pathogenic bacteria: S(45%) < C(75%) Translocation&Sepsis: S = C	[364]
7 AOM-treated rats	S: rat diet + 10^{10} cfu/g probiotic bact. + 10% (I + OF) C: rat diet	15 weeks	Colon tumor markers (MDF per colon, aberrant crypts per MDF): $S^* < C$	[365]

Table 6 (continued)

Subjects	Test groups	Duration	Results	Refs.
64 Ovariectomized rats	S: rat diet + 10% OF + 3×10^6 cfu/d prob. P_1: rat diet + 10% OF P_2: rat diet + 3×10^6 cfu/d probiotic bact. C: rat diet	16 weeks	Ca-absorption: $S \leq P_1^* > P_2 = C$ Vertebra-Ca: $S^* \geq P_1 \geq P_2 \geq C$	[366]
18 Subjects (> 62 years)	B. bifidum/B.lactis plus inulin/OF		Intestinal bifidus flora ↑	[367]
45 Children	Saccharomyces boulardii plus inulin	8 weeks	H. pylori eradication successful in 5 of 45 children	[368]
7 Malnourished subj. with short-bowel-syndrome and enterocolitis	B. breve/L. casei plus GOS	1 year	Anaerobic bacteria ↑ Pathogenic bacteria ↓ Fecal short-chain fatty acids ↑ Body weight ↑	[369]
137 Surgical patients	S: 16 g/d OF + 4×10^9 cfu/g prob.mix C: placebo	~ 3 weeks	No differences in: gut flora, translocation, inflammation, sepsis	[370]

AB = antibiotics; FOS = fructooligosaccharides; OF = oligofructose; I = inulin; S = synbiotic; P = pro- or prebiotic control; L = Lactobacillus/-i, C = control; B = Bifidobacterium/-a; $\leq \geq$= ns.in-/decreased; * = $p < 0.05$ with respect to C, MDF = Mucin-depleted foci; LGG = L. rhamnosus GG; Bb12 = B. animalis Bb12; AOM = azoxymethane; PP = Peyer's patch cells

trointestinal complaints even in the most sensitive persons. Other products combine probiotic lactobacilli with a bifidogenic prebiotic. Other synbiotics were tested only in animal studies, the synbiotic did not show an increased efficacy compared with its pre- and/or probiotic components, or the study design was not appropriate.

Table 6 shows the results of some recent trials investigating effects (modulation of the intestinal flora, immunemodulation, cancer prevention, prevention of sepsis and bacterial translocation) of certain combinations of prebiotic carbohydrates and probiotic bacteria. Although nearly all data did show, that the combinations were more effective than placebo products, only one parameter in one study in rats [318] can be seen as a proof of true synbiotic properties. In other tests the synbioticum showed no advantage over the pre- and/or probiotic products, or no comparisons had been undertaken.

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Improvement of *Saccharomyces* Yeast Strains Used in Brewing, Wine Making and Baking

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Abstract Yeast was the first microorganism domesticated by mankind. Indeed, the production of bread and alcoholic beverages such as beer and wine dates from antiquity, even though the fact that the origin of alcoholic fermentation is a microorganism was not known until the nineteenth century. The use of starter cultures in yeast industries became a common practice after methods for the isolation of pure yeast strains were developed. Moreover, effort has been undertaken to improve these strains, first by classical genetic methods and later by genetic engineering. In general, yeast strain development has aimed at improving the velocity and efficiency of the respective production process and the quality of the final products. This review highlights the achievements in genetic engineering of *Saccharomyces* yeast strains applied in food and beverage industry.

Keywords Baker's yeast · Brewer's yeast · Genetic engineering · Metabolic engineering · *Saccharomyces cerevisiae* · Strain improvement · Wine yeast

1 Introduction

Beer, wine and bread have been produced with the help of microorganisms for thousands of years. Yeast has been found to be responsible for the occurrence of alcoholic fermentation, i.e., the production of ethanol and carbon dioxide from fermentable sugars during brewing, wine making and dough leavening. Apart from these major products, there are a huge number of other minor byproducts of yeast metabolism that determine the flavor of the different products. Indeed, yeast strains used in brewing, wine making and baking differ from each other in terms of their typical characteristic fermentation properties and byproduct formation, even though virtually all belong to *Saccharomyces cerevisiae* or are closely related to this species.

It is strongly assumed that yeast strains which are currently used in these industries are domesticated due to their long-term association with human-induced fermentation. Nevertheless, it is still controversial whether *S. cerevisiae* strains occasionally occurring in nature are derived from domesticated strains or vice versa [1, 2]. Indeed, there is some thought that all *S. cerevisiae* strains originally derive from close relatives occurring in nature. However, several recent genomic studies seem to invalidate this hypothesis by identifying natural *S. cerevisiae* populations not associated with human alcoholic beverage production [3].

As humans have carried out alcoholic fermentation over such a long period of time, it is not surprising that yeast was one of the first microbes to be

studied scientifically. A very comprehensive series of reviews regarding the history of yeast research has been provided by Barnett and colleagues [4–15]. The most important milestones are summarized as follows. The first investigations that suggested that yeast causes fermentation and is a microorganism were published between 1836 and 1838. At this time Schwann [16] identified yeast as a fungus ("Zuckerpilz"). Julius Meyen defined the genus *Saccharomyces* (Latin form of Schwann's *Zuckerpilz*) in 1838 comprising three species, *S. cerevisiae, S. pomorum* and *S. vini*, their names simply indicating where they were found: in beer, fermenting apple juice and wine. The "microbial theory" of alcoholic fermentation evoked a remarkable attack by some leading chemists for a long period of time [17]. It took about 20 years until Pasteur's study led, once and for all, to the general acceptance of the fact that alcoholic fermentation was a process performed by living organisms [18]. Another milestone was the work of Emil Christian Hansen, who developed techniques for obtaining pure yeast cultures in the early 1880s. He named a number of yeast species and made yeast taxonomy a practical proposition. Since that time, the use of isolated yeast strains as starters in different industrial processes has become possible, a practice which is nowadays the rule rather than the exception. Several companies have even established and evolved their own strains in order to guarantee and improve their products quality.

Although it has not often been obvious to consumers, there has been always a demand to improve yeast strains used in brewing, wine making and baking. However, due to the complex genetic structure of industrial yeast strains (see below), mating of industrial yeast strains has been a challenge and often simply not possible. In such cases, a direct analysis of tetrads and the isolation of recessive mutations are not possible. A number of alternative methods of genetic improvement such as rare mating, protoplast fusion, cytoduction and mutagenesis followed by laborious strain selection have been therefore applied for improvements of industrial yeast strains [19].

Owing to the rapid development in recombinant DNA technology (genetic engineering), directed improvements of distinct traits of brewer's, wine and baker's yeast strains have become possible. Recombinant DNA technology enables the targeted manipulation of a cell's genetic information without the accumulation of disadvantageous mutations. The possibility of introducing or deleting genes within yeast chromosomes has even been facilitated the determination of the complete genome sequence of *S. cerevisiae* in 1996 [20].

When genetic engineering for strain improvement targets the modification of metabolic pathways (i.e., "enzymatic, transport and regulatory functions of the cell"), it is considered to be metabolic engineering [21, 22]. Most improvements of brewer's, wine and baker's yeast strains covered in this review are metabolic engineering approaches; however, there are a few cases such as the modification of flocculation or improved stress tolerances where it can be-

come tricky to decide whether the genetic modification can be assigned to metabolic engineering. We have used the term genetic engineering throughout this review for simplicity.

In order to improve relevant traits of yeast strains, a strategy for genetic engineering has often been conducted and first applied in laboratory strains. Indeed, it has been much easier to genetically modify well-characterized haploid laboratory strains than complex industrial yeast strains. Laboratory strains have been derived from naturally occurring *S. cerevisiae* strains and carry mutations or genetic modifications that make them superior for classical and molecular genetics, e.g., they prevent mating type switching (heterothallic strains) or result in a strain's auxotrophy for certain nutrients [23, 24]. Moreover, they are mostly haploid or diploid. This is in contrast to many industrial *Saccharomyces* strains used in baking, brewing and wine making. The latter strains are often homothallic[1], polyploid, aneuploid or even alloploid, and sporulate poorly or not at all [25–27]. Compared to laboratory yeast strains of *S. cerevisiae*, it has been much less efficient to transform industrial yeast strains and establish genomic integrations by homologous recombination. Moreover, such strains are usually not auxotrophic for certain nutrients, and require the use of dominant genetic markers, such as genes conferring resistance to antibiotics or toxic agents. Another possibility is the use of genes which enable the utilization of a substrate such as starch or melibiose that does not belong to those naturally used by *S. cerevisiae* (see Sects. 4.1.2 and 4.1.3).

When a genetic engineering approach has been shown to work well in laboratory yeast strains, it does not necessarily mean that the strategies will also work in industrially relevant conditions. As mentioned above, the genetic background of industrial yeast strains is different to those of laboratory strains; in addition, the relevant practical conditions also strongly differ from laboratory conditions (e.g., brewer's wort or grape must is used instead of the mineral or complex media usually used in the lab). In fact, several cases are known in which a strategy worked well in the laboratory but not in industrial yeast strains [28, 29].

This review gives an overview of the genetic and physiological properties of baker's, wine-maker's and brewer's yeast strains, and highlights the strategies used to improve them by means of genetic engineering. The improvement of both process performance and product quality has been the major goal in industrial yeast strain improvement. Although there are application-specific targets that allow for the specific nature of the product, there has also been a certain amount of overlap between efforts to improve baker's, brewer's and wine yeast strains.

[1] Haploid *S. cerevisiae* cells exist in two different mating types, a or α. The yeast can be either genetically stable (heterothallic yeast) or they can change their mating type (homothallic yeast).

2
Genetic Characteristics of Baker's, Wine and Brewer's Yeast Strains

According to recent taxonomic nomenclature, virtually all industrially used baker's, wine and brewer's yeast strains belong to the *Saccharomyces sensu stricto* species [30, 31]. Baker's yeast, many wine starter yeast strains and top-fermenting brewer's yeast (ale yeast) have been assigned to the species *Saccharomyces cerevisiae* [32–35]. Thus, their genetic information should have a high degree of similarity to laboratory strains and the published genomic sequence of *S. cerevisiae* (obtained from a laboratory strain) [20]. This means that commercial *S. cerevisiae* microarrays can be used for global expression studies, and gene/protein sequences published in public databases such as *Saccharomyces* Genome Database (SGD) [36] and Comprehensive Yeast Genome Database (CYGD) [37] are useful tools for analysis (e.g., transcriptomics and proteomics) and engineering these strains. The situation is different for some wine yeast strains and lager brewer's yeast. Several industrially used wine yeast strains belong to *S. bayanus* or *S. uvarum* [38]. Bottom-fermenting (lager) brewer's yeast originally known as *S. carlsbergensis* according to Hansen [39] were later recognized as part of the *S. pastorianus* species [40].

In terms of their genetic constitution, bottom-fermenting (lager) brewer's yeast are most striking among industrial yeast strains. Several genetic studies have revealed lager brewer's yeast as being a natural allopolyploid interspecies hybrid between *S. cerevisiae* and a non-*S. cerevisiae* yeast. This is in contrast to top-fermenting (ale) yeast which is a diverse group of polyploid yeast strains but closely related to laboratory strains of *S. cerevisiae* [41]. Interestingly, the results of the different studies, which aimed at identifying the second "parent" of lager brewer's yeast, seemed to be contradictory at first glance. For instance, investigations of the restriction fragment polymorphism as well as analysis of the sequence of distinct non-*Saccharomyces* genes pointed to *S. monacensis* being the origin of the second genome of lager brewer's yeast [41–43]. The measurement of the DNA homology between *S. cerevisiae* and other yeast strains suggested that the second genome originates from *S. bayanus* [44, 45]. Two-dimensional (2-D) gel electrophoresis of the proteomes of lager brewer's yeast and type-strains of *S. bayanus*, *S. uvarum*, *S. carlsbergensis*, *S. monascensis* and *S. pastorianus* highlighted the fact that *S. carlsbergensis*, *S. monascensis* and *S. pastorianus* are hybrid yeast strains themselves, also containing *S. cerevisiae*-like proteins [46]. This at least explained the partial homology of lager brewer's yeast with *S. monascensis*. Based on these data, it was even trickier to explain the previously found partial homology to *S. bayanus*. Later, it was found that obviously different strains of *S. bayanus* exist, a few being hybrids containing genes homologous to *S. cerevisiae* and others that do not contain parts of the *S. cerevisiae* genome. The latter strains' (*S. bayanus* isolates IFO539 and IFO1948) might constitute "pure" genetic lines and could probably rep-

resent relatives of the second ancestor of lager brewer's yeast [47]. However, the origin of lager brewer's yeast will certainly continue to be a matter of discussion in the future.

There have been several studies of lager brewer's yeast using *S. cerevisiae* DNA microarrays [48, 49]. When discussing these data, one should however always bear in mind that this method has been a compromise due to the lack of non-*S. cerevisiae* gene sequences. A breakthrough in the molecular analysis of lager brewer's yeast has been the identification of the whole genomic sequence of a commonly used lager brewer's yeast strain, i.e., Weihenstephan No. 34 (34/70) [50]. As announced by the authors, this laid the basis for the development of DNA microarrays that are specifically designed for lager brewer's yeast, allowing reliable gene expression analyses and detailed studies of the genomic constitution of other lager brewer's yeast strains. Although the sequence has not yet been made publicly available, the exact chromosome structure of Weihenstephan No. 34 (34/70) has been published [50, 51]. These data confirmed nicely once and for all the hybrid character of lager brewer's yeast and the coexistence of three types of chromosomes: (1) pure *S. cerevisiae*-type, (2) pure non-*S. cerevisiae*-type and (3) mosaic-type chromosomes, the latter containing parts of both origins.

3 Physiological Properties of Industrial Yeast Strains

3.1 Baker's Yeast

Baker's yeast is responsible for dough leavening by CO_2 production from fermentable sugars, but also influences the taste and texture of bread and other bakery products. Wheat flour is composed mainly of starch, but also of maltose, sucrose, glucose, fructose and glucofructans. Maltose is continuously released from starch during the baking process due to the activity of crop amylases [35, 52]. Efficient fermentation of dough sugars, in particular maltose, by yeast cells is crucial for dough leavening. Moreover, it has been regarded as favorable if baker's yeast could be engineered to directly use starch, the major carbohydrate in dough. With regard to the desired traits of baker's yeast it is, however, not sufficient to solely look at its performance during dough leavening. The production of baker's yeast itself is also an important industrial branch. In this case, the fast propagation of baker's yeast is desired with high biomass yields related to the carbon sources available in the growth medium, which has mainly been molasses. Moreover, a high tolerance to stresses caused by high sugar concentration (sweet dough), drying and freezing (production of dried yeast and frozen dough, respectively) is necessary.

3.2 Wine Yeast

Starter strains in wine making are usually distributed in dried form. Therefore, fast and efficient growth during propagation and high tolerance to drying are important traits that have been already mentioned in the context of baker's yeast. For the wine-making process itself, the pattern of fermentation products determining wine flavor and body is most important to obtain good and reproducible product quality. Moreover, other traits are desirable, such as efficient use of the nutrients in grape must, high ethanol tolerance, optimal flocculation and prevention of contamination. Reduction of wine acidity is another target of yeast engineering. Often, grapes from colder regions have a high concentration of malic acid.

3.3 Brewer's Yeast

Brewer's wort is composed of 25% nonfermentable carbohydrates, mainly dextrins of varying length. Apart from dextrins, wort consists of fermentable monosaccharides (fructose, glucose), disaccharides (maltose, sucrose) and oligosaccharides (maltotriose). Maltose is the major fermentable sugar followed by maltotriose. Efficient utilization of maltose and maltotriose is therefore as important as it is in baking (see above).

In brewing, freshly propagated yeast is usually used for fermentation. A crucial factor for product quality is to inoculate beer fermentation with cells that are well adapted to the conditions used [53, 54]. Cells propagated up to the late logarithmical growth phase in wort, under conditions similar to brewing, are best at fulfilling this requirement. When cells from the stationary phase or even dried yeast were used, the product of the first batch of beer would be much less tasty due to unusual byproduct patterns. Indeed, this metabolic pattern strongly depends on yeast growth conditions and a well-balanced pattern of yeast metabolic products, such as esters and higher alcohols, is extremely important for beer flavor [54]. Brewing fermentations, in particular lager brewing fermentations, are usually carried out at low temperatures of about 8–15 °C. This is important for a well-balanced beer flavor. Higher growth would result in greater production of byproducts of yeast anabolism accompanied by off-flavors.

The production of lager beer requires a long period (up to 3 weeks) for maturation. Green beer contains buttery flavored diacetyl in a concentration above the accepted taste threshold. Diacetyl is a byproduct of yeast anabolism. Reducing yeast's diacetyl formation and shortening the maturation period is a major requirenent in order to maximize a brewery's capacity in beer production.

4 Approaches to Optimize Industrial Yeast Strains Relevant in Baking, Wine Making and Brewing

4.1 Improving and Extending the Utilization of Available Substrates in Industrial Media

4.1.1 Improvement of Sucrose, Maltose and Galactose Utilization: Alleviation of Glucose Repression

Improvements in yeast growth in industrial media such as molasses have been important for both baker's and wine yeast propagation. Molasses is a mixture of sugars such as glucose, fructose, sucrose, raffinose, melibiose, and galactose. Although it is not the major sugar, glucose is always present in complex industrial media and, even at a very low concentrations, prevents the simultaneous utilization of other sugars such as maltose and galactose. This effect has been referred to as "glucose repression" and results in lengthened process times. Apart from being important during yeast propagation in molasses, glucose repression of maltose utilization also occurs during dough leavening and brewing fermentation, as maltose is the most important fermentable sugar in both dough and brewer's wort.

Although much progress has been made in understanding the molecular basis of glucose repression in yeast, it has not yet been completely resolved. It is generally accepted that glucose repression exerts its regulation at the level of transcription by binding regulatory proteins to the promoter region of the regulated genes. Transcriptional repression is caused by a protein complex consisting of Mig1p, Ssn6p, and Tup1p. Ssn6p and Tup1p are responsible for the actual inhibition, whereas Mig1p recruits the SSN6-TUP1 complex to glucose-repressed promoters [55]. Mig1p is a zinc finger protein, whose binding site contains a GC-box with the consensus sequence $^{G}/_{C}$ $^{C}/_{T}$GGGG. Binding sites for Mig1p were found in the promoter region of numerous glucose-regulated genes, including those encoding enzymes involved in utilization of sucrose (*SUC2*), maltose (*MALR, MALS, MALT*) and galactose (*GAL1/3/4/5*) [56]. Hence, *MIG1* has been a promising target to alleviate glucose repression in industrial yeast strains [52].

Sucrose is mainly found in molasses. It is converted to glucose and fructose by the enzyme invertase which is encoded by *SUC2* and secreted into the extracellular medium. The disruption of *MIG1* led to alleviation of glucose repression of *SUC2* in both laboratory and industrial strains. Hence, the lag phase of sucrose consumption in the presence of glucose was significantly reduced [56]. In addition to Mig1p, another zinc finger protein (Mig2p) has been shown to contribute to glucose control of *SUC2* [57]. Compared to

a *mig1* disruptant, an isogenic laboratory *S. cerevisiae* strain deleted in *MIG1* and *MIG2* showed an even greater ability to consume sucrose in the presence of high glucose concentrations [58].

Maltose is the major fermentable sugar in molasses, brewer's wort and dough. This disaccharide is transported into the cell by maltose permease, an active transporter. It is subsequently hydrolyzed into two glucose units and further metabolized by the central carbon catabolism. The *MAL* genes are centralized within the so-called *MAL* loci. One *MAL* locus contains three genes: *MALR*, *MALT* and *MALS*, encoding a transcriptional regulatory protein, maltose permease and maltase, respectively. The expression of these genes is repressed by glucose via *MIG1* and induced by maltose. Glucose influences gene expression regulation both at transcriptional level, and the turnover rate of mRNA [59]. Five *MAL* gene loci exist in industrial strains of *S. cerevisiae*, whereas haploid laboratory yeast only contain one *MAL* locus, which is obviously sufficient for maltose utilization [60].

Expression of *MAL* genes is under dual control: repression by glucose and induction by maltose. Induction is mediated by *MALR*, and Mig1p is involved in glucose repression and also controls *MALR* expression. The deletion of *MIG1* was able to alleviate glucose repression of *MAL* genes, although only in a haploid laboratory yeast strain. Several polyploid industrial strains were also deleted in *MIG1* and an alleviation of glucose repression was found only for sucrose and not for maltose [28]. One of the main differences between genes involved in sucrose and maltose utilization is that the first does not need induction by sucrose, whereas all *MAL* genes require the presence of maltose as an inducer [60]. It has been clearly shown that the polyploid *mig1* disruptants showed increased levels of *MALS* and *MALT*; hence, the stricter glucose control of maltose utilization must be caused by some other factor. There has been some discussion that the inactivation of maltose permease by glucose is the limiting factor for maltose utilization by *mig1* deletion strains in the presence of glucose [28, 56]. There is no plausible explanation for the different effects of *MIG1* deletion in haploid and polyploid strains, except that industrial yeasts are polyploid, i.e., they posses different *MAL* loci, whereas laboratory strains possess only one. One has to mention in this context that there have been other strategies to alleviate the lag in maltose consumption in media containing glucose [35]. For example, *MALS* and *MALT* can be placed under the control of constitutive promoters [61].

Galactose is present in molasses, cheese whey, and lignocellulose. The latter industrial media came into focus as an alternative for molasses in yeast propagation [28, 52, 58, 62]. Galactose is taken up by galactose permease encoded by *GAL2* and is then converted to glucose-6-phosphate by the enzymes of the Leloir pathway encoded by *GAL1*, *GAL7*, *GAL10* and *GAL5*. All of these *GAL* genes are subject to complex regulation involving the regulatory proteins Gal4p, Gal80p and Gal3p [56]. Similarly to maltose, utilization of galactose is under dual control, i.e., repression by glucose and induction by galactose.

Several *GAL* genes (*GAL1, 3, 4* and 5) have been shown to be regulated by Mig1p at transcriptional level. Gal4p is responsible for the transcriptional activation of the *GAL* genes by galactose and Gal80p prevents the action of Gal4p as long as galactose is absent. The use of galactose by *S. cerevisiae* in industrial media encounters two problems: (1) glucose repression of *GAL* genes and (2) a low rate of galactose metabolization, significantly lower than in glucose (even when glucose is absent). Deletion of *MIG1* led only to a slight alleviation of glucose repression on galactose utilization. To improve galactose utilization, *GAL80* was disrupted in addition to *MIG1*. The disruption of *GAL80* led to a constitutive expression of the *GAL* genes [63].

As mentioned above, galactose is utilized very slowly even in the absence of glucose. This problem has recently been solved to a large extent. By means of inverse metabolic engineering, it has been shown that simply an increased level of *PMG2*, encoding the major isoenzyme of phosphoglucomutase, led to a 74% increase in flux through the Leloir pathway [59].

Recently, it has been suggested that glucose repression is mediated by inositol and phosphatidylinositol (PI) [64]. An increase in PI content may cause phosphorylation of Mig1 by a PI-type signaling pathway and its translocation to cytoplasm where it exerts glucose repression. Even though the mechanism of this regulation has not been completely clarified, it opens up a new avenue for the manipulation of glucose repression in yeast.

4.1.2 Utilization of Oligosaccharides such as Raffinose, Melibiose and Maltotriose

Apart from glucose repression, another major problem occurs during yeast propagation in molasses: the oligosaccharides raffinose and melibiose cannot be used by either baker's or wine yeast. Thus, waste streams still contain valuable carbon sources.

Raffinose, a component of molasses (8% of total sugar), is a trisaccharide that is cleaved by the enzyme invertase encoded by *SUC2* (see Sect. 4.1.1) into melibiose and fructose. Melibiose can be cleaved into glucose and galactose by the enzyme melibiase. However, the expression of melibiase encoded by *MEL1* is a specific feature of lager brewer's yeast (*S. pastorianus*) and most baker's yeast strains (*S. cerevisiae*) do not have this gene. Furthermore, *MEL1* expression is also controlled by glucose repression [56]. Therefore, efforts have been aimed at both expressing melibiase in *S. cerevisiae* industrial yeast strains and deregulating the glucose repression of *MEL1*. Both targets have been pursued at the same time by integrating the *MEL1* gene into the genomic loci of *MIG1* and *GAL80* [65]. This strategy resulted in a *S. cerevisiae* strain able to efficiently hydrolyze melibiose.

Another oligosaccharide which is important in brewing fermentation is maltotriose. This trisaccharide accounts for 15–20% of the total sugars in

brewer's wort and is the second most abundant sugar, apart from maltose (50–60%). Maltotriose is only utilized in the later stages of wort fermentation, a fact which is even more manifest in ale yeast. Several transporters are known that account for maltotriose uptake in *S. cerevisiae* [66, 67]. Recently, the *MTTI* gene has been determined to encode another maltotriose transporter, and its overexpression in a lager brewing strain resulted in a significant increase of maltotriose uptake [68].

4.1.3
Utilization of Polysaccharides such as Starch and Dextrins for Improved Dough Leavening and Low-Calorie Beer

Starch is the storage protein in plants and it is made up of glucose molecules joined by α-1,4 linkages (amylose) and additionally joined at branch points by α-1,6 linkages (amylopectin). Starch is the major carbohydrate in dough. Dextrins represent degradation products of starch, i.e., a mixture of large fragments. These starch degradation products result from partial hydrolysis. This hydrolysis is facilitated by the mashing step during brewer's wort production. As mentioned above, nonfermentable dextrins make up about 25% of the carbohydrates in wort. The dextrins in brewer's wort are important for fullness (body) of beer. Nonetheless, utilization of dextrins by brewer's yeast is required when it comes to the production of low-calorie beer.

Neither baker's nor brewer's yeasts are able to hydrolyze starch or dextrins, as they do not excrete starch-decomposing enzymes such as α-amylase (which cleaves α-1,4 glycosidic bonds) and isoamylases (debranching enzymes that hydrolyze α-1,6 glycosidic bonds) [69]. Therefore, α-amylases are often added to dough in the baking industry. In order to produce low-calorie beer, enzymes such as amyloglucosidase from *Aspergillus niger* were added [70]. These practices could be omitted if baker's and brewer's yeasts were provided with starch/dextrin-decomposing enzymes.

S. diastaticus has amylolytic activity, i.e., it is able to excrete amyloglucosidase into the surrounding medium. Amylolytic hybrids between brewer's yeast and dextrin-fermenting yeast have been generated by classical genetic methods. However, some undesirable characters of *S. diastaticus* have also been transferred into the brewing yeast. Thus, for example, the *POF1* gene was cotransferred, leading to the ability to decarboxylate ferulic acid causing a phenolic off-flavor [70]. This effect could be avoided by directly transferring the *DEX1* (=*STA2*) gene of *S. diastaticus*, encoding the glucoamylase into brewer's yeast using recombinant DNA technology. The transformants produced extracellular glucoamylase and showed an improved degradation of carbohydrates [71, 72]. The *S. diastaticus* glucoamylase encoded by *STA2* does not have α-1,6-debranching activity. To obtain the complete degradation of dextrins, an enzyme able to hydrolyze the α-1,4 as well as the

α-1,6 linkages (amyloglucosidase) has therefore been regarded as advantageous. Such an enzyme was identified in *A. niger*. The gene was cloned and integrated into the genome of brewer's yeast [73]. Gopal and Hammond [74] carried out brewing experiments with brewer's yeast secreting the *A. niger* enzyme and demonstrated half of wort dextrins being degraded. One crucial disadvantage of the amyloglucosidase from *A. niger* is its very high thermostability. Thus, it is still active after pasteurization and the beer may become sweet after storage. The amyloglucosidase from *Schwanniomyces occidentalis* is less heat stable. Lancashire et al. [75] isolated this amyloglucosidase gene (*GAM1*) and transferred it into brewer's yeast under the control of the *ADH1* promoter. The resulting transformants were able to degrade dextrins very well and the enzyme was inactivated after pasteurization.

Amylolytic baker's yeast that is able to degrade starch was generated by a similar strategy. For example, Hollenberg and Strasser [76] have transformed industrial *S. cerevisiae* strains with the amyloglucosidase gene (*GAM1*) in combination with the α-amylase gene (*AMY1*) from *Schwanniomyces occidentalis*.

Some other amylolytic brewer's yeast strains have also been constructed. However, such recombinant industrial yeast strains often contained antibiotic (and/or drug) resistance markers from bacteria or yeast that have pathogenic relatives and thus have not been acceptable for applications in the food industry. There has indeed been a general low consumer acceptance of genetically modified organisms, as reviewed in detail using wine yeast as an example [77]. This has always posed a challenge to scientists who work on the genetic modification of industrial yeast strains. Recently, Liu et al. [78] constructed an amylolytic brewer's yeast, *S. pastorianus*, that was free of vector sequence and drug-resistant genes, bearing α-amylase of *Saccharomycopsis fibuligera* [79, 80]. This brewer's yeast was able to utilize starch as its sole carbon source.

4.1.4 Degradation of β-Glucan, Pectin and Xylan for Better Filterability of Beer and Wine

Barley cell walls contain β-glucan, polysaccharides containing β-1,4- and β-1,3-linked glucose molecules. These linkages are cleaved by specific endo-β-glucanases from barley during the malting process. Depending on the conditions used, β-glucan degradation is often incomplete. The enzyme from barley is extremely thermolabile and, due to high temperatures during the drying of malt and mashing, little active β-glucanases are present in wort. Thus, β-glucan is often found in beer, where it reduces filterability by the for-

mation of gels and blocks the filter. Moreover, β-glucan is found in the final product and causes hazes [70, 81].

One can add isolated microbial β-glucanases to mash and wort, respectively, to improve the filterability of beer. Meanwhile, the genes encoding β-glucanases have been cloned from bacteria and fungi, characterized and expressed in brewer's yeast. Cantwell et al. [82] expressed a β-glucanase-encoding gene from *Bacillus subtilis* under the control of the *ADH1* promoter in yeast. However, very little enzyme was secreted by the engineered yeast. Lancashire and Wilde [83] fused the *B. subtilis* gene to the promoter and signal sequence of the *S. cerevisiae* α-factor, a naturally secreted enzyme. Brewer's yeast transformed with this expression cassette secreted functional β-glucanase into the medium, which reduced the β-glucan content of the wort during fermentation.

Enari et al. [84] expressed the *Trichoderma reesei* β-glucanase gene under control of the *PGK1* promoter. Specific yeast-secretion signal sequences were not required, since fungal extracellular enzymes are efficiently excreted by the yeast [85]. Wort β-glucan was effectively degraded and beer filterability was remarkably improved in brewing experiments using the transformed yeast strain [84, 86]. The heat-labile β-glucanase from barley, which is responsible for β-glucan degradation during malting and mashing was fused downstream of a mouse α-amylase-secretion signal sequence and expressed in *S. cerevisiae* under the control of the *ADH1* and *PGK1* promoters, respectively [87, 88]. Brewer's yeast, transformed with this construct, produced beer with reduced β-glucan content in brewing experiments [81]. Recently, the β-glucanase of *Rhizopus microsporus* var. *microsporus* was characterized [89]. This enzyme was shown to be able to hydrolyze barley β-glucan and its maximum activity was detected at temperatures of 50–60 °C. This enzyme was also able to reduce both the viscosity of the brewing mash and the filtration time, and is thus a promising candidate for industrial application.

Glucan, pectin and xylan are polysaccharides that also hamper wine clarification. These polysaccharides are derived from grapes or formed by other microorganisms, respectively. They are responsible for turbidity, viscosity and filter stoppages. Pectinases and glucanases have very often been added to the grape must during wine making in order to improve filterability [25]. Some recombinant yeast strains have been developed that are able to express and secrete these enzymes. For example, a glucanolytic wine yeast was developed expressing fungal or bacterial β-1,4-glucanases [90, 91]. Recently, recombinant wine yeast strains have been constructed that were able to degrade the problem-causing grape polysaccharides, glucan and xylan, by separately integrating the *Trichoderma reesei* X*YN2* xylanase gene and the *Butyrivibrio fibrisolvens END1* glucanase into the genome of a commercial wine yeast strain of *S. cerevisiae* [92].

4.1.5 Improved Utilization of Nitrogen Sources

Apart from the improvement of carbon assimilation, nitrogen uptake has also been challenged. Proline is the most abundant amino acid in brewer's wort and is, oddly enough, the only amino acid that is virtually unassimilated during brewing fermentation. A lager brewer's yeast expressing a modified version of the *PUT4* gene, encoding a highly specific proline permease, was able to efficiently assimilate proline from brewer's wort without any negative impact on beer quality [93]. The site-directed mutagenesis of the Put4 protein resulted in a less degradable form of the permease.

The efficient use of nitrogen sources has also been a major focus for wine yeast improvement, since there is an imbalance between the high level of carbon and low level of nitrogen sources in grape must [94]. Again, proline (together with arginine) accounts for 30–65% of grape amino acids and is also barely utilized during wine fermentation [33]. Salmon and Barre [95] deleted the *URE2* gene encoding a repressor of the *PUT1*-encoded proline permease and the *PUT2*-encoded pyrroline-5-carboxylate dehydrogenase. The mutant strains produced more biomass and exhibited a higher maximum CO_2 production rate than the wild type. An additional effect of nitrogen starvation is the limitation of sugar uptake. Thus, sugar transporters have been assumed to be interesting targets for strain optimization; however, a breakthrough has not yet been achieved in this field [33].

4.2 Optimal Flocculation Behavior of Brewer's and Wine Yeasts for Biomass Separation

Flocculation is an asexual, reversible aggregation of yeast cells to form flocs when lectins on cell walls bind to sugar residues on the walls of neighboring cells [96, 97]. This phenomenon is advantageous in brewing and wine making, since flocculated cells can be easily and efficiently separated after sedimentation. However, the flocculation behavior of many industrial yeast strains is far from optimal; some yeast strains are even unable to flocculate.

It is very important that flocculation starts at the correct time during the fermentation of beer and wine [25, 26, 70]. When the onset of flocculation is too early, fermentation is incomplete which results in an abnormal aroma. When flocculation is too late, yeast cells cannot be separated completely. In general, the flocculation of brewing yeast is associated with the onset of the stationary phase, but the factors which control the precise timing and degree of flocculation are not completely understood. At least it is known that the onset of flocculation depends on many external factors, e.g., ethanol concentration, temperature, osmotic stress and nutrient availability [98–101]. Other

factors involved are pH, calcium and zinc ions, and oxygen, as well as growth phase and cell density [102].

In addition, flocculation is dependent on genetic factors [70, 97, 103]. Three different flocculation phenotypes have been identified, based on the lectins on their cell walls, their sensitivities to sugar inhibition and proteolytic enzymes, and their dependency on growth conditions and pH. These three phenotypes are Flo1, NewFlo [104, 105] and Mannose-Insensitive (MI) [106, 107]. The majority of top-fermenting brewing yeasts belong to the NewFlo phenotype, whereas bottom-fermenting brewing yeast strains are assigned to the Flo1 phenotype [96, 104]. The MI phenotype is rare and occurs only in some top-fermenting yeast strains. The Flo1 phenotype is only inhibited by mannose [102, 108] whereas the NewFlo phenotype is also inhibited by glucose and maltose [107]. Different genes have been identified to be responsible for the flocculation behavior in yeast cells. *FLO* genes encode glycoproteins (lectins), so-called flocculins, which are located at the cell surface and bind specifically to mannose residues at the wall of neighboring cells [109]. The dominant genes *FLO1* and *FLO5* were isolated and characterized. Both genes encode an integral membrane protein that could be the postulated lectin [110, 111]. Nonflocculating brewer's, wine or laboratory yeast strains can attain the ability to flocculate after transformation with *FLO1* and *FLO5*, respectively [26, 110–112]. The Flo1 phenotype is associated with the *FLO1* gene [109, 113] whereas the lectin of NewFlo phenotype is encoded by the *FLO10* gene [114], which is not expressed until the onset of the stationary phase.

There have been some achievements in improving the flocculation of yeast by genetic engineering. The constitutive expression of *FLO1* led to premature flocculation [26]. Thus, it is necessary to regulate the gene expression in such a way that flocculation occurs during the stationary phase at the end of fermentation. The main problem here has been the lack of promoters that can be used for stationary-phase expression under industrial conditions. Therefore, Riou et al. [115] examined gene expression during the stationary phase during wine fermentation and showed that the *HSP30* promoter could be suitable for delayed gene expression during the stationary phase under wine fermenting conditions. Verstrepen et al. [116] replaced the native *FLO1* promoter by the *HSP30* promoter in a laboratory yeast strain and showed that flocculation occurred towards the end of fermentation under laboratory conditions. It still remains to be confirmed whether this strategy can cause flocculation specifically during the stationary phase under industrial conditions. Donalies and Stahl [117] examined the promoters of several genes encoding heat shock proteins and proposed that the *HSP26* and *HSP30* promoters could be suitable candidates for phase-specific gene expression under brewing conditions, since the authors used maltose as a carbon source in their experiments.

4.3 Modification of Byproduct Formation

4.3.1 Reduction of Diacetyl Formation in Brewer's Yeast to Facilitate Maturation of Lager Beer

Vicinal diketones, in particular diacetyl, impart the unwanted butter-like flavor to green beer and cause an off-flavor if present in finished beer. Diacetyl is the product of a nonenzymatic oxidative decarboxylation of α-acetolactate, the latter being an intermediate of the valine biosynthetic pathway as shown in Fig. 1 [70]. The taste threshold of diacetyl in beer is very low, and its presence in green beer is the main reason for the time-consuming secondary fermentation (lagering) required in lager brewing. Diacetyl is metabolized to acetoin and 2,3-butandiol during lagering. There is a high demand to reduce yeast's diacetyl production in order to be able to accelerate lager beer production.

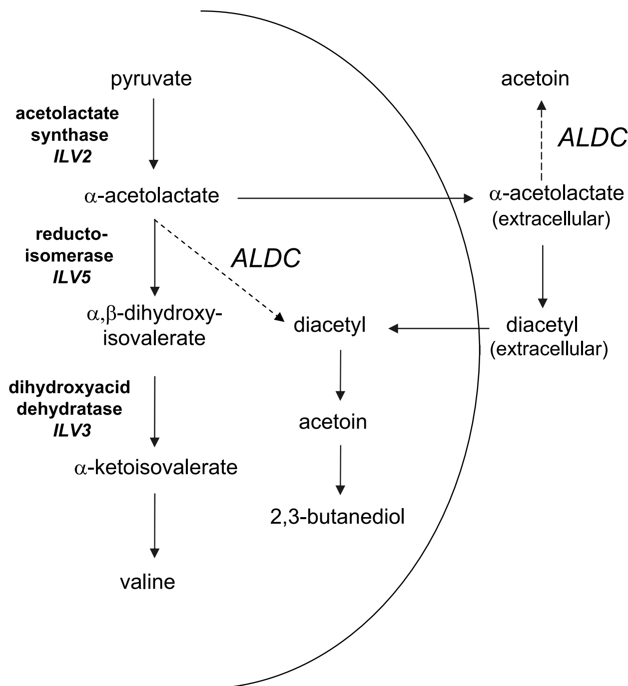

Fig. 1 Diacetyl formation in *Saccharomyces cerevisiae* fermentation originates from α-acetolactate which is an intermediate of the valine biosynthetic pathway. Diacetyl formation can be prevented by heterologous expression of bacterial *ALDC* genes, encoding acetolactate decarboxylase

One strategy has been to introduce a bacterial gene encoding the enzyme acetolactate decarboxylase (ALDC). This enzyme catalyzes the decarboxylation of α-acetolactate to acetoin, and thus prevents oxidative decarboxylation to diacetyl (Fig. 1). Originally, preparations of bacterial ALDC were added to wort; however, this procedure is expensive and not compatible with the German beer-brewing purity laws ("Reinheitsgebot"). Later, the *ALDC* gene from *Enterobacter aerogenes* [118, 119], *Klebsiella terrigena* [120, 121], *Lactococcus lactis* [122] and *Acetobacter aceti* var. *xylinum* [123] were cloned and expressed in brewer's yeast. Diacetyl formation was reduced significantly during fermentation with episomal as well as integrative transformants. Thus, the lagering period became obsolete [120, 124, 125]. The disadvantage of ALDC expression is that yeast is rendered auxotrophic for valine, leucine and isoleucine because of a deficiency of intracellular α-acetolactate. Even though brewer's wort contains amino acids, transformed strains showed reduced growth because of a very slow uptake of these amino acids in brewer's yeast [126]. To solve this problem, the *ALDC* gene was fused to the prepro-sequence of the α-factor leader. Thereby, the expressed ALDC was secreted into the medium and the extracellular α-acetolactate was decarboxylated to acetoin. The transformants showed a reduced diacetyl formation and the yeast was not auxotrophic for the amino acids valine, leucine and isoleucine [126]. It has been suggested that the ALDC genes from *L. lactis* or *Acetobacter* sp. would be preferable for food use as these organisms are already used in food production [122]. It has been assumed that engineered yeast strains without foreign genes ("self-cloned") are more readily accepted by consumers [127–131].

There have been also attempts to reduce diacetyl formation without the use of bacterial genes. For this purpose, two different modifications of the valine biosynthetic pathway were attempted. Firstly, the formation of α-acetolactate was prevented by deleting the *ILV2* gene encoding acetolactate synthase (Fig. 1) This resulted in yeast strains unable to form α-acetolactate [132]. However, these strains were also auxotrophic for the amino acids and grew very badly [85]. Moreover, Falco and Dumas [133] have shown that some *S. cerevisiae* mutants resistant to the herbicide sulfometuron methyl carry an allele of *ILV2*, leading to a less active form of the enzyme acetolactate synthase. Mutant strains forming less α-acetolactate and showing good brewing properties were selected [134, 135]. A reduction of the acetolactate synthase activity was also achieved by employing *ILV2* antisense RNA; Vakeria et al. [136] reported the construction of brewer's yeast strains that formed less α-acetolactate and performed well in brewing trials. A rather recent attempt to reduce acetolactate synthase activity by disrupting *ILV2* in brewing yeast was performed by Liu et al. [78]. The authors employed the α-amylase gene as a reporter gene for detecting the deletion mutant (see Sect. 4.1.3). The obtained strain showed a 30% reduction in acetolactate synthase activity and a 70% reduction in diacetyl in comparison to the reference strain.

Another strategy to modify yeast's valine biosynthetic pathway was to enhance the conversion of α-acetolactate, preventing its accumulation and spontaneous diacetyl formation. The genes *ILV5*, encoding the enzyme reductoisomerase, and *ILV3*, encoding dihydroxy-acid dehydratase, were overexpressed for this purpose (Fig. 1). Brewer's yeast harboring the *ILV5* gene, either on a multicopy plasmid or integrated within the genome, showed increased reductoisomerase activity, resulting in decreased diacetyl formation [137–140]. In contrast, the overexpression of *ILV3* did not lead to a change in diacetyl content, although the encoding dihydroxy-acid dehydratase activity was enhanced [141]. Gjermansen et al. [132] integrated an additional *ILV5* gene into the *ILV2*-locus, leading to a strain with enhanced reductoisomerase activity linked with a reduced acetolactate synthase activity.

4.3.2
Optimization of Formation of Sulfur-Containing Compounds in Beer and Wine

There is a set of volatile sulfur-containing compounds in beer and wine, e.g., sulfite, hydrogen sulfite (H_2S), or dimethyl sulfide (DMS), which have high reactivity, low taste thresholds and different impacts on flavor.

4.3.2.1
Sulfite

S. cerevisiae produces sulfite as an intermediate product during the assimilatory reduction of sulfate to sulfide, which is important for the biosynthesis of the sulfur-containing amino acids methionine and cysteine (Fig. 2). Moderate amounts of sulfite in beer are important for flavor stability. During storage, an ageing aroma, the so-called "cardboard flavor", can be formed in bottled beer as a result of oxidative reactions. This off-flavor is caused mainly by carbonyls such as *trans*-2-nonenal. Sulfite can act as an antioxidant, preventing oxidative reactions. It can also stabilize flavor by forming adducts with carbonyls. These sulfite–carbonyl complexes have a much higher flavor detection threshold than free carbonyls.

To improve the flavor stability of beer, different strategies to increase sulfite formation of the yeast were developed. Korch et al. [142] found that overexpression of *MET14*, encoding APS kinase (Fig. 2), increases sulfite formation in a *met5* mutant (inactive sulfite reductase), but not in a *MET5* prototrophic laboratory strain. In accordance with this, an increase in sulfite production was detected in a sulfite reductase-deficient strain after chromosomal integration of the *MET14* gene under the control of the constitutive *TPI* promoter (Johannesen, personal communication). Moreover, brewer's yeast with reduced *ILV2* gene function produced more than the usual amount of sul-

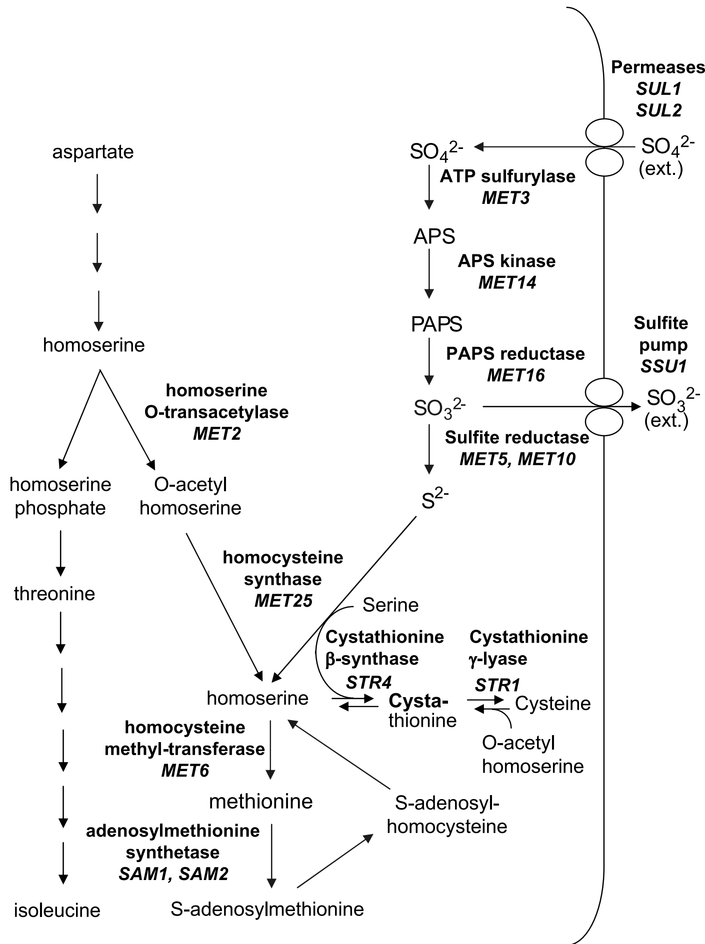

Fig. 2 The assimilatory reduction of sulfate to sulfite in *S. cerevisiae* during the biosynthesis of methionine and cysteine. *APS* Adenosyl phosphosulfate, *PAPS* phosphoadenosylphosphorsulfate *ext.* extracellular

fite [70]. This phenomenon could be explained by an increase in the threonine pool, leading to a derepression of the sulfur assimilation pathway.

Another possibility to increase sulfite accumulation is to prevent the reduction of sulfite to sulfide. A disruption of all the alleles of the *MET10* gene encoding a subunit of sulfite reductase resulted in enhanced sulfite accumulation in the medium compared to the isogenic wild-type strain [143, 144]. Moreover, the inactivation of all copies of the *MET2* gene encoding homoserine *O*-acetyl transferase led to an increase in sulfite formation [145]; *met2* mutants produced more H_2S, an undesirable byproduct (see Sect. 4.3.2.2).

One problem accompanied with the strategies described above is the early onset of sulfite accumulation. When sulfite accumulation has already commenced at the beginning of the fermentation process, it forms complexes with wort carbonyls and prevents the reduction of these carbonyls to the corresponding alcohol, thus negatively influencing beer flavor [146]. This is indeed the reason that some breweries prefer yeast strains which have low sulfite formation. Johannesen et al. [147] inactivated all copies of the *MET14* gene in brewer's yeast for this purpose. Beer produced by such engineered yeast showed accelerated ageing, which can be compensated for by sulfite supplementation before bottling. However, sulfite supplementation is expensive and is not permitted in Germany, because of the German "Reinheitsgebot" (beer purity laws). Hence, it was important to establish a strategy to control sulfite formation during fermentation, i.e., accumulation should not occur until the beginning of the stationary growth phase. Overexpression of *MET14* under the control of the *HSP26* promoter was able to solve this problem [143]. Furthermore, in contrast to the results discussed above, sulfite formation could also be enhanced in the *MET5* wild-type background when using this strong promoter for *MET14* expression.

Sulfite is usually added during wine making. Here, the main function of sulfite is to repress the growth of undesired microorganisms. One problem in this context is that different wine yeast strains differ significantly in their sulfite tolerances. Thus, one task for genetic optimization of wine yeast has been to increase sulfite tolerance [25]. Obviously, sulfite resistance in *S. cerevisiae* is determined by the expression level of the *SSU1* gene, encoding a putative sulfite pump [148–150]. Cells lacking the Ssu1p are sensitive to sulfite, while cells overexpressing this protein are resistant. Park and Bakalinsky [149] hypothesized that Ssu1p is responsible for sulfite resistance by transporting sulfite out of the cell.

As described above, sulfite formation can be increased by overexpression of *MET14*, but sulfite accumulation is limited by an active sulfite reductase converting sulfite to sulfide (Fig. 2). Donalies and Stahl [143] investigated whether the overexpression of the putative sulfite pump could increase sulfite efflux, preventing the reduction to sulfide. The simultaneous overexpression of *SSU1* and *MET14* together did indeed lead to a significant increase in sulfite production. This result also confirmed the hypothesis that *SSU1* encodes a sulfite pump.

4.3.2.2
Sulfide

Sulfide is formed from sulfate, sulfite, sulfur, and cysteine during fermentation [151]. Hydrogen sulfide (H_2S) can negatively influence the organoleptic characteristics of beer and wine due to a very low flavor threshold. H_2S is produced mainly in response to the depletion of nitrogen and the vitamin

panthothenate, respectively [26]. The beer maturation process decreases H_2S concentration. Thus, yeast able to form less H_2S would shorten the beer production process [134]. Tezuka et al. [152] have overexpressed *NHS5* (=*STR4*), encoding the cystathionine β-lyase, resulting in a suppression of H_2S formation by enhancing conversion of homocysteine to cysteine via cystathionine in brewing yeast (Fig. 2). Omura and Shibano [153] overexpressed *MET25* encoding *O*-homocysteine synthase in brewing yeast, which resulted in a strong decrease in H_2S concentration in beer. A third method to reduce H_2S formation has been the disruption of the *MET10* gene, leading to an inactive sulfite reductase [144]. All strategies applied in brewer's yeast to decrease H_2S production could also be used for wine yeast strains [26].

4.3.2.3
Dimethyl Sulfide

DMS is another sulfur-containing compound. It negatively influences the organoleptic properties of beer, especially lager beer. DMS is produced by thermal degradation of *S*-methyl methionine during wort production or by enzymatic reduction of dimethyl sulfoxide (DMSO) during fermentation [154]. Hansen [155] showed that a *mrx1* disruption mutant is unable to reduce DMSO. The *MRX1* gene encodes the enzyme methionine sulfoxide reductase and its disruption in brewing yeast reduces the DMS content in beer [156].

4.3.3
Increasing Glycerol Formation for Improved Wine Viscosity and in Order to Reduce the Ethanol Content in Alcoholic Beverages

Glycerol affects the sensory quality of wine, due to its nonvolatile character and slightly sweet taste. Therefore, a moderate increase in glycerol content is desired; it improves viscosity, sweetness, consistency and the overall body of wine [25]. Glycerol overproduction has been also considered a valuable strategy to produce beverages with decreased ethanol content, as any increase in glycerol production will simultaneously lead to a decrease in ethanol yield. Consumer demand for low-ethanol beer and wine is indeed continuously increasing due to both increased awareness for health and stricter laws regarding drinking and driving.

Glycerol in *S. cerevisiae* is produced by the reduction of dihydroxyacetone phosphate, catalyzed by the glycerol-3-phosphate dehydrogenase (GPDH, Fig. 3). As glycerol overproduction has also been of interest in industrial biotechnology, there have been several approaches to overproduce glycerol in laboratory strains of *S. cerevisiae* [157]. Indeed, engineered *S. cerevisiae* laboratory strains that virtually reached the maximal theoretical yield of

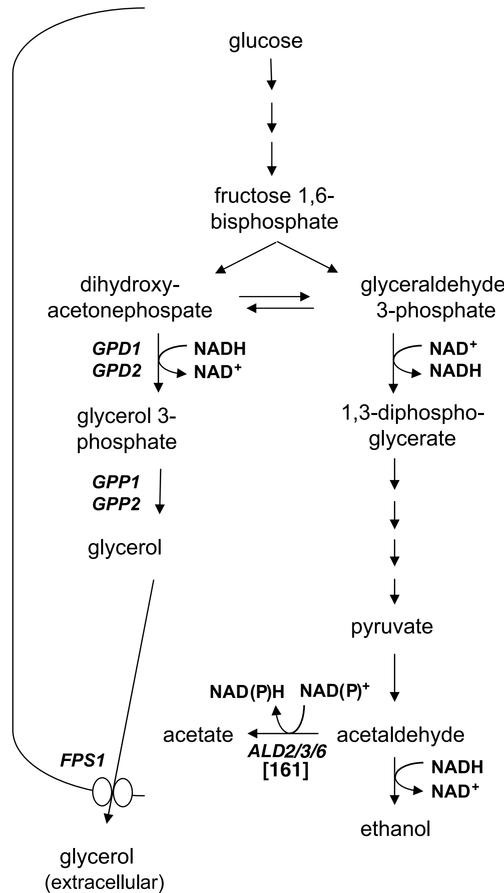

Fig. 3 Glycerol, ethanol and acetate production linked to the glycolytic pathway in *S. cerevisiae*. Genes and enzymes discussed in this review: *GPD1* and *GPD2* isogenes encoding glycerol-3-phosphate dehydrogenase, *GPP1* and *GPP2* isogenes encoding glycerol-3-phosphatase, *ALD2/3/6* genes encoding cytosolic acetaldehyde dehydrogenase, *FPS1* encoding a channel protein which facilitates glycerol transport through the plasma membrane

glycerol per glucose consumed (1 mol mol^{-1}) have been successfully constructed [158–160].

The first strategy to improve glycerol production in brewer's and wine yeasts was the overproduction of glycerol 3-phosphate dehydrogenase (GPD), which is the rate-controlling step in glycerol biosynthesis [161–163]. The increase in glycerol production by overexpression of *GPD1*, one of the isogenes encoding GPD, was first established in a laboratory yeast strain [164]. The overexpression of GPD2, encoding the other isoform of GPDH, had a similar effect. Extracellular glycerol accumulation could be further increased by

the additional constitutive expression of the gene *FPS1*, encoding a channel protein facilitating diffusion of glycerol through the cell membrane [161]. Overexpression of *GPP1*, encoding glycerol-3-phosphatase, did not enhance glycerol production [161]. A general major drawback of glycerol overproduction is the concomitant overproduction of unwanted byproducts. Owing to the altered metabolic flux, the formation of acetate, 2,3-butandiol, succinate, diacetyl and acetoin was increased [29, 162, 163]. The high production of acetate has been the main disadvantage in wine yeast. Cambon et al. [165] therefore combined the overexpression of *GPD1* with the deletion of *ALD6*, which was previously shown to result in lower acetate production in laboratory yeast [166].

A novel avenue to reduce ethanol in beverages could be the use of yeast strains with an abolished Crabtree effect. Based on the approach of Elbing et al. [167], a nonethanol-producing wine yeast strain was developed by modification of hexose transporters [168]. Moreover, introducing heterologous enzymes leading to increased NADH oxidation has been shown to reduce overflow metabolism, i.e., ethanol formation, in wine yeast [169]. As the functionality of such approaches relies on oxygen availability or, at least, microaeration, their use for production of low-alcohol beverages will require sophisticated fermentation strategies. Indeed, full aeration would strongly change yeast's metabolism and byproduct formation in comparison to traditional beer and wine production carried out under oxygen-limited conditions.

4.4 Reduction of Wine Acidity

Wine acidity is an important quality character. Here, the predominant organic acids are tartaric and malic acid. The malic acid content is often very high in must produced by grapes from colder regions. The so-called malolactic fermentation is one way to biologically deacidify wine. During malolactic fermentation, L-malic acid is decarboxylated to L(+)-lactic acid and carbon dioxide by lactic acid bacteria, in particular *Oenococcus oeni* [25]. Lactic acid has a much milder taste than malic acid. This secondary fermentation has been unreliable in numerous situations because wine, e.g. by high sulfite content growth of lactic acid bacteria is inhibited in.

The fission yeast *Schizosaccharomyces pombe* is able to effectively convert malate to ethanol during the so-called maloethanolic fermentation, but also produces off-flavors [170]. In principle, *S. cerevisiae* is also able to assimilate malate; however, conversion is very inefficient. In contrast to *S. pombe*, *S. cerevisiae* lacks an active malate transport system and L-malate enters wine yeast only by simple diffusion. The biochemical pathways for malate degradation in *S. cerevisiae* and *S. pombe* are the same: malate dehydrogenase (malic enzyme) decarboxylates malate to pyruvate which is further degraded

to ethanol and CO_2. The substrate specificity of the *S. cerevisiae* malic enzyme is about 15-fold lower than that of the *S. pombe* enzyme [171].

Using genetic engineering, wine yeast strains were constructed that are able to simultaneously conduct alcoholic fermentation and efficient malate degradation. The malolactic genes (*mle*S) from *Lactococcus lactis* [113, 172–174] and *Lactobacillus delbrueckii* [175] as well as the gene *mle*A from *O. oeni* [176] were cloned and expressed in *S. cerevisiae*. However, due to the absence of an active malate transport system in *S. cerevisiae*, the transformants could not metabolize malate efficiently [171]. Efficient malolactic fermentation was achieved only when the *L. lactis mle*S gene was coexpressed with the *S. pombe mae1* gene, encoding malate permease. Likewise, an efficient maloethanolic *S. cerevisiae* strain was constructed by coexpressing *S. pombe mae1* and *mae2* (the latter gene encoding malic enzyme). Here, the recombinant *S. cerevisiae* strain produced higher amounts of ethanol in comparison to the wild-type strain because the *S. pombe* enzymes converted L-malate to ethanol [171].

Recently, a genetically stable industrial yeast strain was constructed that was able to fully decarboxylate 5.5 g^{-1} of malate in Chardonnay grape must during alcoholic fermentation. This was achieved by integrating a linear cassette containing the *Schizosaccharomyces pombe* malate permease gene (*mae1*) and the *Oenococcus oeni* malolactic gene (*mle*A) under control of the *S. cerevisiae PGK1* promoter and terminator sequences into the *URA3* locus. This malolactic yeast strain enjoys "Generally Regarded As Safe" status from the FDA and is the first genetically enhanced wine yeast that has been commercialized [177].

4.5
Improving Tolerance to Several Types of Stress

Expression studies of suitable marker genes revealed osmotic and oxidative stress as the major causes of stress response under such conditions [178]. Indeed, there have been numerous attempts to improve yeast's tolerance towards these stressors, most of them dating back to the 1990s [35]. Oxidative stress tolerance has also been recently addressed by Chen et al. [179], based on their previous finding that externally added proline was able to protect yeast cells from lethal levels of reactive oxygen species (ROS) generated by paraquat [180]. Their approach, aimed at increasing intracellular proline concentration, is supported by the fact that the deletion of *PUT1* encoding proline dehydrogenase led to increased tolerance to hydrogen peroxide (H_2O_2) [181], whereas overexpression of *PUT1* caused a strongly reduced intracellular proline concentration and a hypersensitivity to oxidants such as H_2O_2 and paraquat [179]. The same authors used the *PUT1*-overexpressing strain background to perform a conditional life/death screen for suppressors of ROS hypersensitivity. Using a tomato cDNA library, they identified

a tomato QM-like protein (tQM) which seems to protect *S. cerevisiae* cells against oxidative stress by regulating intracellular proline levels. The QM protein is a small basic protein which was first identified as a putative tumor suppressor from the Wilms' tumor cell line [182]. It is highly conserved in mammals, plants, worms, insects and yeast [183]. When tQM was expressed in the wild type or in a strain overexpressing *PUT1* and the intracellular proline level was analyzed in the presence of 3 mM H_2O_2, there was a 1.8 times and 7 times increase compared to the corresponding strains without tQM expression, respectively [179]. Phenotypic analysis of a *S. cerevisiae* mutant deficient in *GCR5*, a yeast homologue of QM, suggested that QM is involved in multiple cellular functions, including growth control and proliferation, cytoskeletal function, and energy metabolism [184]. However, it remains to be clarified how tQM increases intracellular proline concentration and protects the *PUT1*-overexpressing strain from oxidative stress, even though a two-hybrid analysis showed a physical interaction between tQM and Put1p [179].

Approaches targeted at stress tolerance of baker's yeast also comprise the improvement of cryoresistnce in frozen dough, as well as osmotolerance in sweet frozen dough. There have been several recent studies to improve freeze–thaw stress tolerance in *S. cerevisiae*. Izawa et al. [185, 186] have reported two engineering approaches to accumulate intracellular glycerol in baker's yeast. Here, the most promising genetic modification is the deletion of *FPS1* encoding a glycerol channel. The engineered cells acquired tolerance to freeze stress and retained high leavening ability, even in dough after frozen storage.

Certain amino acids such as proline, arginine, and glutamate have also been shown to have cryoprotective activity in *S. cerevisiae*. For example, proline accumulation by simultaneous overexpression of a mutant allele of *PRO1* encoding γ-glutamyl kinase and wild-type *PRO2* encoding γ-glutamyl phosphate reductase resulted in a higher tolerance of the engineered strain for freezing [181]. It was later demonstrated that the selected mutant allele of *PRO1* was less sensitive to feedback inhibition by proline [187]. Furthermore, an arginase mutant *car1*Δ accumulated higher levels of arginine and/or glutamate and showed increased leavening ability during the frozen-dough baking process [188]. Yeast strains overexpressing heterologous aquaporin had been regarded as opening new perspectives for the development of freeze-resistant strains [189]. Later studies, however, have shown that they have less potential for use in frozen dough than originally thought [190]. Another recent approach to improve freezing tolerance has been the heterologous expression of antifreeze proteins. An industrial yeast strain expressing the recombinant antifreeze peptide GS-5 from the polar fish grubby sculpin (*Myxocephalus aenaeus*) showed improved viability during frozen storage and an increase in CO_2 production in model liquid dough compared to the control [191]. A very recent approach focused on the impact of unsaturated fatty acids on tolerance to freezing. The multicopy overexpression of either *FAD2-1* or *FAD2-3*, encoding two different desaturases from sunflower, in *S. cerevisiae* increased

the content of dienoic fatty acids, especially 18:2delta(9,12), the unsaturation index, the fluidity of the yeast membrane and the tolerance to freezing [192].

5
Concluding Remarks and Future Prospects

Important progress has been made in the field of recombinant DNA technology in the past 20 years. As the sequence of the complete *S. cerevisiae* genome has become available, targeted and stable genetic modifications can be easily performed in this yeast. Knowledge about the genetics of industrial yeast strains lags behind that of laboratory strains of *S. cerevisiae*. Nevertheless, there have been also recent achievements regarding the genomic structure of industrial yeast strains, in particular brewer's yeast. This will strongly facilitate future strain development programmes and help in understanding the specific nature and evolution of interspecies hybrid yeast strains.

As described in previous chapters, a multiplicity of recombinant yeast strains have been developed in order to improve production of beer, wine and bread. However, the majority of these strains have not been commercialized. The reason for this phenomenon is a lack of public acceptance of the use of genetically modified yeast for the production of food and beverage. The enlightenment of the general public about the advantages, limitations and dangers of genetic engineering, as well as simplified legal regulations, are the most important requirements for the commercial utilization of recombinant yeast strains.

Apart from sequencing industrial yeast strains, it will be crucial for future strain optimization to unravel the function and expression regulation of all genes. *S. cerevisiae* laboratory strains will certainly remain the cutting edge within this research field; however, the knowledge will also facilitate the understanding and improvement of industrial yeast. It has been generally accepted that the coordinated expression and/or inhibition of several genes is required for optimizing phenotypic traits rather than modifying a single or a few gene(s). Moreover, genetic/metabolic engineers have become aware of the necessity to consider the cell as a whole when modifying certain metabolic pathways. Indeed, the modern tools of global analysis (e.g., for the analysis of genomes, transcriptomes, proteomes, metabolomes and fluxomes) will have an important impact in yeast strain improvement [193, 194].

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Filamentous Fungi for Production of Food Additives and Processing Aids

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Abstract Filamentous fungi are metabolically versatile organisms with a very wide distribution in nature. They exist in association with other species, e.g. as lichens or mycorrhiza, as pathogens of animals and plants or as free-living species. Many are regarded as nature's primary degraders because they secrete a wide variety of hydrolytic enzymes that degrade waste organic materials. Many species produce secondary metabolites such as polyketides or peptides and an increasing range of fungal species is exploited commercially as sources of enzymes and metabolites for food or pharmaceutical applications. The recent availability of fungal genome sequences has provided a major

opportunity to explore and further exploit fungi as sources of enzymes and metabolites. In this review chapter we focus on the use of fungi in the production of food additives but take a largely pre-genomic, albeit a mainly molecular, view of the topic.

Keywords Acids · Enzymes · Food · Fungus · Vitamins

1 Introduction

Filamentous fungi have long been used in the production of foods and beverages. Although only a small fraction of all fungal species is either eaten or used as a source of food additives or processing aids, there are still many species used for those purposes and there is considerable experience of handling those species at the commercial scale. We focus here on the fungal products used in food production and these include enzymes and smaller metabolites such as fatty acids, other organic acids, vitamins and flavour compounds. In some cases, the fungal products that are important parts of food manufacture are provided in situ rather than added exogenously. Examples of such foods include many fermented soy-based foods, and many mould-ripened foods where the fungus provides flavour. For the purposes of this chapter we adopt the current European Commission definitions of "food ingredient", "food additive" and "processing aid". A food ingredient is a component of the food that characterises that food. Some fungal products are classed as "additives". In that case, the additive is not a component that particularly characterises the food but, rather, the additive is provided for a specific purpose such as manufacture, processing, colouration or preservation of the food and the additive remains in the food. Many fungal products fall into this category and are discussed in this chapter. Several fungal enzymes are used in food processing but, in the main, they do not class as additives because they do not generally remain in the food with functionality – rather, they remain as residues from an early stage in the food manufacture. In that case, they are processing aids rather than additives. We include discussion of fungal enzymes in this chapter and the title is therefore widened to include processing aids.

2 Enzymes from Fungi

The range of native fungal enzymes used in foods is impressive and the enzymes have a large variety of applications. As reviewed previously [1, 2], fungi probably account for ca. 40% of available commercial enzymes and there are many published lists of enzymes and their applications [3, 4]. As more fungal genomes are sequenced and annotated, an even broader range of fungal enzymes with food applications will emerge. In addition, fungi are promising

Table 1 Fungal enzymes approved for use in food production (enzyme list taken from http://www.amfep.org). Note that the source fungus is provided even though, in some cases, the enzyme might be produced in recombinant form from a different fungus

Enzyme	Fungal genus	Typical application
Aminoacylase	*Aspergillus*	Synthesis of L-amino acids
Aminopeptidase	*Aspergillus* *Rhizopus*	Protein hydrolysis: flavour enhancement, debittering
α-Amylase	*Aspergillus* *Thermomonspora*	Starch liquefaction, alcohol production
Arabinanase	*Aspergillus*	Lignocellulose degradation, e.g. for vegetable processing and bioethanol
Asparaginase	*Aspergillus*	Reduction of acrylamide formation: applications in baking
Carboxypeptidase	*Aspergillus*	Protein hydrolysis: flavour enhancement, debittering
Catalase	*Aspergillus*	Removal of hydrogen peroxide, used together with glucose oxidase to improve storage of foods and to produce gluconic acid from glucose
Cellulase	*Aspergillus* *Humicola* *Penicillium* *Trichoderma*	Lignocellulose degradation, e.g. for vegetable processing, malting and brewing, and production of bioethanol
Dextranase	*Chaetomium*	Used in sugar industry and as stabilizers
Esterase	*Rhizomucor*	Feruloyl esterases are used in lignocellulose degradation for a variety of applications
α-Galactosidase	*Aspergillus*	Raffinose hydrolysis; used in brewing and to reduce bloating
β-Galactosidase	*Aspergillus*	Lactose hydrolysis; used in some milk products, whey utilisation
β-Glucanase	*Aspergillus* *Disporotrichum* *Humicola* *Penicillium* *Trichoderma*	Lignocellulose degradation; used in brewing, juice clarification
Glucoamylase	*Aspergillus* *Rhizopus*	Starch degradation for release of glucose as substrate for fermentations; used in starch and brewing industries
Glucose oxidase	*Aspergillus* *Penicillium*	Removal of glucose. Often used with catalase, e.g. to produce gluconic acid from glucose
α-Glucosidase	*Aspergillus*	Starch processing enzyme
β-Glucosidase	*Aspergillus* *Penicillium*	Lignocelluose degradation and also flavour enhancement in wines
Glucosyltransferase	*Aspergillus*	Production of oligosaccharides with food-relevant properties, e.g. low calorie glycans

Table 1 (continued)

Enzyme	Fungal genus	Typical application
Hemicellulase	*Aspergillus*	Lignocellulose degradation, and applications in starch and baking industries
Inulase	*Aspergillus*	Production of fructose and oligomers. May be used as a prebiotic
Laccase	*Myceliopthora* *Polyporus* *Thielavia* *Trametes*	Lignocellulose degradation; used in brewing and other beverage industries
Lipase	*Aspergillus* *Penicillium* *Mucor* *Rhizomucor* *Rhizopus*	Various lipase specificities, e.g. in cheese manufacture
Mannanase	*Aspergillus* *Trichoderma*	Lignocellulose degradation
Pectin lyase	*Aspergillus* *Penicillium* *Rhizopus* *Trichoderma*	Degradation and modification of pectins; used in fruit and wine industries
Pectin methylesterase	*Aspergillus* *Penicillium* *Rhizopus* *Trichoderma*	Degradation and modification of pectins; used in fruit and wine industries
Pentosanase	*Aspergillus* *Humicola* *Trichoderma*	Breakdown of pentosan for reduction in viscosity; used in baking
Phosphatase	*Aspergillus*	Dephosphorylation, e.g. in legume processing and baking
Phosphodiesterase	*Leptographium* *Penicillium*	Used as a flavour enhancer
Phospholipases A and B	*Aspergillus* *Trichoderma*	Processing aid, used mainly in the cheese industry
Phytase	*Aspergillus*	Dephosphorylation, e.g. in legume processing and baking
Polygalacturonase	*Aspergillus* *Penicillium* *Trichoderma*	Degradation and modification of pectins; used in fruit and wine industries
Protease	*Aspergillus* *Cryphonectria* *Penicillium* *Rhizomucor* *Rhizopus*	Variety of applications including the baking and cheese (chymosin) industries
Pullunanase	*Trichoderma*	Used in starch (amylopectin) processing
Rhamnosidase	*Penicillium*	Used in flavour development (e.g. in wines) and debittering

Table 1 (continued)

Enzyme	Fungal genus	Typical application
Tannase	*Aspergillus*	Use in tea products and to enhance anti-oxidant properties
Transglutaminase	*Streptoverticillium*	Used to cross-link proteins in a variety of applications, e.g. in meat and cheese industries
Xylanase	*Aspergillus* *Disporotrichum* *Humicola* *Penicillium* *Trichoderma*	Xylan degradation, e.g. in brewing

hosts as cell factories for the production of heterologous enzymes [5–8] and many of those enzymes have food applications. In addition to being added exogenously during food manufacture, the provision of fungal enzymes is a key component in the manufacture of fermented soy products [9, 10] and we therefore discuss in this chapter enzymes that are synthesised in situ or added separately. There is a distinction made between enzymes used as processing aids (the majority of food enzymes) and those approved as food additives (e.g. invertase in soft-centered chocolates). For approval of food ingredients as additives, a history of safe use has been a guiding principle, although the addition of new compounds to approved lists requires evaluation governed by strict procedures. In Europe, the Association of Manufacturers of Fermentation Enzyme Products (AMFEP) provides a catalogue of enzymes approved for use in food within Europe (http://www.amfep.org). Filamentous fungal-derived approved enzymes, taken from AMFEP, are listed in Table 1. Their data includes both native and recombinant fungal enzymes, and a subset of the recombinant (heterologous) enzymes produced by fungi was tabulated in a recent review [11]. In the US, the Enzyme Technical Association (http://www.enzymetechnicalassoc.org) provides a list of enzymes approved as food additives either for specific uses or as generally regarded as safe (GRAS) substances. Most enzymes are used in practice, however, as processing aids.

2.1 Native Enzymes Expressed by Filamentous Fungi

2.1.1 Submerged Liquid Culture

Enzymes are produced from fungi grown in a variety of formats and these mainly involve submerged liquid culture. The use of solid or semi-solid sub-

strates is discussed separately in the coming sections. Because most of the fungal species used to supply industrial enzymes are naturally adept at degrading polymeric material, the spectrum of enzymes produced from fungi is dominated by hydrolytic enzymes for the degradation of proteins and the major plant-derived polysaccharides that include cellulose, hemicellulose, pectin and starch. The other major carbonaceous polymer is lignin, which is impervious to hydrolytic degradation but its degradation by some species of fungi is also possible through the action of peroxidases and oxidases. The genome sequence of the lignin-degrading "white-rot" Basidiomycete *Phanerochaete chrysosporium* is now publicly available [12] and is likely to lead to a better knowledge of the range of enzymes available for degradation of lignin. These enzymes are primarily used in applications such as waste treatment and environmental clean-up, which are beyond the scope of this chapter (for further details of lignin degradation using fungal enzymes see [13, 14]).

The degradation of plant cell wall polysaccharides, and the ferulic acid-containing cross links between the polysaccharides, by enzymes from *Aspergillus* spp. has been described recently in detail by de Vries and Visser [15]. This review provides a detailed description of the *Aspergillus* enzyme systems which degrade cellulose, different hemicelluloses (with a focus on the xylans and (galacto)glucomannans) and the pectins. Recent genome sequence data can also predict genes likely to encode enzymes that degrade plant cell wall polymers [16]. Indeed, the number of available fungal genome sequences is increasing (http://www.broad.mit.edu/annotation/, and other sites) and several genome sequences have been annotated and published (e.g. [17, 18]). In addition to encoding enzymes capable of degrading plant cell wall polymers, *Aspergillus* spp. are sources of starch-degrading enzymes [19, 20]. *Aspergillus* spp. are not alone in secreting industrially useful polysaccharide-degrading enzymes and, in particular, *Trichoderma* [21, 22] and *Penicillium* [23, 24] are genera that include species noted for production of commercial levels of cellulases and xylanases [22–25], and *Rhizopus* spp. [20, 26, 27] join *Aspergillus* spp. as important sources of commercial glucoamylase, lipase and pectinases. Plant cell wall polysaccharides are linked through both covalent (e.g. ferulic acid links) and non-covalent bonds (especially hydrogen bonds). Fungal ferulic acid esterases have been summarised elsewhere [15, 28, 29] and another enzyme activity, glucuronyl esterase, may cleave susceptible links between lignin and hemicellulose [30]. Recently, a protein was described from *Trichoderma reesei* that has similarity to plant expansins which have a role in disrupting hydrogen bonds between plant cell wall polymers without itself causing hydrolysis. The fungal protein, which had a cellulose-binding domain at the N-terminus, was named swollenin [31] and it remains to be seen if this and other related proteins will find applications in food processing. Already, a fusion protein of swollenin and feruloyl esterase has been constructed and used [32]. Many of the plant cell wall-degrading enzymes from fungi find a wide variety of applications as aids during the processing

of fruits and vegetables [15]. For example, xylanases are used to improve the quality of bread doughs, in clarifying juices and in the production of xylose and oligomers of xylose used as food additives. Similarly, pectinases are used in juice clarification and for production of monomeric components of pectins that serve as the starting materials for conversion to other products (e.g. galacturonic acid to ascorbic acid). The use of fungal amylases is important in the starch processing industries to provide glucose syrups used in many fermentation processes. As well as being sources of enzymes that degrade plant cell wall polymers, fungi provide many other enzymes used as food processing aids and these include galactosidase, catalase, glucose oxidase, lactase, lipase, phospholipase, phytase and, importantly, proteases where *Aspergillus* and *Rhizopus* spp. provide the bulk of these commercial enzymes.

The search is always on for enzymes with new properties (e.g. in terms of pH optimum, salt tolerance, proteolytic resistance or thermal stability) and for strains that produce enzymes at improved yield or, at least, under production conditions where the cost is lowered (which might include the use of cheaper substrates for growth or improved bioreactor formats). In addition, the availability of fungal genome sequences is providing a resource for the identification of genes that may encode interesting new activities. So, for example, many cellulose-binding and starch-binding domains have sequence similarities that encourage a search of databases for genes encoding familial enzymes. This type of strategy can be taken to levels of greater detail and ingenuity, dictated only by knowledge of key sequence motifs or functional domains in target enzymes (e.g. in the cellulases and xylanases [33–35]) and also by availability and access to fungal genome sequences. The approach is also tempered by the knowledge that sequence is not a guarantor of functionality, therefore it is necessary to clone and express genes of interest so that the encoded enzymes can be characterised. A recent example of this approach is provided by the cloning of the gene encoding a prolidase (a protease) from *Aspergillus nidulans* [36]. The approach of purifying enzyme activities and then resorting to reverse genetics for gene cloning has been the principal recombinant DNA-based strategy alongside classical strain improvement by mutagenesis and screening. Although a flurry of genome sequence data mining is inevitable in the immediate future and mid-term, and likely to be productive, the more classical approaches should not be overlooked. Xylanases have been purified and characterised from several fungi (especially species of *Aspergillus, Trichoderma* and *Agaricus*) and, in some cases, the encoding genes have been cloned and aspects of their transcriptional regulation studied [15, 37–41]. In addition to xylanases from *Penicillium funiculosum*, xylanases and their genes have been described from *Penicillium purpurogenum* (see [24] and references therein).

The transcriptional regulation of the main groups of genes encoding hydrolytic enzymes from fungi has been well described [42, 43] and will not be covered in detail again here. The cellulolytic and xylanolytic genes from

fungi are, for example, subject to transcriptional regulation through substrate induction and carbon catabolite repression, and there is interplay between the two systems. The roles of the transcriptional activator XlnR and the carbon catabolite repressor protein CreA, in *Aspergillus* spp. have been described recently in detail [15, 44]. XlnR (thought originally to be a specific regulator of xylanase-encoding genes but now known to regulate other genes including some cellulases) binds to a UAS (upstream activator sequence) with the core consensus GGCTAA whereas CreA binds to a URS (upstream repressing sequence) with the consensus SYGGRG (where S = C or G, Y = C or T, R = A or G). A summary of inducing and repressing mechanisms of fungal cellulases has recently been provided [45] and the expression of ten hemicellulases from *T. reesei* grown in the presence of various di- and monomeric sugars as well as some polymeric carbon sources was also reported [46]. The role of XynR as a regulator of genes encoding xylanases and cellulases in *Aspergillus oryzae* has also recently been reported [41, 47]. We continue to learn more of the mechanisms of both induction and repression of the fungal cellulases. For example, it has recently been shown that phosphorylation of Cre1 is necessary for binding to its recognition site and subsequent action as the mediator of carbon catabolite repression in *T. reesei* [48]. The isolation of putative transcriptional regulators (in addition to the Cre1 repressor) of cellulase-encoding genes in *T. reesei* has recently been successful with the description of two genes *ace1* [49] and *ace2* [50] that encode transcriptional regulators that contribute to the transcriptional regulation of some cellulase- and xylanase-encoding genes. It has been hypothesised that regulation of the xylanase gene *xyn1* in *T. reesei* is orchestrated by an interplay of the Cre1 and Ace1 regulators with Xyr1 (homologue of the *A. niger* XlnR and both regulators have closely related consensus binding motifs) acting as a transactivator [51]. Not all enzymes involved in the complete degradation of cellulose to glucose need be extracellular as demonstrated by the description of an intracellular β-glucosidase in *T. reesei* [52] and prediction of intracellular hydrolases in *A. nidulans* [53]. The induction of genes encoding pectinases and ferulic acid esterases [15] requires the presence of substrate and, in the case of pectinases for example, it appears that D-galacturonic and/or glucuronic acids may be the key components that lead to transcriptional induction via a mechanism yet to be described. Expression profiling of the known pectinase- and ferulic acid esterase-encoding genes in *A. niger* confirmed the importance of D-galacturonic acid (or a derived metabolite) as a general regulator of pectinase gene expression [54]. This study went further and showed that, in addition, subsets of genes encoding pectinolytic activities respond to L-arabinose, L-rhamnose or ferulic acid. Regulation of pentose catabolic pathway genes has been described further recently [55].

The secreted production of cellulases to high yields [22] by *T. reesei* is well known and underpins commercial activities with the system. The cellulases secreted by *T. reesei* are glycosylated and a combined approach of metabolic

labelling and proteomics has enabled a kinetic analysis of the assembly and secretion of these glycoproteins [56]. The heterogeneity and kinetics of secretion of cellobiohydrolase I (Cbh1) with respect to glycosylation during its assembly within the secretory pathway was demonstrated. While it took on average about 4 min to synthesise a Cbh1 glycoprotein, it took about 11 min for its secretion. This type of analysis may prove to be useful in identifying bottlenecks in secreted production of an enzyme and may be particularly useful when applied to heterologous proteins. Global analyses of protein production using proteomic approaches with *T. reesei* are proving useful and use can also be made of cDNA microarrays in global analyses of transcriptional regulation [57]. Studies with *T. reesei* have also shown that O-linked glycosylation of cellulases during the secretory pathway has an impact on secreted yields [58], i.e. affects the passage of glycoproteins.

Fungi (especially *A. niger*, *A. oryzae* and *Rhizopus* spp.) are used to produce proteases for use as processing aids and proteases are important activities in the solid state koji fermentation (see below). As an example, *A. niger* encodes a very large number of different proteases and, to date, these have been mainly studied as purified enzymes or in mutagenised strains that have lost an activity [59–61]. Not all of these proteases are secreted or lend themselves to commercial applications. Even so, removal of a contaminating protease activity may be just as useful, depending on the application, as overproducing a target protease so continued exploration of fungal proteases and, in particular, the regulation of their expression is warranted. Protease-encoding genes are subject to transcriptional regulation in response to nitrogen and carbon sources (reviewed in [59]) and pH is also a potent regulator of proteases and mediated by the PacC regulator [62]. The pH response and regulatory system appears to operate with close similarity in many fungal species (reviewed in [63]) and leads to transcriptional regulation of a wide variety of genes used in food processing, e.g. xylanases and pectinases [15].

Fungi are sources of commercial quantities of enzymes for degradation of starch. These include both *endo*-acting and *exo*-acting amylases, and some with de-branching activity. In order to explain the mechanism of induction (e.g. by starch or maltose) and repression (e.g. by glucose or xylose) of the starch-degrading enzymes, there has been extensive analysis of promoters through deletion studies [64, 65]. Overexpression studies have suggested that the various amylase-encoding genes share common transcriptional regulators and have shown that titration of transcription factors can occur [66–71]. The CreA protein that mediates carbon catabolite repression of cellulases also operates to repress transcription of amylases in fungi [44, 70]. Transcriptional activation is mediated through the AmyR zinc-finger protein [71, 72]. A review summarises the functions of both the XlnR and AmyR transcriptional activators as well as the role of the CCAAT-binding protein complex in enhancing promoter activity of many fungal genes, including the genes encoding cellulose- and starch-degrading enzymes in fungi [73].

2.1.2 Solid State Culture

The growth of microorganisms on surfaces can lead to growth characteristics and products formed that differ from those of the same cultures grown in submerged liquid culture [74, 75]. The capacity of some species of filamentous fungi to secrete degradative enzymes is exploited in solid state fermentations (SSF) for production of fungal biomass (i.e. edible mushrooms) and a variety of foods and beverages [9, 61, 76]. The manufacture of products from fermented soy, rice and other plant materials is widespread in far Eastern countries where species of *Aspergillus* and *Rhizopus* are most commonly exploited to initiate the initial breakdown of complex organic matter [9, 76]. Increasingly, SSF is being used in Europe and the USA for the manufacture of fermented products. SSF is seen as a means of making a range of valuable products in a more cost effective manner as it enables the synthesis of valuable products from substrates which have a low or even a negative value, e.g. waste agricultural products which incur a high cost for either transport and disposal or for conversion into animal feed. The fungi are particularly well-adapted for SSF as they have a high capacity for the secretion of hydrolytic enzymes necessary for the degradation of plant cell walls and storage carbohydrates. Secondary fermentations can then be carried out by yeasts and bacteria, which utilise the partially degraded organic matter produced by the action of fungal enzymes.

Several comparisons have been made between fungal strains grown in SSF and liquid cultures (reviewed in [74, 75]). For example, depending on the substrate used, secretion of specific degradative enzymes such as amylases, phytases and pectinases is commonly higher in SSF than in liquid cultures [77–79]. The differential expression of two glucoamylase-encoding genes by *A. oryzae* is particularly marked with the *glaB* gene being strongly up-regulated in SSF [80, 81]. In addition, SSF-grown *Aspergillus* is reported to be less sensitive to carbon catabolite repression than when grown in liquid culture [82]. The synthesis of non-enzymic metabolites by fungi can also be enhanced in SSF (e.g. [83]).

A recent review of the koji process [9], that employs *A. oryzae* enzymes secreted from the fungus during growth on the solid substrate (e.g. soy or cereals), updates some earlier reviews (e.g. [76]) and provides a clear analysis of the process and the key role of fungal enzymes in degrading the plant materials prior to subsequent fermentation by other microorganisms. The recently published genome sequence of *A. oryzae* [84] offers major promise for further development of *A. oryzae* in solid-substrate fermentations (SSF) [10]. The expectation is that a combination of genome sequence, gene microarrays and proteomic studies will provide the scientific community and commercial users of *A. oryzae* with a major resource for understanding the science of solid state fermentations [85] in relation to the transcription of genes en-

coding enzymes important for the koji process. Analogous facilities will also be available for other key industrial enzyme-producing fungi. The *A. oryzae* genome sequence should aid investigations into the molecular basis of regulatory processes that are specific to solid state cultivation of the fungus. At present, as mentioned previously, we know that solid state cultivation leads to altered patterns of transcription compared to that when the same fungus is grown in submerged culture, and we already have examples of SSF-specific promoters that have been used in the construction of new hybrid promoters [86]. The lower water activity in solid state cultivation may be the trigger to altered transcriptional regulation [9, 10] but we are far from explaining at the molecular level all the differences in morphology and transcriptional regulation in *A. oryzae* grown under the two different cultivation conditions. The solid state system is characterised by low water activity, the presence of surfaces and also gradients in nutrients [74, 75, 87] that combine to present a highly complex system. Unravelling the system will require its physical characterisation alongside the use of genomic approaches. A recent comparison between SSF (on wheat bran) and submerged cultivation (using a wheat bran extract to support growth) of *A. oryzae* [88] used subtractive cloning of genes differentially expressed under the two different regimes. Sequences were compared with the *A. oryzae* EST database as well as other gene databases in order to identify putative genes that were specifically transcribed or repressed in SSF compared to submerged culture. The database generated by this approach will require further analysis but it is clear that SSF leads to the specific expression of a large number of genes. *A. oryzae* has a further attraction for being the target of detailed genomic and proteomic studies [10]. Not only is it a species used widely in solid state fermentations but it is also very closely related to other food-use fermentative species (*Aspergillus sojae*) and aflatoxigenic species (*Aspergillus flavus* and *Aspergillus parasiticus*) [89–91]. This is not to suggest that koji moulds are likely to synthesise aflatoxins because they do not, and there are known gene-based reasons underlying the lack of toxin synthesis by *A. sojae* [92] and *A. oryzae* [93]. But, as a group of species that are important for both their enzymes and their capacity for secondary metabolite production, the genomic and proteomic approaches now available present us with unparalleled opportunities for detailed mechanistic studies.

2.2
Heterologous Proteins Expressed by Filamentous Fungi

Fungal species that have been used successfully as cell factories for commercial enzymes that are used in food manufacture appear to be attractive hosts for production of any protein, whether it be encoded by a native or heterologous gene. The fungi lend themselves to commercial-scale operations (i.e. the fungi are industrially friendly and produce profitable yields of target

enzymes) and produce enzymes in formulations that have a history of safe use. Most of the enzymes produced in this way are secreted so there is no recourse to mycelial breakage and, for many formulations, the mycelium is simply removed from the culture to leave a saleable product. It has not, however, been so straightforward to develop fungi as hosts for the production of heterologous enzymes on the commercial scale. This chapter is concerned with fungal products used in foods so, in the case of enzymes, we are dealing primarily with a bulk process where there is no commercial sense in secreting a poor yield of a target protein (unlike in some clinical applications where a high price for the enzyme might mitigate against a low yield). In the main, and despite some success stories, e.g. chymosin from *Aspergillus awamori* [94] and lactoferrin from *A. oryzae* [95], heterologous protein production from fungi has led to strains that secrete far lower (often 1–3 orders of magnitude) levels of heterologous enzymes than can be achieved with native enzymes. The reasons underlying this lack of success have been evaluated in detail in recent reviews [1, 7, 8] so will only be summarised here.

The first major success story was the secreted production of chymosin from *A. awamori* which has been summarised [94]. The approaches used during this work to produce a production strain have been adopted in most of the subsequent, and less successful, attempts to produce heterologous proteins at high secreted yields. The key elements to the success with chymosin were the use of a strong native fungal promoter (from the glucoamylase gene, *glaA*) driving the expression of a translational fusion of the pro-chymosin gene with the gene encoding glucoamylase (as a carrier protein), deletion of the gene encoding the major aspartyl protease (aspergillopepsin, *pepA*) and then rounds of mutagenesis and screening (including selection for resistance to deoxyglucose). Pro-chymosin, being a protease, was able to cleave the glucoamylase-pro-chymsoin fusion protein post-secretion to release mature chymosin. This, "fusion strategy" has been widely adopted and modified somewhat (summarised in [1, 5–7]) for use with target proteins that are not proteases. In those cases, an endoproteolytic dibasic amino acid cleavage site that is recognised by the intracellular KEX2-like protease [96, 97] is incorporated during construction of the expression construct at the fusion site between the coding regions for the carrier protein and target protein – incorporated, for example, in the high level expression of lactoferrin from *A. oryzae* [98]. The dibasic amino acid cleavage sites are generally, but not always [99, 100] cleaved faithfully during secretion to release the target protein. As many fungal species are excellent protease producers, it is not surprising that deletion of protease activity, either by mutagenesis or targetted gene disruption, can lead to improvements in the yields of protease-sensitive proteins. The use of a strong fungal promoter and optimised gene copies (this does not necessarily mean highest number [66]) has also been adopted quite generally as a strategy, although there have been few attempts to tailor promoters for particular uses. One recent and welcome addition to the list of

available promoters has been the construction of a strain of *A. niger* able to use the *A. nidulans alcA* promoter [101], a strong and tightly regulated promoter which is increasingly well understood in terms of the mechanism of transcriptional regulation [102]. With the exception of fungal genes expressed "heterologously" in a related fungal species, it is sobering to note that there has been no significant advance that has led to the reliable secreted production at high yield of all heterologous proteins in fungi. Some target proteins are more easily expressed and secreted than others, so the nature of the protein itself is important. There are issues relating to codon usage and mRNA stability that are important for the expression of some heterologous proteins but it appears that many problems are post-translational and most probably reside in the secretory pathway [1,5–7]. Therefore, in those cases, it will be necessary to understand the very nature of what makes the target protein appear as foreign to the fungal secretory system before strategies to overcome the difficulties can be devised. It may therefore be necessary to tailor the fungus to the particular target protein although it is hoped that new understanding will be generally applicable.

Most current studies aimed at improving the secreted yield of heterologous proteins from fungi are with target proteins that are not destined for food use. It is likely though that knowledge gained in those systems will be advantageous in the production of food-use proteins. The secretion process itself, involving protein folding, glycosylation and vesicular transport to the hyphal tip, is a major target of current research effort [5,7]. Accordingly, the morphology of the producer strain is likely to be a key issue in optimising secreted yields of proteins, especially under production conditions where physical properties of a mycelium are important. Folding of secretory proteins takes place within the lumen of the endoplamic reticulum (ER) and is assisted by foldases and chaperones that are resident in the lumen. Activation of the unfolded protein response (UPR) [103,104] by heterologous proteins indicates that the lumenal environment is not conducive to folding that particular target protein with the kinetics expected by the fungus (i.e. compared to its native proteins). The UPR leads to the transcriptional up-regulation of a wide range of genes (e.g. in *Saccharomyces cerevisiae* [105], *T. reesei* [106] and *A. niger* [107]), some of which encode chaperones and foldases that are resident in the lumen of the endoplasmic reticulum, i.e. a stress response mechanism within the cell to overcome a detected difficulty in protein folding. The UPR also leads to proteolytic degradation (via the proteasome) of poorly folding proteins within the ER: this is termed ERAD (endoplasmic reticulum associated protein degradation) [108]. Current knowledge on the detail of the UPR in filamentous fungi has recently been summarised [5,7]. An issue relevant to the discussion in this chapter is whether, and how, knowledge of the UPR has helped to improve the folding of heterologous proteins in the ER. One strategy, for example, is to deliberately induce the UPR in the expectation

of a folding problem when expressing a heterologous protein. General up-regulation of the UPR through overexpression of the mediating transcription factor Hac showed some promise with some proteins [109] and there have been various examinations of altered expression of individual chaperones or foldases [110–113]. This approach has not generally been as useful as anticipated although there are some indications that it can work for some chaperone (or foldase)/heterologous protein combinations. Few such studies have been with food-use proteins, a notable exception being the expression of the sweet protein thaumatin from *A. awamori* [114]. Thaumatin is a 22 kDa plant-derived protein that contains eight disulphide bonds and can be expressed in *A. awamori* (a close relative of *A. niger*). Co-expression of additional copies of *pdiA* (encoding protein disulphide isomerase) was studied and an optimal level of PdiA protein (between two and four-fold compared to the natural level) was shown to improve the secreted yield of thaumatin by five-fold whereas the secreted levels of two native proteins were unaffected [114]. In contrast, lysozyme is a smaller (ca. 14 kDa) protein containing four disulphide bonds but co-expression of additional *pdiA* gene copies did not affect secreted lysozyme levels [110]. Lysozyme is an interesting target protein for food applications as an antimicrobial [115–117] and, like the antimicrobial lactoferrin [95, 118, 119] has been successfully produced from *Aspergillus* spp. [120, 121]. In both cases, the structures of recombinant lysozyme [120] and lactoferrin [122] have been shown to be authentic. In an interesting aside, variant lysozymes have also been secreted from *A. niger* and there has been a recent focus on variant forms of lysozyme that form amyloids that are associated with disease and death in humans [123]. Thus, the production system for variant forms of lysozyme [124, 125] underpins studies of the key protein folding events that lead to a variety of amyloid diseases [126].

3 Organic Acids

Filamentous fungi produce a number of organic acids that are used as additives in the food industry (Table 2) and [127, 128]. Although the basic biochemical pathways for their synthesis are known, the mechanisms by which overproduction is achieved in industrial applications are still under investigation in many cases. Acidogenesis is considered to be one energy-efficient way of dealing with excess carbon when other nutrients, particularly nitrogen, are limiting [129]. Another strategy is to divert excess carbon to the biosynthesis of fatty acids and storage lipids [130]. Several fungi are rich sources of fatty acids (Table 2), especially organisms that produce high yields of triacyglycerol (TAG) oil, the so-called oleaginous fungi [131, 132]. In this section, production of organic acids will be reviewed and recent genetic and molecu-

Table 2 Organic acids, including fatty acids, produced by filamentous fungi for use, or with potential use, as food additives

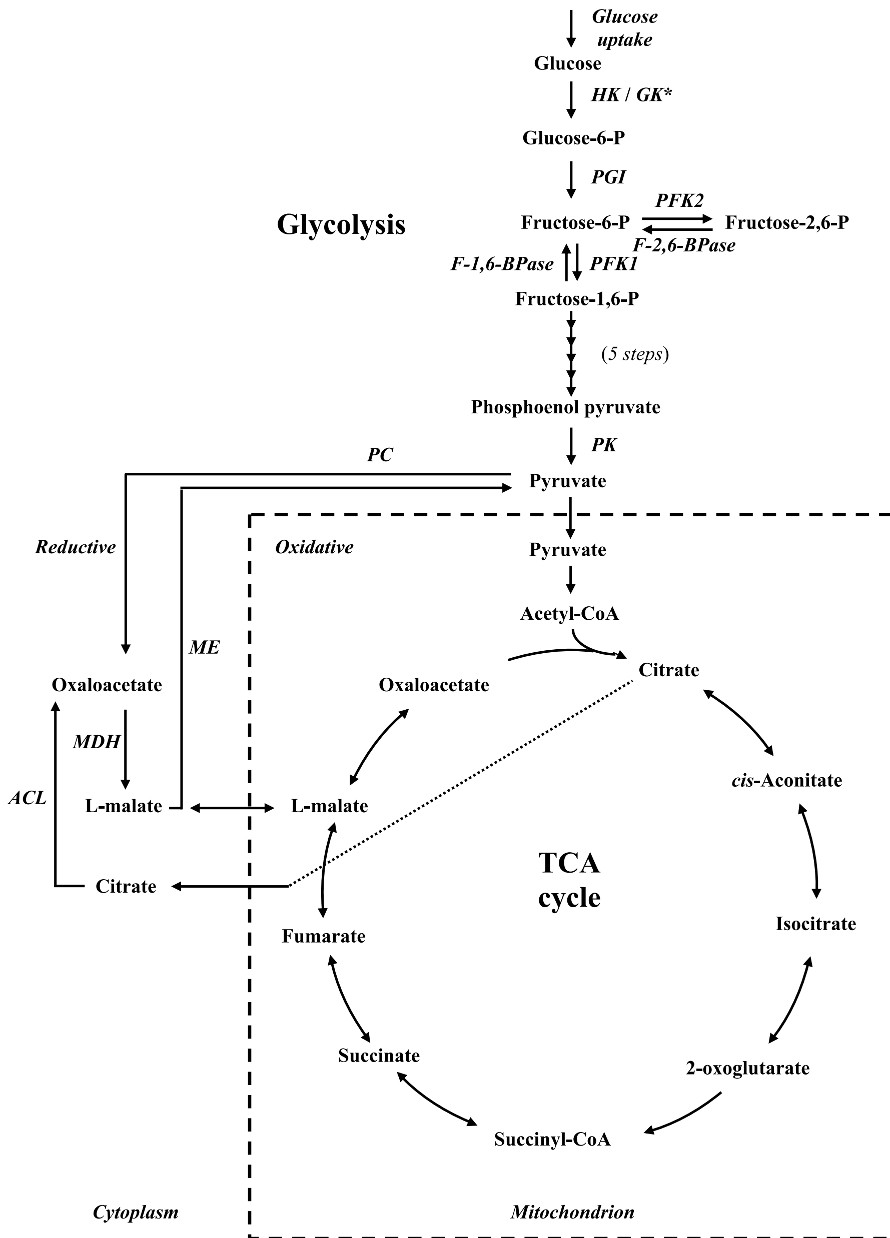
Product	Use(s)	Fungal source
Citric acid	pH regulator; acidulant; acid flavour; preservative; lipid antioxidant; emulsifier	*A. niger*
Gluconic acid (derivatives and δ-gluconolactone)	Chelating agent (for mineral enrichment); slow-acting acidulant; preservative	*A. niger*; *Penicillium* spp.
L-Malic acid	Acidulant; preservative; "smooth" acid flavour	*Schizophyllum commune*; *Paecilomyces varioti*
Lactic acid	Acidulant; preservative	*Rhizopus oryzae*
Fumaric acid[a]	Acidulant; long-lasting "strong" acid flavour	*Rhizopus* spp.
Succinic acid[a]	Flavouring agent	*Rhizopus* spp. (mixed fermentation with bacteria)
Tartaric acid[a]	Flavouring agent	*Aspergillus* spp.; *Penicillium notatum*
iso-Ascorbic acid[a]	Preservative	*Aspergillus* spp.; *Torula* spp.; *Penicillium* spp.; *Fusarium* spp.
Kojic acid[b]	Antibrowning agent; antioxidant; preservative	*A. oryzae*; *A. sojae*; *Penicillium* spp.
γ-Linolenic acid[c,d]	Nutritional supplement	*Mucor circinelloides*; *Mortierella isabellina*
Arachidonic acid[c,e]	Baby milk supplement	*Mortierella alpina*

[a] Produced primarily by chemical synthesis
[b] Use in foods now banned because of mycotoxin properties
[c] Produced as a fatty acid component of triacylglycerol oil
[d] Produced now from plants
[e] Blended with an algal docosahexaenoic-rich oil

lar biological approaches to understand and manipulate these pathways will be discussed where applicable.

3.1 Citric Acid

Many of the organic acids excreted by filamentous fungi are intermediates of the tricarboxylic acid (TCA) cycle (Fig. 1). Their efflux into the growth medium is considered an "energy-spilling" phenomenon which avoids the synthesis of excess NADH from TCA cycling. Also termed "overflow metabolism", the fungi in question are able to uncouple catabolic and anabolic processes in order to maintain a high flux of carbon when the car-



bon source is in excess and some other factor is limiting [133]. Organic acid excretion can reduce the pH of the culture medium to below 2.0 which is tolerated by these fungi because of their extremely efficient intracellular pH-homeostatic system [134]. Citric acid, by far the most important econom-

◀ **Fig. 1** Schematic diagram of glycolysis, the tricarboxylic acid (TCA) cycle and associated pathways showing the biosynthesis of many of the important organic acids excreted by fungi (modified from [127, 130, 154]). The following glycolytic and associated enzymes are abbreviated: *HK*, hexokinase; *GK*, glucokinase (* less active during citrate production [151]); *PGI*, phosphoglucose isomerase; *PFK1*, 6-phosphofructo-1-kinase; *PFK2*, 6-phosphofructo-2-kinase; *F-1,6-BPase*, fructose-1,6-bisphosphatase; *F-2,6-BPase*, fructose-2,6-bisphosphatase; *PK*, pyruvate kinase; *PC*, pyruvate carboxylase; *MDH*, malate dehydrogenase; *ME*, malic enzyme (malate decarboxylase); *ACL*, ATP:citrate lyase

ically, is used as a pH regulator and acidulant that imparts acid flavour in a wide variety of foods ranging from carbonated soft drinks to processed meats [127]. The chief commercial source of citric acid used in the food industry is *A. niger*, accounting for an estimated 0.4 million tonnes worldwide worth £0.5 billion in 2001 [135–137], with demand continuing to increase by about 5% annually. This acid has GRAS status, being regarded as an extremely safe food additive, and is used as the standard against which other acidulants are measured in food formulations. The favoured production method is large-scale submerged fermentation using pure sugar or molasses as the carbon source [138]. In the past, a small proportion was made in Japan by solid state koji fermentation on substrates such as wheat bran or sweet potato waste pulp [139, 140] but this method is now uneconomic. Improvements in citric acid yield by *A. niger* have been achieved by both strain development, using standard mutagenesis and selection, and optimising culture conditions [127, 141, 142]. Metabolic engineering to improve strains by recombinant DNA technology is less advanced but offers future rewards once the key enzymes that regulate flux through the pathway have been identified [128].

The attainment of high citric acid yields in industrial fermentations is associated with a number of interacting nutritional and physiological factors, including high sugar and oxygen concentrations, low pH and deficiency in manganese and iron ions [127, 135, 143]. Of these parameters, sugar concentration is thought to be one of the most important because of the induction of a low-affinity glucose transporter and the subsequent phosphorylation of glucose by hexokinase, two significant flux control points at the start of the glycolytic pathway [143] and (Fig. 1). A number of biochemical studies on the control of citric acid production have identified regulatory roles for phosphofructokinase I, pyruvate kinase, pyruvate carboxylase, citrate synthase, isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase [127, 129]. During overproduction of citric acid, it was believed that the TCA cycle was interrupted at one or more steps after citrate biosynthesis but this is in dispute [129, 144]. However, several of the TCA cycle enzymes are subject to tight regulation, one example being inhibition of the irreversible 2-oxoglutarate dehydrogenase step. A recent investigation of this reaction by flux analysis has implicated a 4-aminobutyrate (GABA) shunt moving carbon from glutamate to succinate rather than via 2-oxoglutarate [145]. During acidogenesis, GABA

levels increased by about four-fold and flux from glutamate to succinate was greatly reduced. This therefore represents another flux control point at which genetic modification could elevate citric acid yields. If the fermentation process is not strictly controlled during citric acid biosynthesis, oxalic acid may occur as an unwanted, toxic by-product from the breakdown of oxaloacetate. A mutant strain of *A. niger* has been isolated that is defective in both glucose oxidase and oxaloacetate acetylhydrolase (OAH), the latter being responsible for oxalate accumulation, and interestingly, this mutant produced citric acid in a medium with a much higher pH and manganese ion concentration than normal [146]. The basis for this phenomenon has yet to be established but it confirms that the regulation of citric acid production can be modified. As already discussed, industrial citric acid-producing strains of *A. niger* have undergone several rounds of mutagenesis and many are defective in OAH activity, making them oxalate-negative strains.

To date, molecular genetic strategies to increase overall yields of citric acid or productivity rates have proved largely unsuccessful. The gene encoding citrate synthase (*citA*), which forms citrate from oxaloacetate and acetyl-CoA and which in the past has been considered a key step in the citric acid cycle, has been cloned from *A. niger* and overexpressed in this fungus with no apparent effect on citrate accumulation or on the levels of any other TCA cycle intermediate [147], which may or may not have been foreseen [148]. Similarly, overexpression of the *A. niger* phosphofructokinase I (*pfkA*) and pyruvate kinase (*pkiA*) genes, either individually or together, did not increase the rate of citric acid production suggesting that these enzymes also do not contribute significantly to regulating flux through the pathway [149]. One genetic modification in *A. niger* that did have a beneficial effect on citric acid synthesis was the disruption of one of the two differentially regulated trehalose-6-phosphate synthase genes (*tpsA*, otherwise designated *ggsA*) whose metabolic product, trehalose-6-phosphate, was shown to inhibit hexokinase activity, albeit to differing degrees in vitro [150, 151], and therefore to have a role in regulating glycolytic flux in this organism. Increased initial rates of citric acid formation were only evident in the *tpsA* disruptant when sugar concentrations in the medium were $\geq$5% (w/v), which would have resulted in sufficiently inhibitory cellular concentrations of trehalose-6-phosphate in the wild-type strain [150]. The final yield of citric acid produced was however unchanged. Interestingly, multicopy *hxkA* transformants of *A. niger* have been created that produced 50 times the wild-type level of hexokinase but no data have yet been presented on citric acid biosynthesis in these strains [151]. *A. niger* possesses a second respiratory pathway that is catalysed by the alternative oxidase (AOX) in addition to the normal cytochrome pathway and this has been implicated in citric acid biosynthesis as a way of continuing glycolysis during reduced TCA cycle activity [129, 152]. The gene encoding AOX, *aox1*, has been cloned [153] but its regulation during citrate production is still under study. Mathematical modelling has recently highlighted three main

flux control points for citric acid production, viz. sugar uptake into the cell, mitochondrial transport of pyruvate and citrate and citrate excretion from the cell, which correlates well with current experimental evidence [154]. In order to optimise citric acid productivity, this study indicated that the levels of at least 13 biosynthetic enzymes and transport proteins would have to be modified for a significant increase in yield to be achieved.

3.2 Gluconic, Malic and Kojic Acids

Gluconic acid is added to food as a slow-acting acidulant but only to a limited extent and even then mainly as the biosynthetic precursor, δ-gluconolactone which is used in combination with sodium bicarbonate to control the leavening of baked products. Because of its effective chelating properties, gluconic acid and its derivatives are being increasingly used to enrich foods with minerals such as calcium or iron but its main, non-food uses are in cleaning applications and concrete admixtures. A large proportion of the approximately 45 000 tonnes of gluconic acid used annually worldwide [155] is produced by submerged fermentation using *A. niger* [127, 141] or more recently by an in vitro process utilising concentrated glucose solutions and glucose oxidase and catalase enzymes isolated from *A. niger* [156]. Biosynthesis of δ-gluconolactone from glucose is catalysed extracellularly at pH values >3.5 by glucose oxidase and subsequent gluconolactonase-mediated hydrolysis of this intermediate yields gluconic acid [157] which can itself act as a carbon source for the fungus [158]. Increased biosynthetic yields of gluconic acid have been achieved mainly by optimising fermentation parameters and by strain improvement in a similar fashion to citric acid production [127, 141, 159–162]. In both approaches, this is mainly achieved by increasing glucose oxidase levels. Gluconic acid can also be synthesised chemically by the direct electrochemical oxidation of glucose.

The use of malic acid as an alternative acidulant in food and drink has gained importance over the last 10–20 years with the increasing popularity of artificial sweeteners such as saccharin and aspartame. Malic acid provides a slower-acting acid taste compared with citric acid. When present either on its own or in combination with citric acid, it can impart a smoother, more palatable flavour without the aftertaste associated with the sweetener and can also reduce the amount of sweetener required [163, 164]. Although malic acid for the food industry is primarily made by chemical means, resulting in a DL-racemic mixture, fungal fermentation giving L-malic acid remains a possibility, especially with strains of *Schizophyllum commune* and *Paecilomyces varioti* which can produce relatively high yields of this TCA cycle intermediate [127]. Lactic and fumaric acids are two other organic acids used as food acidulants with different taste characteristics which can be excreted by fungi such as *Rhizopus* spp. [127, 165] but both are obtained from other sources at present.

Kojic acid (5-hydroxy-2-hydroxymethyl-γ-pyrone) is produced by a number of fungi, particularly those of the *A. flavus*–*A. oryzae* group [166, 167]. This secondary metabolite has been associated in the past with several traditional Japanese koji-fermented foods and food products, such as *miso*, *shoyu* (soy sauce), *sake*, *amazake*, *shoucha* and *mirin*, but most *Aspergillus* strains used in koji have now been selected for their inability to produce this acid because of its mycotoxin properties [168]. Until recently, kojic acid was also added to a variety of processed foods as an antioxidant or antibrowning agent [169] and was promoted as a component of health foods. Although it has a long history of human consumption with no apparent medical problems, kojic acid is now considered to be a mycotoxin and recent evidence from animal studies has implicated it in the induction of thyroid cancers by affecting thyroid function [170]. The effect on thyroid function is reversible and is only associated with continuous high doses of kojic acid. Because of this concern, a number of foods have been screened for the presence of this metabolite and most proven to be negative [168, 171]. In addition, kojic acid has been shown to be approximately 90% inactivated by cooking [172]. Nevertheless, its use in foods has now been stopped even although it is not thought to present a major problem for human health [166]. The biosynthetic pathway is not understood completely, perhaps involving three separate routes, but the primary pathway is thought to be direct synthesis from glucose via reduction to gluconolactone and oxidation to 3-ketogluconic acid lactone. The latter then undergoes two dehydration steps resulting in the loss of two water molecules and the reduction of one double bond to form kojic acid. The genes encoding the kojic acid biosynthetic enzymes have yet to be identified. Recent studies have concentrated on increasing kojic acid yields from *A. oryzae* and *A. flavus* for non-food applications (e.g. in cosmetics, insecticides and biodegradable polymers). The approach has been to optimise fermentation parameters [173, 174] or improve strains by chemical mutagenesis [175].

3.3 Polyunsaturated Fatty Acids

In human nutrition, two polyunsaturated fatty acids (PUFAs), linoleic acid ($18:2n-6$) and α-linolenic acid ($18:3n-3$), are classed as essential fatty acids and are sometimes referred to as vitamin F, or the vitamin F group when some of their PUFA derivatives are included [176, 177]. Their absolute requirement for a healthy diet stems from the lack of fatty acid $\Delta12$-desaturase activity in humans (Fig. 2). Some filamentous fungi are used to produce fatty acids, particularly long-chain PUFAs [130, 179–181] that have importance both nutritionally and pharmacologically to humans. Long-chain PUFAs, arachidonic acid (ARA, $20:4n-6$) and docosahexaenoic acid (DHA, $22:6n-3$), are components of human milk and are incorporated into mem-

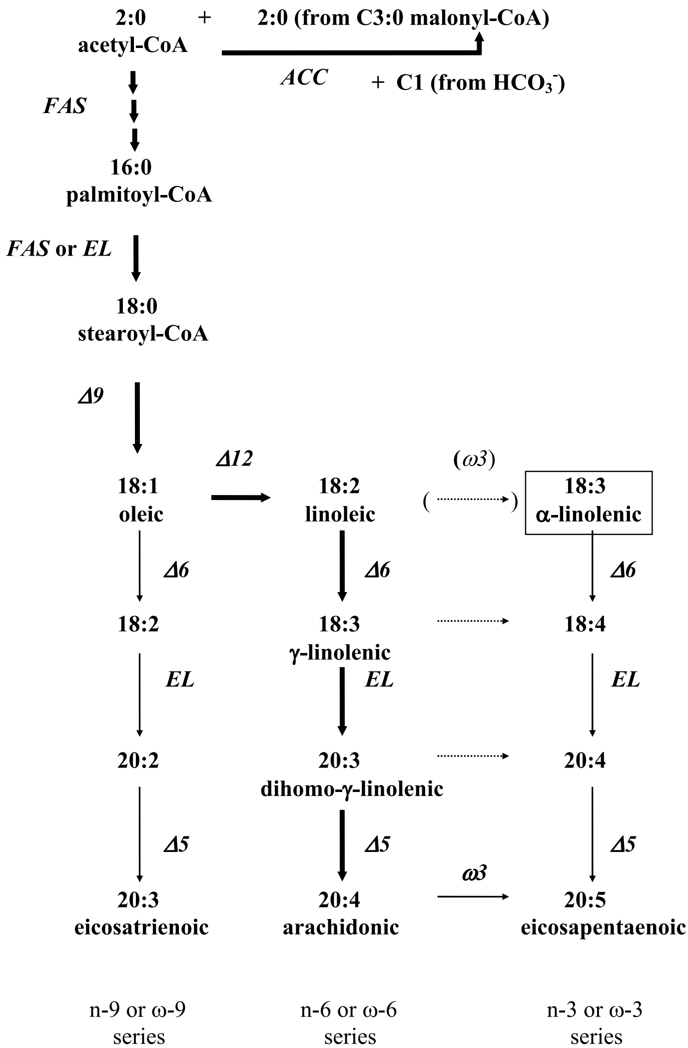


Fig. 2 PUFA biosynthetic pathway in *Mt. alpina*. Fatty acid biosynthetic pathway from acetyl-CoA (2 : 0) to polyunsaturated EPA (20 : 5n – 3), showing reactions catalysed by acetyl-CoA carboxylase (*ACC*), fatty acid synthase (*FAS*), fatty acid elongase (*EL*) and fatty acid desaturases (Δx or ωx). The major pathway of ARA biosynthesis is indicated by bold arrows whereas side reactions are shown by thinner arrows. Desaturation of ARA to EPA by ω3-desaturase is stimulated by growth at low temperature. The ω3-desaturation of 18 : 2n – 6 to 18 : 3n – 3 is bracketed as it occurs only at very low levels. The pathway for desaturation and elongation of exogenously supplied α-linolenic acid (boxed) to EPA is also shown. Pathway modified from [130, 131, 178]

branes of nerve cells and brain tissue. They are required for normal development and functioning of the central nervous system (CNS), especially in neonatal infants [182, 183], and are now added to a number of baby milk formulations. An ARA-rich oil supplement (ARASCO®) produced from the oleaginous fungus, *Mortierella alpina*, received GRAS approval in 2001 from the US Food and Drug Administration (FDA) for this purpose ([184] and http://www.martekbio.com). Oil from this fungus has been tested extensively for negative side effects and has been passed as safe [185–188]. FDA approval was also given to a DHA-rich oil (DHASCO®) [184] made by a heterotrophic marine alga, *Crypthecodinium cohnii* [189]. The ARA- and DHA-containing oils are blended together before addition to a range of baby milks. DHA is also produced by a group of marine microorganisms known as thraustochytrids that were once thought to be distantly related to filamentous fungi but which have now been reclassified [190, 191]. Oil from these organisms is not yet used in baby milk formulations but the whole cells are added to poultry feed to produce "ω-3 enriched" eggs and to farmed fish feed [130]. Besides its role in CNS development and function, ARA, along with other long-chain PUFAs, γ-linolenic acid (GLA, $18:3n-6$), dihomo-γ-linolenic acid ($20:3n-6$) and eicosapentaenoic acid (EPA, $20:5n-3$), act as precursors to eicosanoid hormones (prostaglandins, leukotrienes and thromboxanes), some of which play an important role in combating or preventing a number of human diseases [192]. Interestingly, some species belonging to the Zygomycota, including *Mortierella* spp. and *Cunninghamella elegans*, also appear to synthesise some of these human hormones [193].

A GLA-rich oil was produced commercially by *Mucor circinelloides* as a human nutritional supplement but this has now been replaced by more competitively priced plant-derived products, evening primrose and borage oils, even although the fungal oil appeared to be a "healthier" product in terms of higher oleic acid ($18:1n-9$) and lower linoleic acid contents [130, 131]. As in most fungal biotechnological processes, PUFA production is carried out by submerged fermentation and many studies have reported improvements in oil yield and alterations in fatty acid composition by optimising and modifying culture parameters such as carbon source, nitrogen source, growth temperature, mineral concentrations and dissolved oxygen [194–203]. The production of PUFAs by fungi growing on solid substrates [204, 205] or immobilised in fluidised-bed fermenters [206] has also been described.

A simplified pathway for PUFA biosynthesis is outlined in Fig. 2. Typically, fungi synthesise saturated fatty acids with chain lengths of 16 and 18 carbon atoms up to a chain length of at least 26 carbon atoms, the latter albeit at very low levels, but most produce unsaturated fatty acids only up to 18 carbon atoms in length ($18:2n-6$ and traces of $18:3n-3$) [132]. Several fungi make longer-chained, more unsaturated fatty acids up to 20 carbon atoms in length with up to five double bonds [131, 203, 207–209].

In most cases, these PUFAs are components of TAGs and some fungi can synthesise as much as 50% of their biomass as oil. A proportion of PUFAs are also components of membrane phospholipids and of more "exotic" lipids such as sphingolipids and cerebrosides that play important roles in membrane function [210]. Fatty acid synthesis is initiated by the formation of malonyl-CoA from acetyl-CoA by acetyl-CoA carboxylase (ACC) which is considered to be an important flux control point for fatty acid and lipid biosynthesis in many organisms [211, 212]. Subsequent elongations of the acyl moiety, each by two carbon units at a time, is carried out by the multifunctional fatty acid synthase (FAS) until palmitic (16 : 0) or stearic (18 : 0) acid is produced. At this stage the fatty acid is present in the fungal cytoplasm as the acyl-CoA ester which acts as the substrate for the ER-membrane bound fatty acid Δ9-desaturase. Desaturation occurs on the cytoplasmic face of the ER membrane to produce primarily oleoyl-CoA (18 : 1n – 9) in most organisms although palmitolyl-CoA (16 : 1n – 7) may predominate in a few cases. The fatty acid then becomes incorporated into membrane phospholipids by the action of acyltransferases such as lysophosphatidylcholine:acyl-CoA acyltransferase (LPCAT) or glycerol-3-phosphate acyltransferase (GPAT) whose specificity determines at which position on the glycerol backbone the acyl group will be transferred [213, 214]. All subsequent desaturations indicated in Fig. 2 occur using the acyl moieties of phospholipids as substrates [213]. In relation to the elongation step prior to Δ5-desaturation, the acyl group must first be removed from the phospholipid molecule by an as yet unknown mechanism, but most likely involving the formation of an acyl-CoA ester, before elongation can proceed [130]. Recent evidence has indicated that this elongation step is important in regulating the overall rate of ARA biosynthesis in *Mt. alpina* [215]. As stated above, there are at least two possible biosynthetic routes for the transfer of PUFAs to the final TAG storage oil product, with the specificity of each acyltransferase determining the complexity of the mix of TAGs formed. Phospholipids can be converted to diacylglycerols (DAGs), which are then used as substrates by acyl-CoA:diacylglycerol acyltransferase (DGAT) to form TAGs. Alternatively, de novo synthesis of DAGs by the Kennedy pathway starts with the synthesis of lysophosphatidic acid from glycerol-3-phosphate by GPAT, followed by the formation of phosphatidic acid by another acyltransferase, lysophosphatidic acid acyltransferase. Finally, phosphatidic acid is converted to DAG by the action of phosphatidic acid phosphohydrolase, summarised in [130, 180].

In oleaginous fungi, the enzymes considered to be key in determining high lipid yields are ACC, ATP:citrate lyase (ACL) and malic enzyme (ME) [130, 216–218] and (Fig. 1). A "lipogenic metabolon" has been proposed where several of the enzymes involved in lipogenesis are intimately associated to effect tighter channeling of particular substrates [130]. In this model, ME is thought of as being the most important enzyme in supplying the reducing

power of NADPH for fatty acid synthesis by FAS. To date, there is no direct evidence for these protein–protein interactions, which might be quite weak but the yeast two-hybrid system might be a useful tool to test the metabolon hypothesis [219]. Nevertheless, overexpressing either the *Mc. circinelloides* or *Mt. alpina* genes that encode the fatty acid biosynthetic ME isoform in *Mc. circinelloides* resulted in a 150% increase in lipid accumulation, which strongly suggests that such an interaction occurs in vivo [220].

Initially, much of the PUFA biosynthetic pathway was elucidated from the isolation of mutant strains defective in a particular step and by the use of fatty acid desaturase inhibitors ([221] and Table 3). Some of the mutant strains have indeed proved useful in producing fungal oils with modified PUFA compositions, particularly some double or triple mutants. One drawback, however, with the use of mutants is that many are "leaky" in nature when the block in the pathway may not always be 100% effective. In cases where more defined blocks in the pathway or the overproduction of a particular enzyme activity are required, manipulation of the gene in question is more desirable.

The ultimate aim in most biotechnological applications is to improve productivity through a combination of metabolic engineering and strain improvement by mutagenesis and screening. With this in mind, much effort has been spent in isolating and characterising as many fungal genes as possible whose gene products act in the PUFA biosynthetic pathway and whose manipulation could lead to increased oil yields or modified TAG fatty acid composition for particular applications. Most of these genes are listed in Table 4, although not all are from oleaginous fungi and some, including those encoding LPCAT and other acyltransferases with distinct specificities, await isolation. The ACC-catalysed synthesis of malonyl-CoA has been manipulated in some organisms, including plants, to improve overall fatty acid and lipid yields [264]. To date, although the gene encoding ACC has been cloned from *A. nidulans* and its expression analysed [238], little if anything has been done to enhance lipid accumulation by manipulating *ACC* gene levels in fungi. All the fatty acid desaturase genes isolated so far from fungi encode proteins with similar characteristics to the membrane bound enzymes from other eukaryotes [265, 266]. Their enzymic activities have been confirmed in vivo by introducing each gene concerned into *S. cerevisiae* or *A. oryzae*, sometimes previously transformed with fatty acid desaturase genes acting earlier in the PUFA biosynthetic pathway or supplemented with the appropriate fatty acid substrate. Similarly, the in vivo function of the GLA elongase condensing subunit (GLELO) from *Mt. alpina* was confirmed in yeast transformants that were supplemented with GLA [261]. The ability to specifically redesign fatty acid desaturases to alter their substrate specificities and desaturation sites [267, 268] increases the feasibility of creating novel PUFAs, which may not occur naturally. Moreover, the bifunctionality of some fungal fatty acid

Table 3 Altering fatty acid composition in vivo using chemical inhibitors or genetic mutations in *Mt. alpina*

Treatment	Fatty acid desaturase(s) or other enzyme affected[a]	PUFA composition[b]	Refs.
Mut T4[c]	Δ9	18 : 0 ↑; all PUFAs ↓	[222]
Mut48[c]	Δ12	18 : 1*n* – 9 ↑; 20 : 3*n* – 9 ↑; all *n* – 6 and *n* – 3 PUFAs ↓	[223]
	Δ12	20 : 5*n* – 3[d] ↑	[224]
Mut M226-9[c]	Δ12 and Δ5	20 : 4*n* – 3[d] ↑	[225]
	Δ12 and Δ5	20 : 2*n* – 9 ↑	[226]
Mut M209-7[c]	Δ12; Δ6 (enhanced)	20 : 3*n* – 9 ↑	[227]
Mut JT-180[c]	Δ12; Δ5 and Δ6 (both enhanced)	20 : 3*n* – 9 ↑	[228]
Mut49[c]	Δ6	18 : 2*n* – 6 ↑; 20 : 3*n* – 6 ↓; 20 : 4*n* – 6 ↓	[223]
	Δ6	20 : 3(Δ5)*n* – 6[e] ↑; 20 : 4(Δ5)*n* – 3[f] ↑	[229]
Mut44[c]	Δ5	20 : 3*n* – 6 ↑; 20 : 4*n* – 6 ↓	[223]
Mut S14[c]	Δ5	20 : 3*n* – 6 ↑; 20 : 4*n* – 6 ↓	[202]
	Δ5	20 : 4*n* – 3[d] ↑	[178]
Sesame non-oil fraction (sesamin)	Δ5	20 : 3*n* – 6 ↑; (+/–20 : 4*n* – 6 ↓)	[188, 230]
	LPCAT[g]	20 : 3*n* – 6 ↑ at *sn*-2 of *sn*-PtdCho[h]	[231]
Sesamin and episesamin	Δ5	20 : 3*n* – 6 ↑; 20 : 4*n* – 6 ↓	[232]
Curcumin	Δ5 (and Δ6)	20 : 3*n* – 6 ↑	[233]
Alkyl gallate	Δ5 and Δ6	18 : 1*n* – 9 ↑; 18 : 2*n* – 6 ↑; 20 : 4*n* – 6 ↓	[234]
Mut K1[c]	Δ5 and *ω*3	All *n* – 3 PUFAs ↓	[235]
Aspirin	Cyclooxygenases	20 : 4*n* – 6 ↑	[236, 237]

[a] Unless stated, all activities were reduced or completely inhibited
[b] Major fatty acid levels showing an increase (↑) or decrease (↓), primarily in the TAG lipid fraction
[c] *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine-derived mutant
[d] Grown with 18 : 3*n* – 3 (linseed oil)
[e] Only when grown at 12–28 °C
[f] Only when grown at <24 °C or when supplemented with 18 : 3*n* – 3 or 20 : 3*n* – 3
[g] Lysophosphatidylcholine : acyl-CoA acyltransferase
[h] *sn*-Phosphatidylcholine

Table 4 Cloned filamentous fungal genes required for fatty acid biosynthesis and lipid accumulation

Gene	Encoded enzyme activity	Accession number(s) and references
accA	Acetyl-CoA carboxylase	Y15996 [238]
acl1	ATP:citrate lyase subunit 1	AJ243817 [239]
acl2	ATP:citrate lyase subunit 2	AJ243817 [239]
fasA	Fatty acid synthase α subunit	U75347 [240]
fasB	Fatty acid synthase β subunit	U75347 [240]
ole1 (*sdeA*)	Fatty acid Δ9-desaturase I	Y18553 [241]; AF085500 [241]; AB015612 [242]; AF026401 [243]; AF510861 [244]
ole2	Fatty acid Δ9-desaturase II	Y18554 [241]; AB195980 [245]
Δ12 (*odeA*)	Fatty acid Δ12-desaturase	AB020033 [246]; AF110509 [247]; AF161219 [248]; AF262955 [249]
$\Delta 6-1$	Fatty acid Δ6-desaturase I	AF110510 [247]; AB020032 [250]; AB052086 [251]; AY392409[a] [252]
$\Delta 6-2$	Fatty acid Δ6-desaturase II	AB090360 [251]; AF290983[b] [253]
Δ5	Fatty acid Δ5-desaturase	AF054824 [254]; AF067654 [255]
ω3	Fatty acid ω3-desaturase	AB182163 [256]
ω9 (*scd3*)	Fatty acid ω9-desaturase	AB196774 [245]; AJ278339 [257]
cytb5	Cytochrome b_5	AB022444 [258]
cbr1	NADH-cytochrome b_5 reductase I	AB020035 [259]
cbr2	NADH-cytochrome b_5 reductase II	NA[c] [260]
glelo	γ-linolenic acid elongase CS[d]	AF206662 [261, 262]
maelo	Fatty acid elongase CS[d,e]	AF268031 [262]
dgat2A	Diacylglycerol acyltransferase 2	AF391089 [263]
dgat2B	Diacylglycerol acyltransferase 2	AF391090 [263]
malEM (*maeA*)	Malic enzyme (NADP-malate decarboxylase)	AF529885 [Szewczyk E, unpublished]; EAA57954 [17]; DQ973624 [220]; DQ975377 [220]

[a] Originally described as isoform II
[b] Originally described as isoform I
[c] Not available
[d] Condensing subunit
[e] Most likely elongates 16 : 0 to 18 : 0

desaturases adds further complexity to the range of unsaturated fatty acids that can be synthesised [269–271].

In order to genetically manipulate an organism, it is first necessary to develop an efficient DNA transformation system for gene deletion or gene copy number amplification. In only two cases has such a system been reported for oil-producing fungi, viz. that of *Mc. circinelloides* where an extremely efficient transformation system has existed for several years [272–274] and *Mt. alpina* [275–277]. In *Mt. alpina*, the levels of ARA in relation to total fatty acids were elevated by about 10–30% after overexpressing the endogenous GLELO fatty acid elongase gene by genetic transformation [277, 278]. Gene silencing by RNA interference technology, which is also dependent on an efficient transformation system was used to reduce expression of the Δ12-desaturase gene to modify fatty acid composition in this fungus [279]. Although the genetic manipulation of PUFA biosynthesis in oleaginous fungi is yet in its infancy, use has been made of the fungal fatty acid desaturase and elongase genes in modifying fatty acid composition in a number of other organisms, especially oil-seed crop plants [280–285], and several patents now exist for this purpose [262, 286–290].

4 Vitamins

Vitamins are essential micronutrients in human and animal diets. These compounds cannot be synthesised by mammals but are required to maintain normal metabolic and physiological functions. Plants and microorganisms, including filamentous fungi, can require vitamins as essential components for metabolism but are also capable of synthesising some of these compounds that are required by humans. The production of vitamins in traditional fermented foods involving fungi clearly benefit from their versatile metabolic abilities, either through direct biosynthesis or through the provision of precursor molecules and culture conditions to assist vitamin synthesis by co-fermenting microbial species. Tempe, for example, is a traditional Indonesian food prepared by inoculating pre-cooked soybeans with the spores of *Rhizopus* species. The resulting solid substrate fermentation will generate nutritionally significant quantities of the water-soluble vitamins: vitamin B_6, riboflavin, nicotinic acid and nicotinamide [291]. In general, vitamins are produced on an industrial scale for use as food and feed additives in addition to their use in numerous health care and cosmetic products. Vitamins are mainly prepared by chemical synthesis but there is an increasing trend towards the efficient use of natural and environmentally sensitive production methods. Therefore there are examples where biotechnological production routes, either by fermentation or microbial/enzymatic transformation, are beginning to make a contribution.

4.1
Riboflavin (Vitamin B_2)

Eremothecium ashbyii and *Ashbya gossypii* are closely related filamentous hemiascomycetes that have been used for the industrial production of riboflavin [292–294]. Yields up to 15 g of riboflavin per liter of culture have been reported from aerobic submerged fermentation using growth media containing industrial grade molasses or plant oils as carbon sources. Alternative waste organic materials have also been investigated for their ability to support riboflavin production by *E. ashbyii* [295]. As early as 1940 *E. ashbyii* was used for the commercial production of riboflavin to be replaced in 1946 by *A. gossypii* [296–298]. Both of these fermentations were later to become redundant as synthetic chemistry routes became economically favourable. This remained the case until Merck in 1974 and BASF in 1990 recommenced riboflavin production using *A. gossypii* [294]. Several *Aspergilli* have also been evaluated for their ability to produce riboflavin [299, 300]. *A. niger* and *Aspergillus terreus* have been shown to produce riboflavin upon fermentation with various carbon sources albeit at significantly lower levels [301]. *A. niger* and *A. terreus*, in common with *E. ashbyii* and *A. gossypii*, and in contrast to yeast species, are able to produce riboflavin in fermentation without being adversely affected by iron concentrations required to support growth [302].

The non-specific precursors of riboflavin biosynthesis are guanosine triphosphate and ribulose-5-phosphate. Therefore the supplementary addition of GTP synthesis precursors (purine biosynthesis precursors such as hypoxanthine and glycine) to the growth media of *A. gossypii* and *E. ashbyii*, have been reported to increase the production of riboflavin [299, 303, 304]. Several other strategies have been employed to improve the production of riboflavin, these include the selection of mutants with improved performance in fermentation. These approaches require not only the initial mutant selection but also a reappraisal of the growth conditions to optimise riboflavin production, as reported for a UV-mutant of *E. ashbyii* [305]. However, the majority of this work has focussed on *A. gossypii* since *E. ashbyii* displays innate genetic instability [299]. To this end the genes encoding the enzymes necessary for riboflavin biosynthesis have been cloned from *A. gossypii* [306]. The utilisation of plant oils as carbon and energy sources requires the β-oxidation pathway of filamentous fungi to operate in order to supply acetyl-CoA for energy and to support biosynthetic needs. *A. gossypii* and *E. ashbyii*, like many other filamentous fungi, will induce the key enzymes of the glyoxylate bypass, isocitrate lyase and malate synthase [307, 308]. These enzymes are peroxisomally located, and it is in the peroxisome that malate is produced as precursor of GTP synthesis [309]. Itaconate is an inhibitor of isocitrate lyase. Itaconate resistant mutants of *A. gossypii* overcome the effects of this inhibitor by producing higher levels of isocitrate lyase, and the increased metabolic flux possible because of this is thought to enhance the yields of

riboflavin [310, 311]. However, since riboflavin yields were also increased under conditions when isocitrate lyase was repressed the mutations are likely to exhibit pleiotropic effects beneficial to riboflavin production. To take a rational approach to metabolically enhance riboflavin production the genetic tools necessary to manipulate *A. gossypii* have been developed, including DNA-mediated transformation with integrative and replicative vectors [312–314]. Using these techniques, isocitrate lyase levels of *A. gossypii* were also increased by the introduction of a second copy of the *ICL1* gene leading to increased riboflavin production upon fermentation with soybean oil [315]. Similarly, overexpression of the *GLY1* gene led to a ten-fold increase in threonine aldolase, which allowed an increase in precursor GTP synthesis in response to threonine supplementation and thereby increased riboflavin production [316]. To reduce the metabolic flux from glycine to serine the *SHM2* gene, encoding a serine hydroxymethyltransferase, was targeted for disruption. The mutation resulted in a significant increase in riboflavin productivity. Evidence for elevated glycine levels in these mutant strains was obtained from ^{13}C-labelling experiments [317]. These studies showed that *SHM1* disruption (encoding the mitochondrial isozyme) had no detectable effect on serine labelling, but disruption of *SHM2* led to a decrease in serine (2–5%) and an increase in glycine (59–67%) labelling.

Riboflavin efflux from *A. gossypii* is an active process supported by a specific carrier, the activity for which is up-regulated in production strains [318]. However, riboflavin in these strains has been found to accumulate in vacuoles leading to unwanted product retention. To circumvent this problem the vacuolar ATPase was inactivated by disruption of the *VMA1* gene that encodes one of the enzyme subunits [319]. The resulting mutant excreted all the riboflavin into the culture supernatant to improve product recovery.

We can expect further improvements in the production of riboflavin in *A. gossypii* in the near future as the genetic mechanisms that control the synthesis components are elucidated. Targets for the metabolic engineering of *A. gossypii* have been highlighted using genome-wide transcript expression analyses to follow the staged development of commercial production strains [320]. Initial findings show that 3,4-dihydroxy-2-butanone 4-phosphate (DHBP) synthase, the first enzyme in the biosynthetic pathway for riboflavin, increases co-ordinately with transcription of its structural gene *RIB3* during stationary phase culture [321]. Moreover phosphoribosylamine biosynthesis in *A. gossypii* has been reported to be repressed by extracellular purines, where ATP and GTP inhibit phosphoribosyl pyrophosphate (PRPP) amidotransferase as the first enzyme committed to the precursor purine biosynthesis pathway required for riboflavin production. The constitutive overproduction of a mutant form of PRPP amidotransferase that was no longer subject to feedback inhibition, could abolish adenine-mediated transcriptional repression and enhance metabolic flow through the purine pathway, and thereby increase the production of riboflavin [322].

4.2
Pantothenic Acid (Vitamin B_5)

Commercial production of D-pantothenate involves initial reactions that generate racemic pantolactone from isobutyraldehyde, formaldehyde, and cyanide. The optical resolution of this racemate to D-pantolactone is a costly step in the synthesis before condensation with alanine to form D-pantothenic acid or 3-aminopropanol to form D-pantothenyl alcohol. As a cost-effective alternative to expensive synthetic alkaloids that are used in the chemical process to resolve DL-pantolactone, a biotransformation with a fungal enzyme, lactonohydrolase, has been proposed to produce D-pantoic acid directly [323]. Fungi of the genera *Fusarium*, *Gibberella* and *Cylindrocarpon* have been shown to produce lactonohydrolase activities capable of performing the optical resolution with D-pantoic acid yields in 96% enantiomeric excess [324]. These enzymes catalyse the stereospecific hydrolysis of a variety of D-lactones, of which D-pantolactone is a favoured substrate leaving L-pantolactone unchanged to be racemized and recycled as substrate. *Fusarium oxysporum* cells contain sufficient enzyme activity to carry out the biotransformation. Once prepared, these cells are also sufficiently robust that they may be immobilised in calcium alginate gels, in which form they retain around 90% of their lactonohydrolase activity over a long period, and may be conveniently recovered and reused in the process [325].

4.3
β-Carotene (Pro-Vitamin A)

β-carotene is used as a natural vitamin additive, as a functional antioxidant and as an orange/red colorant in food, animal feeds and cosmetics. Vitamin A deficiency leading to blindness remains a problem in developing countries. Filamentous fungi are a rich potential source of carotenes suitable for most applications [326]. In fungi, carotenoids are synthesised from prenyl pyrophosphates that are generated from the mevalonate biosynthetic pathway. Three molecules of acetyl-CoA are utilised to make 3-hydroxy-3-methylglutaryl-CoA which is converted via mevalonate to isopentenyl-pyrophosphate (IPP). IPP is isomerised to dimethylallyl pyrophosphate, a common precursor of carotenoid biosynthesis that is condensed with IPP or an alternative homoallylic pyrophosphate to elongate the structure [327]. In several filamentous fungal species this addition is performed by a single enzyme, farnesyl pyrophosphate synthase [328]. Successive "head-to-tail" additions by this reaction form C_{20} geranylgeranyl pyrophosphate, which then combines with itself in a "head-to-tail" addition catalysed by phytoene synthase to form C_{40} phytoene [327]. It is from this colourless precursor that β-carotene is formed by four consecutive dehydrations and two cyclization reactions [329]. Genes encoding the phytoene synthase and carotene cyclase

activities have been cloned from a number of fungi, including *Neurospora crassa* [330], *Xanthophyllomyces dendrorhous* (the perfect, yeast state of *Phaffia rhodozyma*) [331], *Mc. circinelloides* [332], *Phycomyces blakesleeanus* [333] and *Fusarium fujikuroi* [334]. In all cases the phytoene synthase and carotene cyclase functions are performed by a single bifunctional protein, an arrangement that contrasts with bacterial and plant kingdoms where the enzymic activities are associated with two separate protein products encoded on separate genes [333, 335]. Again in contrast to bacteria and plants that possess two phytoene dehydrogenase enzymes, fungi maintain a single enzyme that is capable of performing all dehydrogenase steps [329]. The structural genes encoding phytoene synthase/carotene cyclase and phytoene dehydrogenase are closely linked as a gene cluster in *Mc. circinelloides*, *P. blakesleeanus* and *F. fujikuroi* but, although on the same linkage group, the genes are somewhat distant in *N. crassa*. More recently overexpression of the *crtYB* (encoding bifunctional phytoene synthase and carotene cyclase) and *crtI* (phytoene desaturase) genes from *X. dendrorhous* was demonstrated to be sufficient to enable carotenoid production in the yeast *S. cerevisiae* [336].

β-carotene production using the fungus *Blakeslea trispora* has been industrialised for many years in Russia to yield both purified β-carotene and β-carotene-enriched mycelium for use as an animal feed additive [337]. The fungus exists in two mating types (+) and (–), of which the (+) mating type produces trisporic acid, a precursor of β-carotene biosynthesis that stimulates the (–) mating type to produce β-carotene when they are brought together in an optimised ratio. β-carotene overproducing mutants have been selected on the basis of resistance to lovastatin and acetoanilide. Using these mutants in aerobic submerged batch fermentations has yielded up to 7 g/l of β-carotene extracted from mycelia after a 7-day fermentation.

4.4 Prospective Vitamin Production

Fungi produce analogues of the vitamin C molecule L-ascorbic acid based on the molecule D-erythroascorbic acid [338], which confer similar antioxidant properties to vitamin C in these organisms [339]. In this pathway D-erythroascorbic acid is synthesised from D-arabinose in a three-step process as observed for the phytopathogen *Sclerotinia sclerotiorum* [340]. It has been demonstrated that *S. cerevisiae* can synthesise L-ascorbic acid when furnished with non-physiological substrates such as L-galactonolactone and L-galactose [341], which has led to the speculation that yeast could be engineered to make L-ascorbic acid in a direct fermentation, and can thereby replace some of the chemical synthetic steps currently required for the Reichstein process of production [342, 343]. Given the breadth of substrates available to filamentous fungi and their versatile metabolism it can only be a matter of time until production of vitamin C from these sources is investigated.

Vitamin B_6 compounds, mainly pyridoxine and pyridoxal phosphate, are currently produced by chemical synthesis. The complete biosynthetic pathway of vitamin B_6 is not known for fungi but recent work with the phytopathogenic fungus *Cercospora nicotianae* has identified the first genes involved in the synthesis of pyridoxine that appear to be conserved in Eubacteria and the Archaea [344, 345]. The initial gene was isolated on the basis that it conferred resistance of the fungus to a singlet-oxygen-generating toxin, cercosporin, which it produces to parasitise plants. The gene was therefore given the name *SOR1* but it became evident that the gene could rescue pyridoxine auxotrophs of both *C. nicotianae* and *A. flavus* [344]. This observation was subsequently confirmed in *A. nidulans* and *N. crassa*, in which homologues of *SOR1* were identified as the structural genes for *PYROA* and *pdx-1*, respectively, mutants of which require pyridoxine for growth [346, 347]. Analysis of the complete and emerging genome sequences in which PDX1 homologues appear, reveals that they are clustered with a second highly conserved gene which has also been confirmed as a functional component of the pyridoxine biosynthetic pathways of *C. nicotianae* and *N. crassa* by the complementation of closely linked pyridoxine-requiring mutants [345, 347, 348]. The gene pair is therefore known as *PDX1* and *PDX2*. Studies on the products of these may well help to establish a common biosynthetic route for pyrodoxine biosynthesis, which could then be exploited to enable biotechnological production.

5
Flavour Compounds

Fermentation processes for the production of palatable foods has taken place since ancient times. Filamentous fungi of the genera *Aspergillus*, *Rhizopus*, *Mucor*, *Actinomucor*, *Amylomyces* and *Neurospora* are used in the manufacture of fermented foods from carbohydrate-rich but often otherwise bland staple cereal and vegetable crops. However, *Penicillium* species predominate in the ripening of richly flavoured cheeses and meats [349]. In these fermentations filamentous fungi often form part of a mixed microbial flora that give rise to the final organoleptic properties of the products. The additional microflora notwithstanding, it is clear that filamentous fungi produce a wide variety of taste and aroma active compounds that are of potential commercial value.

As noted in Sect. 3.1 above, citric acid is by far the largest volume flavour compound produced by filamentous fungi but the premium prices commanded by specialist flavour and fragrance ingredients have galvanised the search for new sources of these chemicals, which has included prospecting in the products of fungal metabolism [350]. Coupled with the customer demand for a wider variety of flavour compounds, there is a preference to produce these from natural sources or through natural processes [351]. For legislative

purposes "natural flavours" have a common definition in the European Union and the USA, being defined as compounds obtained from living cells, including food-grade microorganisms and their enzymes. This definition offers considerable opportunity for the biotechnological production of flavours in compatible microorganisms like filamentous fungi. In essence production may be accomplished through the recovery of aroma-active compounds from mixtures of fungal biosynthetic products (de novo synthesis) or by using fungi to perform a specific biotransformation/bioconversion process from an inactive or cheaper precursor.

5.1 De Novo Synthesis

The production of natural flavour compounds by fungi has been exploited for many years. Examples of recognised flavour chemicals produced de novo by filamentous fungi are presented in Table 5. However, the full biosynthetic

Table 5 De novo synthesis of flavour compounds in fungi

Source	Chemicals	Flavour notes
Penicillium camemberti *Penicillium caseicolum*	2-methoxy-3-isopropyl pyrazine	Earthy, nutty flavour of surface ripened cheeses – Brie [352]
A. sojae	2-hydroxy-3-6-substituted pyrazines	Roasted nutty, potato-flavour and soy sauce [353]
Ceratocystis variospora *Ceratocystis moniliformis* *Ceratocystis fimbriata* *E. ashbyii*	Acyclic terpenoids: geraniol and citronellol	Essential oils [354, 355]
Penicillium roqueforti	Methyl ketones	Blue cheese flavour [356, 357]
A. oryzae *C. moniliformis* *Fusarium poae*	γ-decalactone and other lactones	Peach and other fruit flavours [358–360]
Tyromyces sambuceus		Passion fruit [361]
Polyporus durus		Coconut/pineapple [362, 363]
Trichoderma spp.	6-pentyl-α-pyrone	Coconut [364]
Agaricus spp. *Polyporus* spp. *Pleurotus sapidus*	Benzaldehyde	Cherry and almond [365]
Basidiomycetes *Morchellaceae*	Octanols and octanals	Mushroom flavours [349, 366]

capacities of a number of species have not been realised in any commercial sense. This can be due to the low quantities of flavourants produced by fungi under the growth conditions used or problems of a technical nature relating to poor product recovery following fermentation. Species of the genus *Ceratocystis*, for example, are not only notable for their pathogenicity to trees but also for their ability to produce a wide range of flavour compounds, including aromas of peach, banana, pear, rose and citrus [354, 364, 367–370]. Typically, the headspace of these cultures contains the volatiles acetaldehyde, ethanol, isopropanol, ethyl acetate, ethyl isobutyrate, isoamyl acetate and ethyl-3-hexanoate. These cultures can also produce γ-decalactone when cultured in glycerol-urea medium or terpenoids such as citronellol, geraniol and nerol in response to galactose-urea [358]. *Ceratocystis fimbriata* and *Ceratocystis moniliformis* have been the focus of much of this research owing to their attractive growth rates and their ability to utilise agro-industrial wastes as substrates for aroma production [371]. Solid state fermentations of *C. fimbriata* with cassava bagasse, apple pomace or soya bean supported the production of strong fruity aromas, whereas amaranth or coffee husks produced a distinctive pineapple aroma [372, 373]. The yields of the flavour compounds from these fermentations are often limited because of the toxic nature of the compounds themselves. To recover these hydrophobic compounds and circumvent their inhibitory effects on growth, fermentation processes have been configured to allow in situ product removal (ISPR). To accomplish this, the fermentation vessel (bioreactor) was interlinked with a pervaporation membrane module to create an integrated bioprocess (IBP). Implementing an IBP with *C. moniliformis* and *C. fimbriata* resulted in higher growth yields of the fungi, and significantly more aroma compounds that were enriched in the permeates obtained from pervaporation, from which they could be conveniently recovered [374, 375].

5.2
Biotransformation and Bioconversion

Biotransformation and bioconversion processes are becoming commercially realistic alternatives to purely chemical synthesis routes for the production of chemicals. In particular the ability of biological systems to confer stereo- and *regio*-selectivity to synthetic routes is of great benefit. The use of biotransformation and bioconversion has a relatively long history in the development of flavours. As long ago as 1924 the synthesis of methyl ketones by moulds from triglycerides and fatty acid was examined with respect to formation of off-flavours in cocoa fat [376], and later with respect to the blue cheese flavours produced by *Penicillium roqueforti* [377]. In recent years there has been considerable progress in the use of precursor molecules to programme the synthesis of flavour compounds by microbes or their isolated enzymes. These studies are the subject of two recent treatises [355, 378]. Table 6 presents

Table 6 Production of flavour compounds by bioprocessing and biotransformation

Source	Precursor	Product	Flavour notes
Penicillium digitatum *Lasiodiplodia theobromae*	Limonene	Cyclic terpenoids: α-terpineol and jasmonate	Essential oils [355, 379]
A. niger *Penicillium chrysogenum* *Penicillium rugulosum*	Geraniol	Linalool and α-terpineol	
P. roqueforti	Fatty acids and short chain esters	Methyl ketones	Blue cheese flavour [377, 380]
Aspergillus spp.	Transformation of α-ionones	*trans*- and *cis*-3-hydroxy-α-ionone	Tobacco odours [381]
Rhizopus spp. *Aspergillus* spp.	Transformation of β-ionones	4- and 2-hydroxy-β-ionone	Floral – violet and rose-like [382, 383]
A. niger and *Pycnoporus cinnabarinus*	Sugar beet pulp (ferulic acid)	Vanillin (3-methoxy-4-hydroxybenzaldehyde)	Vanilla flavour [384]
A. niger *Phanerochaete chrysosporium* *Cladosporium suaveolens* *Mucor* spp.	Castor oil or ricinoleic acid	4-decanolide	Fruit and creamy dairy flavours [385]
Polyporus spp.	Castor oil and lignin	Acetophenone	Orange blossom [386]

several examples of flavour chemicals produced by filamentous fungi from precursor molecules by biotransformation or bioconversion protocols. The use of two species of filamentous fungi in the development of a two-step process to produce the flavour compound vanillin is outlined below.

Vanillin or 3-methoxy-4-hydroxybenzaldehyde is used extensively in the food industry, most notably in ice cream and confectionery. The majority of the 12 000 tonnes consumed each year is the product of chemical syntheses, either from petrochemicals or lignin [386]. A small fraction of this production (0.5%) is extracted from the fermented pods of *Vanilla* orchids in association with a few additional compounds that add to the flavour intensity of natural vanilla. Natural vanillin attracts a price that is 80-fold or more that of the synthetic chemical product and it is this premium market that is the target for biotechnological production [387]. Several biotechnological routes leading to the production of vanillin have been investigated but in particular *A. niger* and *Pycnoporus cinnabarinus* have been used in a two-step process to produce vanillin [384]. The process utilises *A. niger* to convert ferulic acid to vanillic acid and then a laccase deficient strain of *P. cinnabarinus* to reduce vanillic acid to vanillin. Use of the laccase deficient strain was adopted to prevent the formation of a deleterious ferulic acid polymer [331]. Cheap

sources of ferulic acid for this process are the agro-industrial waste products sugar beet pulp, maize bran and waste residues of rice bran oil [388–390]. However, the decarboxylation of vanillic acid to methoxyhydroquinone during the second step of the process limited the production of vanillin [391]. Addition of cellobiose was found to overcome this by channeling the vanillic acid into vanillin production [392]. *Phanerochaete chrysosporium* has been investigated as an alternative to *P. cinnabarinus* in the second step of the process but this fungus preferentially converted vanillic acid to vanillyl alcohol via vanillin. The implementation of ISPR for vanillin by selective adsorption with a polystyrenic resin was reported to yield 500 mg/l of vanillin [393]. Sensory evaluation of the vanillin made in this way identified that the vanilla aroma was accompanied a secondary pleasant chocolate odour [388]. It is only with natural sources of vanillin that such secondary organoleptic notes are perceived.

6 Conclusions

Filamentous fungi are metabolically versatile organisms that are exploited commercially as cell factories for the production of enzymes and a wide variety of metabolites. Although this chapter has focused on food applications for the fungus-derived products, many products find applications in the clinical area. Fungi are an immensely rich resource of enzymes and other natural products but it is a resource that is barely tapped because only a fraction of the world's fungal species is known to science. Many of those species that are known are not cultivable in the laboratory and, historically, this has restricted their use apart from harvesting natural populations of the fungus for culinary or medicinal purposes. This is about to change due to advances in genomic, proteomic and metabolomic ("omic") technologies. The driver for exploitation of fungi in this way is primarily as sources of valuable clinical compounds but the principle of exploring the metabolic capacity of fungi using global approaches applies irrespective of the end-use of the product. Most lichen fungi, for example, are not readily cultivated in the laboratory and, in the wild, grow slowly. Although lichens are a major component of some ecosystems, and could be harvested for extraction of a product, there is potential in applying modern molecular approaches to clone pathway genes and express them in an amenable host [394]. Despite the potential, this is not an easy way forward but it serves to illustrate the point that emerging methodologies provide a potential that was not appreciated just a few years ago. Cloning and expressing pathway genes is aided by the clustering of many genes for secondary metabolite synthesis but their cloning and heterologous expression remains far more of a technical challenge than that for a single gene. For that reason, uncultivable (or poorly cultivable) fungi are likely to be a more immediate source

of genes for the production of novel enzymes and many of these could provide interesting activities for use as processing aids in foods.

We have taken an unashamedly molecular approach in this chapter and have paid scant attention either to the importance of the industrial process, i.e. the large-scale cultivation of fungi and down-stream processing, or to the regulatory aspects for approval of the use of additives in foods. In many respects, these facets of an industrial process will be the prime determinant of its success. We anticipate, however, the application of "omic" approaches in the area of processing to optimise the productive capacity of a chosen fungal cell factory in an industrial context. The ability to determine the flux of carbon, for example, into the desired product, and to identify and overcome bottlenecks in its production, will require a combination of bioreactor technology and global methods of analyses that are only now becoming possible. This capacity heralds the advent of new possibilities for fungi, because of their inherent metabolic diversity, in the provision of food additives.

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Plant Biotechnology: Transgenic Crops

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Abstract Transgenesis is an important adjunct to classical plant breeding, in that it allows the targeted manipulation of specific characters using genes from a range of sources. The current status of crop transformation is reviewed, including methods of gene transfer, the selection of transformed plants and control of transgene expression. The application of genetic modification technology to specific traits is then discussed, including input traits relating to crop production (herbicide tolerance and resistance to insects, pathogens and abiotic stresses) and output traits relating to the composition and quality of the harvested organs. The latter include improving the nutritional quality for consumers as well as the improvement of functional properties for food processing.

Keywords Crop improvement · Genetic manipulation · Input traits · Output traits · Transformation technology

Abbreviations

AA	Arachidonic acid
ACC	Aminocyclopropane-1-carboxylic acid
AK	Aspartate kinase
CI-2	Chymotrypsin inhibitor 2
DHA	Docosahexaenoic acid
DHDPS	Dihydrodipicolinate synthase
dpa	Days post anthesis
EPA	Eicosapentaenoic acid
EPSPS	5-Enolpyruvoylshikimate 3-phosphate synthase
GLA	γ-Linolenic acid
HMG-CoA	3-Hydroxy-3-methylglutaryl-coenzyme A
HMW	High molecular weight
IPP	Isopentenyl diphosphate
PAT	Phosphinothricin acetyltransferase
PEG	Polyethylene glycol
PG	Polygalacturonase
Phytic acid	*Myo*-inositol-1,2,3,4,5,6-hexakisphosphate
PIG	Particle inflow gun
PLRV	Potato leaf roll virus
PMI	Phosphomannose isomerase
PPT	Phosphinothricin
PRSV	Papaya ringspot virus
PUFAs	Polyunsaturated fatty acids
SAM	*S*-Adenosyl methionine

1 Introduction

Much has been written about the projected increase in the world population, from six billion in 1999 to a predicted ten billion by the middle of this century [1]. This increase is clearly not sustainable with current practices in agricultural production, particularly in developing countries where much of the population increase is predicted to occur. Although it has been argued that the genetic yield potential of many crops is currently being approached [2], this is rarely reached in most countries due to the limited availability of nutrients and water, unsuitable climatic conditions and poor control of pests and pathogens. For example, the world yields of bread wheat average less than three tonnes per hectare, compared with about eight tonnes per hectare in the UK. However, high yields currently require high inputs in terms of fertiliser and of chemicals to control weeds, pests and pathogens. It is crucial to reduce this requirement and genetic modification technology is currently the most promising way to do this.

Although yield is still the major target in many countries, the end use properties are also important, particularly when the crop is used in complex food processing systems. Although many crops are used in this way, the two

that are most pervasive in foods are wheat and soybean. Wheat is the prime ingredient in a wide range of breads, other baked goods (cakes, cookies etc.) and in pasta and noodles. In addition, either wheat flour or wheat-derived ingredients (chiefly starch and gluten) are used in a wide range of other foods to confer specific functional properties. Soybean is also widely used in foods, although in this case it is mainly the protein fraction that is used. This can be extruded or textured and can be used to confer a wide range of functional properties including gelation (e.g. tofu), foaming, emulsification, viscosity and absorption of water, fat and flavours [3].

Food processing quality is clearly an important target for improvement with a high financial incentive. However, of greater potential importance is the improvement of the nutritional quality of crops. The emphasis so far has been on delivering increased contents of vitamins and minerals, such as vitamin A [4], iron [5] and folate [6], particularly for nutrition in developing countries. However, it is also important to consider the composition of crops in relation to major diet-related conditions in developed countries, such as bowel cancer, cardiovascular diseases, type 2 diabetes and obesity. Improved diets are required to alleviate these problems in large populations via widely consumed staple foods (e.g. bread, rice and potatoes) as well as via increasing the consumption of fruits and vegetables.

The techniques available to the plant breeder have been augmented in the last two decades by the development of genetic modification. Whereas all plant breeding involves altering the genetic constitution of crops, be it through sexual crossing, selection, or chemical and radiation mutagenesis, the term genetic modification has been applied specifically to the technique of inserting a single gene or small group of genes into the DNA of an organism artificially. Genetic modification has become established in plant breeding because it has advantages over other techniques: it allows genes to be introduced into a crop plant from any source; it is relatively precise in that single genes can be transferred; genes and their products can be tested before use to ensure their safety; and genes can be altered and assessed under laboratory conditions to change their properties before being introduced into a plant.

Genetically modified (GM) crops were first grown commercially in 1994 but it was not until 1996 that large-scale cultivation of major commodity crops began. GM crops are now being grown commercially on approximately 100 million hectares in 22 countries: Argentina, Australia, Brazil, Bulgaria, Canada, China, Colombia, Czech Republic, France, Germany, Honduras, India, Indonesia, Mexico, the Philippines, Portugal, Romania, Slovakia, South Africa, Spain, Uruguay and the USA [7].

In the present article we initially provide a brief introduction to the technology of genetic modification and its application to crop plants. We then discuss its application to two broad classes of trait: “input traits”, which relate to the performance and protection of the crop in the field, and “output

traits", which relate to the composition and end use quality of the harvested organs.

2 Technologies for Crop Genetic Manipulation

2.1 Methods for DNA Delivery

Crop genetic manipulation depends on two key processes, the ability to insert DNA into the host genome (transformation) and the ability to regenerate fertile, adult plants from those transformed cells. In a few crop species, the requirement for a tissue culture phase has been obviated by the development of germ-line transformation methods (discussed below) but in the majority, it is a bottleneck in genetic manipulation. Many DNA-transfer methods have been tried with varying success, including electroporation, micro-injection, silicon carbide fibres, polyethylene glycol (PEG) and laser-mediated uptake, but two methods now predominate in crop species: transformation via particle bombardment and *Agrobacterium*.

Particle bombardment (also called biolistics) involves the adsorption of naked DNA, usually in the form of circular bacterial plasmids, onto the surface of submicron particles of metal which are driven at high velocity into recipient plant cells using an acceleration device [8, 9]. It has also been used to deliver DNA into the chloroplast and mitochondrion genomes (for review see [9]). Effective DNA transfer has also been demonstrated using *Escherichia coli* or *Agrobacterium* cells as micro-projectiles [10]. The helium-driven particle delivery system first developed by DuPont then subsequently marketed by BioRad as the PDS1000/He has been widely used, but other devices, such as the particle inflow gun (PIG) and the ACCELL™ electrical discharge technology, have also been used successfully.

Particle bombardment effectively distributes DNA over a wide area of the target tissue and is relatively genotype independent. For many important crop species, particle bombardment technology was used to produce the first transgenic plants. Breakthroughs using this DNA-delivery method were reported for maize [11, 12], sugarcane [13], wheat [14] and papaya [15]. In addition, particle bombardment removed the germplasm dependency of other systems, enabling the transformation of elite varieties of indica and japonica rice [16] and cotton [17].

Although *Agrobacterium* transformation has always been the method of choice for some crops, for others it has overtaken direct DNA transfer as improved protocols have been developed [18–23]. *Agrobacterium tumefaciens* is the causative agent for crown gall disease and is well adapted for transferring DNA to its host plant cell. A major advance in the application of

Agrobacterium to plant genetic engineering was made in the early 1980s when the large, tumour-inducing (Ti) plasmid was modified into the more easily manipulated disarmed binary vector system in common use today [24, 25]. These vector systems comprise two plasmids, one with a convenient multiple cloning site flanked by T-border sequences, a selectable marker gene and an origin of replication for easy maintenance in *E. coli* and the other, a disarmed Ti plasmid, lacking the tumour-inducing genes but retaining the *vir* loci whose products interact with the T-strand and facilitate DNA transfer to the plant cell.

Characterisation of large numbers of transgene loci resulting from various transformation methods reveals differences in copy numbers and patterns of integration. *Agrobacterium* is perceived to have advantages over other forms of transformation including biolistics because it can introduce larger segments of DNA with minimal rearrangement and with fewer copies of inserted transgenes at higher efficiencies and at lower cost [19, 26–29]. Regardless of the DNA-delivery process and with the exception of the few crop species for which germ-line transformation methods have been developed, the production of fertile adult transgenic plants requires a tissue culture phase.

2.2 Tissue Culture and Selection

The two principal routes to recover plants from transformed somatic cells are via somatic embryogenesis or organogenesis. In most crops one method prevails but in some, such as sugar beet, either regeneration method can be used. In cereals and some dicotyledonous plants, regeneration is achieved via the production of somatic embryos which under certain conditions can be "germinated" to form shoot and root structures and give rise to fertile adult plants. Reliable regeneration protocols for a wide range of cereals have been developed using embryogenic callus derived from the immature scutella of zygotic embryos as the starting explant. Viable alternative explants include immature inflorescences for wheat [30] and tritordium (a fertile amphidiploid between bread or durum wheat and the wild barley *Hordeum chilense*) [31], shoot meristem cultures for barley, oats and maize [32, 33], and protoplasts for barley, maize and rice [34–36].

Compared to the cereals, dicotyledonous crops offer a broader range of starter explants and highly specialised tissue culture routes for regeneration. Transgenic soybean plants have been produced using *Agrobacterium* and biolistic methods in conjunction with shoot meristems [37], cotyledonary nodes [38], embryogenic suspension cultures [39] and immature cotyledonary explants [40]. Brassica species are commonly transformed using hypocotyl segments, with efficiencies reaching 25% [41], but other explants have been used successfully including stem internodes [42], stem seg-

ments [43] and cotyledonary petioles [44]. Nuclear transgenic and transplastomic tobacco, which have been used for pharmaceuticals production and "molecular farming", are most effectively regenerated via shoot organogenesis from leaf explants [45–48]. A rapid method of sugar beet transformation taking only 8–9 weeks has been developed using PEG-mediated DNA delivery into protoplasts from stomatal guard cells [49].

Selection systems are imposed during the tissue culture phase to kill or compromise the growth of untransformed tissues and allow the preferential survival of transformed plants. Regimes based on negative selection are used widely but alternatives based on positive selection are becoming important in some crops. Positive and negative chemical selection systems comprise two components: a selection agent additive in the tissue culture medium, such as an antibiotic, herbicide or a particular carbon source, and the incorporation into the transformation cassette of a gene conferring selective advantage under those media conditions. A commonly used selection agent for cereals is the herbicide phosphinothricin (PPT), which acts by irreversibly inhibiting glutamine synthetase, a key enzyme for ammonium assimilation and nitrogen metabolism in plants [50] resulting in increasing levels of ammonia [51]. Two genes isolated from different soil microorganisms, *bar* (Basta resistance) [52, 53] and *pat* [54], encode the enzyme phosphinothricin acetyltransferase (PAT) which converts PPT to the non-toxic acetylated form and allows growth of transformed cells in the presence of PPT or commercial glufosinate ammonium-based formulations, such as Basta, Bialaphos, Challenge, Harvest and Dash. This selection system also forms the basis of the Bayer "LibertyLink" range of herbicide-tolerant crops.

Another commonly applied selection system utilises aminoglycoside antibiotics, such as kanamycin, neomycin, gentamycin, G418 and hygromycin, which inhibit protein synthesis. These antibiotics can be inactivated by phosphotransferases encoded by various genes including *npt*II (*neo)* [55] and *hpt* (*aph*IV) [56, 57]. Kanamycin selection became a laboratory standard for many dicotyledon species but the cereals and other grasses demonstrated innate tolerance to relatively high levels of this antibiotic [58]. Instead, hygromycin and G418 proved useful alternatives for maize, wheat, barley, rice, and *Lolium* (for reviews, see [59, 60]).

Driven partly by perceived risks of horizontal and vertical gene transfer, a range of more environmentally benign selection systems has been developed. The most advanced of these is the phosphomannose isomerase (PMI) system (Syngenta) which utilises the *manA* gene to convert the predominant carbon source, mannose-6-phosphate, to fructose-6-phosphate for respiration [61, 62]. Not only are the untransformed cells deprived of a carbon source, but the unutilised mannose-6-phosphate also accumulates and has additional negative effects including inhibition of glycolysis, possibly due to phosphate starvation. PMI selection has been used successfully to produce transgenic maize [63, 64], cassava [65], sugar beet [66], orange [67] and pearl

millet [68] and has been shown to be superior to antibiotic and herbicide selection for maize, wheat and sugar beet [63, 69, 70].

2.3 Germ-Line Transformation

The significant bottleneck created by the tissue culture phase in plant transformation has created a powerful driver to develop germ-line (also known as "*in planta*") methods that obviate the need for regeneration via tissue culture. *Arabidopsis* can be transformed by *Agrobacterium* using well-developed "vacuum infiltration" and "floral dip" methods [71, 72] that target the unfertilised ovules [73–75]. Recent studies have demonstrated that delivery of DNA directly into germ-line tissues of other model and crop species can lead to transformed seed embryos. The model legumes *Medicago truncate* and *Brassica campestris* (pak choi) have been transformed by *Agrobacterium* infiltration of flowering plants [76, 77]. Efficient male germ-line transformation by biolistics has been achieved in tobacco [78] and there are reports that *in planta* transformation of soybean has been achieved in China [79].

2.4 Targeted Manipulation of Gene Expression

The core promoter, together with enhancers and other *cis*-acting regulatory elements, are non-coding sequences that form part of the mechanisms that control gene expression. The ability to make chimeric expression cassettes, which combine the promoter from one gene and the coding region from another, forms an important design facet of transformation-based experimentation. Promoters from the cauliflower mosaic virus 35S gene [80], maize polyubiquitin [81] and rice actin [82] have been used widely in dicotyledonous and cereal crops to give more or less constitutive expression of introduced genes. The desire to localise transgene products in crop plants, either temporally or spatially, is driving research to identify and characterise tissue-specific, developmentally regulated or inducible promoters for functionally important tissues and organs. The cereal grain is a well-adapted organ that stably accumulates proteins, lipids and starch to high levels in discrete bodies, and of particular interest are promoters that drive expression in specific grain tissues, such as the starchy endosperm or aleurone. Promoters conferring these expression profiles allow the targeted modification of seed storage components that are important in the human diet and food processing and have the potential to convert the seed into a bioreactor for the production of recombinant pharmaceutical proteins. There are a number of useful candidates, including a high molecular weight glutenin subunit (1Dx 5) promoter which gives strong endosperm-specific GUS reporter gene expression in wheat beginning 10 days post anthesis (dpa) (Fig. 1) [83]. Constructs

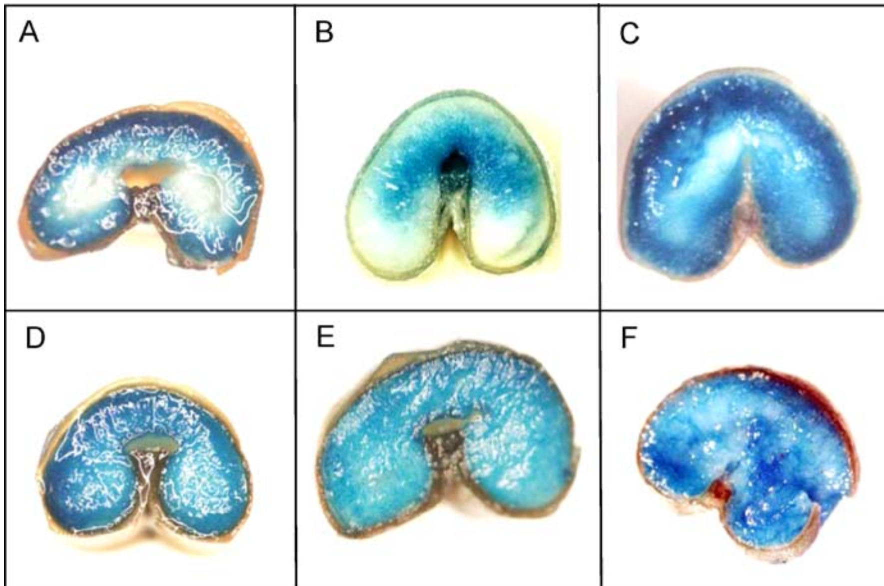

Fig. 1 GUS expression patterns in mature wheat seeds from plants transformed with the promoter *UidA* reporter constructs listed below (Sparks CA and Jones HD, unpublished results). **a** Rice tungro bacilliform virus promoter plus leader sequence [200]. **b** Wheat Bx17 high molecular weight subunit promoter (*Glu-B1x*) [201] (Laszlo Tamas, unpublished results). **c** Wheat 1Dx5 high molecular weight subunit promoter (*Glu-D1x*) [83]. **d** Rice actin promoter plus first intron (*Act1*) [82]. **e** Maize polyubiquitin plus first intron (*Ubi1*) [81]. **f** Wheat ADPglucose pyrophosphorylase promoter [86]

incorporating this or other promoters of genes encoding glutenin proteins have been successfully used to modify gluten protein quality as discussed below (see also [84, 85]). In addition, the promoter of the wheat ADPglucose pyrophosphorylase gene (which encodes an enzyme of starch synthesis) has been shown to drive strong expression in embryos and guard cells of transgenic tobacco and in the endosperm and aleurone of transgenic wheat plants starting at 5 days after flowering [86]. Stable transgenic rice plants were used to analyse the promoters of 15 genes which demonstrated the spatial and temporal expression patterns in rice seed, and which demonstrated the potential of some to promote the expression of recombinant proteins in seeds [87]. Promoters of the storage albumin and globulin gene family have also been shown to confer high levels of endosperm expression in transgenic tobacco [88], flax [89], rice [90] and wheat (authors' unpublished data).

The ability to localise the products of introduced sequences, both at the level of transcription/translation and by manipulating intracellular protein trafficking, is set to become an important tool in research and application of crop genetic modification.

2.5 RNA Interference

Most applications of plant genetic engineering involve the transformation and over-expression of a heterologous gene cassette to produce a "foreign" protein. However, it is also possible to down-regulate the levels of native proteins using a targeted gene silencing approach such as RNA interference (RNAi) [91, 92]. RNAi uses DNA constructs designed to generate hairpin-shaped, double-stranded RNA (dsRNA) which triggers sequence-specific down-regulation of endogenous genes. An RNase III-like enzyme dicer-x cleaves the long dsRNAs into 21–23 nucleotide lengths of double-stranded, small interfering RNA (siRNA), which are assembled into RISC (RNA-induced silencing) complexes and destroy any matching cytoplasmic mRNA. The specificity and robustness of RNAi-mediated gene silencing means that it is now preferred to earlier approaches such as co-suppression and antisense.

3 Manipulation of Input Traits

3.1 Herbicide Tolerance

Herbicide-tolerant GM crops have been developed to simplify weed control and to cut input costs. They enable farmers to use a single herbicide instead of many, reducing application costs. They also simplify crop rotation and improve farm safety, because the herbicides that are used with them degrade rapidly in the soil and are less poisonous to humans than those used on conventional crops. Furthermore, their use has led to a significant increase in the adoption of no-till farming, in which weeds and stubble are left undisturbed over winter. This reduces soil erosion and nitrate run-off.

The first GM herbicide-tolerant plants to be grown commercially were glyphosate-tolerant soybeans [93]. Glyphosate is a broad-range herbicide that targets 5-enolpyruvoylshikimate 3-phosphate synthase (EPSPS), an enzyme that is required for the synthesis of many aromatic plant metabolites, including some amino acids. The gene that confers tolerance of the herbicide is from the soil bacterium *Agrobacterium tumefaciens* and encodes an EPSPS that is not affected by glyphosate.

Two other broad-range herbicide-tolerant GM systems are also in use, conferring resistance to the herbicides glufosinate and bromoxynil. Glufosinate tolerance is conferred by a gene from the bacterium *Streptomyces hygroscopicus* that encodes PAT, an enzyme that detoxifies glufosinate. Bromoxynil tolerance is conferred by a gene isolated from the bacterium *Klebsiella pneu-*

moniae ozanae. This gene encodes an enzyme called nitrilase, which converts bromoxynil into a non-toxic compound. Glufosinate- and bromoxynil-tolerant varieties of oilseed rape have been particularly successful in the USA and Canada [50, 94].

Herbicide tolerance has now been engineered into many crop species, including varieties of oilseed rape, maize, soybeans, sugar beet, fodder beet, cotton and rice. In the USA in 2002, 81% of the US soybean crop, 59% of the upland cotton and 15% of the maize was GM herbicide tolerant [95–97]. In the same year 95% of Argentine soybean and 66% of Canadian oilseed rape (canola) was GM herbicide tolerant.

3.2 Insect Resistance

A naturally occurring soil bacterium called *Bacillus thuringiensis* has been used as a pesticide by organic and salad farmers for several decades. The insecticidal properties of the bacterium are imparted by the Cry protein, with different types of Cry protein produced by different strains of the bacterium being effective against different types of insects. Cry1 proteins, for example, are effective against the larvae of butterflies and moths, while Cry3 proteins are effective against beetles. The Cry proteins have no toxicity to mammals, birds or fish.

Cry1-encoding genes have been introduced into several crop species [98], and the modified varieties are generally referred to as Bt varieties. Commercial varieties of Bt maize and cotton have been successful in many parts of the USA and Bt cotton has proved popular in regions of Australia, China, India and the Philippines. Farmers who use these varieties cite reduced insecticide use and/or increased yields as the major benefits [99]. A further, unexpected benefit of Bt maize varieties is that the grain contains lower amounts of fungal toxins (mycotoxins) [100, 101]. However, the benefits of using Bt crops depend on many factors, most obviously the nature of the major insect pests in the area (not all are controlled by Bt) and the insect pressure in a given season.

3.3 Resistance to Pathogens

Viruses and fungi that infect crop plants are a serious threat to the livelihoods of farmers in developed countries and they cause the deaths of millions of people in developing countries through the destruction of food crops. This makes the development of virus- and fungus-resistant GM crops particularly exciting and important, and there are already examples of commercial virus-resistant GM varieties.

The first virus-resistant variety to be grown was papaya ringspot virus (PRSV)-resistant papaya [102, 103]. An epidemic of PRSV in the Puna district of Hawaii in the 1990s almost destroyed the papaya industry until the GM variety was introduced in 1998. The GM variety contains a gene that encodes a PRSV coat protein, a strategy that mimics the phenomenon of cross protection. In true cross protection, infection by a mild strain of a virus induces resistance to subsequent infection by a more virulent strain (reviewed in [104]).

A commercial potato variety resistant to potato leaf roll virus (PLRV) as well as carrying a Bt gene was launched in the 1990s but has since been withdrawn due to reluctance to use it within the highly lucrative fast food industry. In this case resistance was imparted by engineering the plant to block the activity of a viral replicase gene [105].

Currently, there are no fungus-resistant GM crops on the market. However, a number have shown promising results in field trials. One example is a potato line that is resistant to late blight [106]. Late blight is caused by the oomycete *Phytophthora infestans* and is infamous as the cause of the Irish potato famine of the nineteenth century. However, it still causes serious crop losses around the world today. The gene that was introduced into the potato line was called *RB* and came from a wild Mexican potato species called *Solanum bulbocastanum*.

3.4 Tolerance to Abiotic Stresses

The ability of crops to tolerate abiotic stresses, such as drought, salinity and extreme temperatures, is likely to become increasingly important as the world population increases, competition with other land uses pushes agriculture into harsher environments, fresh water becomes scarcer and the climate change predicted by some scientists increases environmental stress.

The genetic basis for abiotic stress tolerance is complex but some genetic modification approaches have shown promising results. For example, plants often respond to abiotic stresses by changing their metabolism to produce sugars or similar compounds that act as osmoprotectants. One such compound is trehalose, a disaccharide similar to sucrose. Trehalose levels have been increased in GM rice by over-expressing genes encoding trehalose biosynthetic enzymes from the bacterium *E. coli* [107]. This resulted in plants that showed improved performance under salt, drought and low-temperature stress conditions.

Another possible solution to the problem of salt pollution, which affects millions of acres of otherwise fertile land, usually as a result of irrigation, involves the over-expression of a gene that encodes a vacuolar Na^+/H^+ antiport pump [108]. This increases the rate at which a plant can remove salt from its cytoplasm and transfer to its vacuole. Tomato plants modified in this way

can tolerate salt concentrations several times higher than non-GM plants and should survive in the salt concentrations of soils that are currently considered unusable. Furthermore, the fruit does not accumulate salt and is edible. Similar technologies are being developed to address the problem of contamination of soils with heavy metals.

4 Manipulation of Output Traits

4.1 Fruit Ripening

One criticism of the application of genetic modification technology to crop improvement is that biotechnology companies have focused on input traits, with benefits for farmers, rather than output traits that would benefit consumers. In fact, the first GM plants to be grown commercially were tomatoes with improved shelf life, a trait of benefit to consumers as well as producers and processors. A major problem in fruit production is that consumers want to buy ripe fruit but ripening is often followed quite rapidly by deterioration and decay. Fruit ripening is a complex process that brings about the softening of cell walls, sweetening and the production of compounds that impart colour, flavour and aroma. The process is induced by the production of a plant hormone, ethylene. Genetic modification has been used to slow ripening or to lengthen the shelf life of ripe fruit by interfering either with ethylene production or with the processes that respond to ethylene.

The first GM tomatoes with increased shelf life had reduced activity of an enzyme called polygalacturonase (PG), which contributes to cell wall softening. A fresh fruit GM tomato with this trait was marketed in the mid 1990s under the trade name "Flavr Savr" but did not prove popular with consumers. However, the trait was also introduced into tomatoes used for processing into paste, resulting in higher solid content, improved (thicker) consistency, reduced waste and lower processing costs. This product proved very popular with consumers in the UK from its introduction in 1996 until 1999 when retailers withdrew it in response to anti-GM hostility.

However, some GM tomato varieties with delayed ripening are still on the market in the USA. They have reduced activity of the enzyme aminocyclopropane-1-carboxylic acid (ACC) synthase, which is required for ethylene synthesis. ACC has also been targeted using a gene from a bacterium, *Pseudomonas chlororaphis*, which encodes an enzyme called ACC deaminase that breaks down ACC [109]. A similar strategy has been adopted to break down another of the precursors of ethylene, *S*-adenosyl methionine (SAM), using a gene encoding an enzyme called SAM hydrolase.

Genetic modification to delay ripening and improve post-harvest shelf life is also being used in papaya, mango, pineapple and other fruits but there are no commercially available varieties yet.

4.2 Seed Protein Quality for Food and Feed

Proteins consist of 20 amino acids, of which only 10 can be synthesised by mammals. The remainder (methionine, lysine, leucine, isoleucine, phenylalanine, tyrosine, threonine, histidine, valine and tryptophan) must be provided in the diet and are therefore termed as essential. In addition, cysteine can only be synthesised from methionine, which is itself essential. Hence cysteine and methionine are usually combined when considering protein nutritional quality. If only one of these amino acids is limiting in a diet, the others will be broken down and excreted. A further consideration is that the concept of essential and non-essential amino acids does not apply to ruminants, as the microflora present in the rumen can synthesise all amino acids from other compounds.

The protein quality of the major seed crops is determined by the storage proteins which account for half or more of the total seed proteins. This results in legume seeds being deficient in cysteine and methionine, and most cereals, but not oats and rice, in lysine, methionine and, in maize, also tryptophan. Consequently it is necessary to use mixtures of seeds with different compositions of essential amino acids or to supplement with fish meal or amino acids produced by fermentation. Because lysine and methionine are the two major limiting amino acids, attention has been focussed on these. In both cases two major approaches have been used: to increase the pools of free amino acids and to express additional high-quality proteins.

The pools of free amino acids in seeds are generally very low, accounting for small proportions of the total amino acids present. Furthermore, their amounts are regulated at the level of biosynthesis by complex feedback mechanisms. These mechanisms need to be overcome and this has been achieved by transformation to express feedback-insensitive enzymes from bacteria. Lysine, threonine and methionine are all products in plants of the aspartate biosynthetic pathway, which is summarised in Fig. 2. The entry into the pathway is controlled by the enzyme aspartate kinase (AK) while the branch point to lysine is catalysed by dihydrodipicolinate synthase (DHDPS). Increases in total free lysine of $\times 2$ have been achieved by transforming canola to express a feedback-insensitive form of DHDPS from *Corynebacterium* and of $\times 5$ by transforming soybean with the same gene and an AK gene from *E. coli* [110]. However, the situation was found to be more complicated in maize [111]. Expression of the *Corynebacterium* DHDPS under the control of the globulin 1 (*Glb1*) gene promoter, which confers expression in the embryo and aleurone layer, resulted in increases in free lysine which were sufficient to

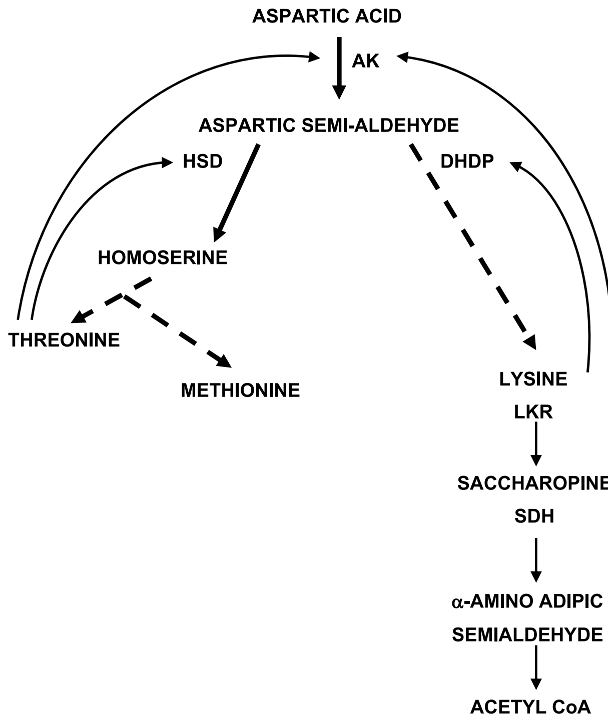

Fig. 2 Outline of the biosynthetic pathway leading from aspartic acid to lysine, threonine and methionine and of lysine catabolism. *Thick lines* show enzymic steps with *broken lines* showing several steps. *Thin lines* show feedback regulatory loops. AK—aspartate kinase; HSD—homoserine dehydrogenase; DHDP—dihydrodipicolinate synthase; LKR—lysine ketoglutarate reductase; SDH—saccharopine reductase

increase the total grain lysine content by 50–100%. However, no increase in lysine was observed when the same gene was expressed under control of the starchy endosperm-specific glutelin 2 promoter. This was ascribed to increased catabolism of lysine as the degradation product α-aminoadipic acid was found to accumulate. The accumulation of α-aminoadipic acid and saccharopine (a second degradation product) was also observed in canola and soybean [110, 111]. This effect was most extreme in tobacco seeds, in which expression of the *Corynebacterium* DHDPS resulted in increased synthesis of lysine but also increased activity of lysine ketoglutarate reductase, the enzyme catalysing its degradation to saccharopine (Fig. 2). The net result of these changes was no increase in free lysine [112, 113]. More recent work from the same group has shown that this effect can be overcome in *Arabidopsis* by expressing the *Corynebacterium* DHDPS in a knockout mutant lacking activity of lysine ketoglutarate reductase and saccharopine dehydrogenase [114]. Whereas the DHDPS transgenic and knockout mutant produced ×12 and ×5 the levels of free lysine, respectively, compared with the wild-type seed,

the combination of the two traits gave an 80-fold increase. The level of free methionine was also increased in the various lines by up to 38-fold, indicating the existence of complex regulatory networks in amino acid biosynthesis in seeds.

In 2006 the Monsanto Company introduced the first commercial high lysine corn, combining the improved feed quality with resistances to herbicide and European corn borer. This line expresses the feedback-insensitive DHDPS gene from *Corynebacterium* controlled by the maize *G1b1* gene primate, and is reported to have an increase in total grain lysine from 2500–2800 ppm dry weight to 3500–5300 ppm dry weight (Monsanto petition number 04-CR-114U). Wasaka et al. [115] recently used a similar approach to increase free tryptophan in seeds of rice, with transgenic expression of a mutant feedback-insensitive form of anthranilate synthase leading to an increase in total grain tryptophan of between $\times 2$ and $\times 12$.

A number of lysine-rich and methionine-rich plant proteins have been identified providing a basis for their use to engineer crops with improved nutritional quality. The chymotrypsin inhibitor CI-2 was initially identified as one of four proteins which occur in large amounts and therefore contribute to the high lysine phenotype in the barley line Hiproly [116]. However, despite its biological activity, it does not appear to have anti-nutritional properties. The major form of CI-2 contains eight lysines out of 83 amino acid residues (i.e. 9.5 mol %). Roesler and Rao [117] designed five mutant forms containing 20–25 mol % lysine and demonstrated that one of these was sufficiently stable (after expression and purification from *E. coli*) to be a candidate for expression in transgenic plants. More recently, the same authors have made substitutions to over a third of the total residues (introducing eleven lysines, five methionines, three threonines, two tryptophans and one glycine) and restored stability to the protein via a single disulphide bond [118].

Lysine residues have also been introduced into two different parts of the CI-2 protein, with one mutant containing three additional lysines being sufficiently stable for future exploitation [119]. However, none of these proteins has yet been expressed in transgenic plants.

A second high lysine plant protein is the barley seed protein hordothionin. This contains five lysines out of 45 residues but molecular modelling has been used to design mutants with up to 27% lysine which have subsequently been characterised using synthetic peptides [120]. However, although hordothionines are present in barley grain and related proteins in other cereals, it is unlikely that they will be acceptable for expression in transgenic plants. This is because the purified proteins are highly toxic in vitro to a range of microorganisms (bacteria, fungi, yeasts), invertebrates and animal cells [121].

Methionine-rich proteins appear to be much more widespread in plants than lysine-rich proteins, with methionine contents ranging up to over 25 mol %. Most of these belong to two families of seed storage proteins, the 2S albumins of

dicotyledonous plants and the β- and δ-prolamins of maize and related tropical panicoid cereals.

The 2S albumin fraction of Brazil nuts (i.e. seeds) comprises several methionine-rich proteins, including a major component containing 18.8 mol % methionine. The sequence encoding this protein has been widely used in transgenic crops and model plant species in order to increase the methionine content. The expression level in canola was reported as between 1.7 and 4% of the total protein resulting in an increase in seed methionine of up to a third [122]. Similarly, expression in two legumes, soybean and Narbon bean, was reported as 10 and 5% of the total protein leading to 50% and twofold increases in methionine, respectively [123–125].

Methionine-rich 2S albumins are also present in sunflower seeds, although in this case only two components out of 11–13 are methionine-rich [126]. One of these, SFA8, contains 16 methionines out of 103 residues [127]. Expression of this protein in lupin seeds resulted in a 94% increase in seed methionine but no impact on total seed sulphur, the increase being at the expense of cysteine and sulphate [128]. Consequently the combined amounts of cysteine and methionine were only increased by 19%. Similarly, in transgenic rice the methionine content was increased by about 27% but the cysteine content fell by about 15% [129]. As a result, there was little impact on the overall nutritional quality of the seed protein in either species. A similar result was observed in Narbon bean which was doubly transformed to express the Brazil nut 2S albumin and a feedback-insensitive form of AK which led to larger pools of free methionine. Protein-bound methionine was increased by 2- to 2.4-fold but at the expense of cysteine, sulphate and other sulphur-containing compounds [130]. These results are all consistent with a recent study [131] in which wild-type lupins and lines expressing SFA8 were grown under varying conditions of sulphur nutrition. This showed that the total sulphur content of the seed was determined by the supply to the seed (i.e. the source) rather than the expression of genes for sulphur-rich proteins in the seed (i.e. the sink). Hence, the availability of sulphur in the soil and the uptake and transport to the grain must also be considered.

More recently, a gene encoding a sulphur-rich 2S albumin from sesame (containing 15 residues of methionine and eight of cysteine) was expressed in the bran [132] and starchy endosperm [133] of rice, using oleosin and glutelin promoters, respectively. The transgenic grain contained 24–38% more methionine and 50–62% more cysteine in the bran [132] and 29–76% more methionine and 31–75% more cysteine in the whole seed [133], but the total S content of the seed was not reported.

However, it is unlikely that these 2S albumins will ever be expressed in transgenic crops for human consumption. The 2S albumin proteins are often allergenic to humans [134] and the Brazil nut albumin has been shown to be a major allergen [135] and to retain its immunological properties when expressed in transgenic soybean [136]. SFA8 has also been reported to be

allergenic [137, 138], although reaction appears to be fairly rare, while the sesame 2S albumin is also an established allergen (Ses i 1) [139].

The β-zeins and δ-zeins of maize have methionine contents ranging from 11.4 (β-zein) to 26.9% (M_r 18 000 δ-zein) [129, 130] and have no known allergenic or anti-nutritional properties. It is therefore surprising that there have been few attempts to exploit them. However, it has been reported [142] that transformation of maize with additional copies of a δ-zein gene led to 30% increases in methionine. It has similarly been reported [143] that the amounts of δ-zein and total grain methionine can be increased by modifying the stability of its mRNA. However, no data on total grain sulphur were reported although cysteine was slightly lower.

It is clear from these reports that the protein quality of cereal and legume crops can be improved by transformation. However, it is important to ensure that the modifications do not result in unwanted anti-nutritional or allergenic properties, and in the case of methionine to ensure that adequate sulphur is available to support the increased amount without compensatory decreases in cysteine.

4.3 Food Allergies and Intolerances

Food allergy is considered to affect 1–2% of the general population, and up to 8% of children below three years of age. Furthermore, it is clear that the incidence has risen in recent years and there is no indication that this increase will not continue. Allergenic reactions to food may affect the skin (urticaria, atopic dermatitis), the gastrointestinal tract (oral allergy, gastrointestinal anaphylaxis) and the respiratory system but may also be generalised (anaphylaxis, food-dependent exercise-induced anaphylaxis). Also, sensitisation may occur via the gastrointestinal tract, via inhalation of related proteins (sensitisation to pollen resulting in reactions to fruit and vegetables) or, more rarely, via contact (the latex-fruit syndrome).

In principle genetic modification provides an exciting opportunity to manipulate the expression and/or sequences of plant proteins to reduce or eliminate their allergenicity. In practice, this may be difficult to achieve for several reasons. Firstly, many plant foods that result in severe allergic reactions contain multiple allergenic proteins, which may be unrelated in their structures and sequences. A good example of this is peanut in which the characterised allergens include 2S albumins (Ara h 2, 6 and 7), 7S globulin (Ara h 1), 11S globulins (Ara h 3 and 4) and a profilin-related protein (Ara h 5). Similarly, soybean allergens include 7S globulins, 11S globulins and a cysteine proteinase called P34 (see [144, 145]). Furthermore, multiple IgE-binding sites appear to be present on most if not all of these proteins. For example, at least 23 are present on Ara h 1, 16 on P34 and 11 on the soybean 11S globulin [145]. It is certainly possible to remove at least some epitopes by protein engineer-

ing, as demonstrated for the major cherry allergen Pru a 1 [146]. In this case, mutation of a single serine residue to proline resulted in reduced binding of IgE from sera of most patients. However, it is difficult to predict whether multiple epitopes can be removed in this way without adversely affecting the structure and biological properties of the protein. Also, it would be necessary to replace the endogenous gene with that encoding the mutated protein. At present the technology for routine gene replacement is not available.

The most widespread food intolerance is coeliac disease, which is a T-cell mediated autoimmune response that is triggered by a range of proteins present in wheat gluten and related proteins in rye and barley [147]. As with multiple allergens, eliminating the "toxic" properties would require the removal of many proteins from the grain.

Beyond the practical problems of removing multiple proteins from wheat and other species it is also necessary to consider the effects on the functional properties of the crop. The wheat gluten proteins are the major determinants of the functional properties of wheat (as discussed elsewhere), while the proteins of soybean confer a range of functional properties which allow their exploitation in food systems (see above). Nevertheless, some progress has been made.

The major dietary allergens in rice grain are inhibitors of human α-amylase with molecular masses ranging from about 14 000 to 16 000 [148]. At least seven immunologically cross-reactive proteins have been identified and more than ten different but homologous cDNAs [148–150]. Nevertheless, the antisense expression of a single sequence resulted in an 80% reduction in sense transcripts and a decrease in total allergen content from about 300 μg/seed to about 60–70 μg/seed [151]. Although the transgenic rice can be described as "hypoallergenic" it clearly still contains substantial amounts of the allergenic proteins and would not be suitable for consumption by allergic individuals. Transgene-induced gene silencing has been used to remove the Gly m Bb 30 k allergen from seeds of soybean [152], while antisense technology has been used to down-regulate the production of the Lol p 1, Lol p 2 and Lol p 3 allergens in pollen of ryegrass, which are the major source of grass pollen allergy in cool temperate climates [153, 154].

The RNA interference (RNAi) approach is proving more reliable for silencing specific genes than other approaches (as discussed above). A recent report demonstrates the potential of RNAi to silence specific allergens in crop plants. In areas where birch pollen is endemic, allergy to apple is dominated by IgE antibodies against the protein Mal d 1. When the gene encoding Mal d 1 was silenced using RNAi in transgenic apple plants, there was reduced expression as measured by immunoblotting and in vivo allergenicity was significantly reduced [155].

A final important consideration in removing allergens or epitopes is that the effects must be permanent without any chance of reversal. The long-term stability of mRNA-mediated gene suppression is still not known and a more

satisfactory option would be to use gene replacement, or in vivo mutagenesis via chimeraplasty [156]. Neither of these technologies is currently routinely applicable to crop plants.

4.4
Wheat Quality for Breadmaking

The ability to make bread and a range of other processed foods (pasta, noodles, cakes, biscuits etc.) from wheat flour, but not from other cereal flours, is determined by the unique properties of the grain storage proteins. These are deposited in discrete protein bodies in the cells of the starchy endosperm, but during the later stages of grain maturation these protein bodies coalesce to form a continuous matrix, or network, in the cell. When flour is mixed with water to form dough, the gluten proteins in the individual flour particles come together to form a continuous network in the dough. This network confers cohesiveness to the dough and a unique combination of elasticity (strength) and extensibility. These properties allow the gluten network to be expanded by carbon dioxide released by yeast during proving, conferring a light porous crumb structure to the loaf. Although it is possible to make bread with all types of wheat there is massive variation in the quality of the product, which is related to genetic differences between cultivars, environmental factors and interactions between these. In general, wheats grown in the UK and northern Europe tend to produce weaker gluten and dough than those grown in hotter, drier regions of North America and southern/central Europe and require fortification (i.e. blending) with stronger wheats to produce acceptable bread.

A massive volume of research has been carried out on wheat gluten proteins, over a period in excess of 250 years. This has established that the most important factor determining dough strength is the ability of some gluten proteins (called glutenins) to form polymeric complexes, which are stabilised by inter-chain disulphide bonds and may have molecular masses up to about ten million. Furthermore, it is clear that one group of glutenin proteins, called the high molecular weight (HMW) subunits, are particularly important in determining the proportion of polymers which have high molecular masses and that this proportion is related to differences in dough strength [157–161]. Consequently, although the HMW subunits account for only about 8–12% of the total gluten proteins they have been calculated to account for between about 45 and 70% of the variation in breadmaking quality within European wheats [157, 158]. Cultivars of bread wheat express either three, four or five HMW subunits, encoded by loci (*Glu-1*) on the long arms of chromosomes 1A, 1B and 1D. In fact, each of these loci comprises two genes, encoding one low M_r y-type and one high M_r x-type subunit. However, between one and three of these genes are silent ("null") in bread wheat. Consequently all cultivars contain 1Bx, 1Dx and 1Dy subunits with 1By and/or

1Ax subunits being also present in some cultivars. Furthermore, all expressed subunits occur in two or more allelic forms which are usually numbered based on their relative mobility in SDS-PAGE. For example, subunit 1Dx 5 is allelic to but faster than subunit 1Dx 2. Finally, because the genes for x-type and y-type subunits are tightly linked, the encoded subunits are inherited as "allelic pairs" (e.g. subunits 1Dx 2 + 1Dy 12 are allelic to 1Dx 5 + 1Dy 10). This allelic variation in gene expression and in the encoded proteins appears to result in two types of effect on dough strength: qualitative and quantitative [161, 162].

Quantitative effects relate to gene expression, particularly at the *Glu-A1* locus. Thus the expression of a 1Ax subunit (1Ax 1 or 1Ax 2*) results in an increase in the total amount of HMW subunit protein by about 2% [163]. Qualitative differences are less well understood but are thought to result from differences in the structures and properties of allelic proteins. The most well-established qualitative effect is associated with the *Glu-D1* locus, with subunits 1Dx 5 + 1Dy 10 being associated with good quality compared with other widely occurring alleles (1Dx 2 + 1Dy 12, 1Dx 3 + 1Dy 12, 1Dx 4 + 1Dy 12). A number of HMW subunit genes have been isolated from bread wheat, including those encoding 1Ax (1, 2*), 1Bx (7, 17), 1Dx (2, 5), 1By (9) and 1Dy (10, 12) subunits. This has facilitated attempts to improve wheat processing properties by genetic engineering.

A number of studies on the expression of HMW subunit genes in transgenic wheat have been published over the past decade, with broadly similar results [169–175]. Expression of a 1Ax 1 subunit in lines which have the null allele at the *Glu-A1* locus results in increased dough strength and improved breadmaking quality [164, 165, 168–175], to a similar extent to that associated with the expression of an endogenous 1Ax subunit. Similarly, it was reported that a substantial increase in mixing time (i.e. dough strength) occurred in lines in which the 1Dx 5 and 1Dy 10 transgenes were expressed together [166]. In contrast, the expression of subunit 1Dx 5 in the absence of subunit 1Dy 10 (with which it is invariably associated in European cultivars) resulted in extreme and unpredictable effects. Flour made from such lines failed to absorb water and form a normal dough in the Mixograph and, as a result, the Mixograph curve was flat and loaves baked from the dough had low volume and dense texture [165, 167, 170, 171] (Fig. 3). Fractionation of the gluten from these lines showed a high proportion of insoluble glutenin polymers, while rheological analysis showed that the gluten fraction had similar properties to those which were observed when transglutaminase was used to introduce additional cross-linking [170]. Subunit 1Dx 5 differs from all other x-type subunits in that an additional cysteine residue is present in its sequence, and it was concluded that this led to an unusually high level of cross-linking within and between the glutenin polymers, particularly in the absence of subunit 1Dy 10. This would account for the insolubility of the polymers and their failure to hydrate normally

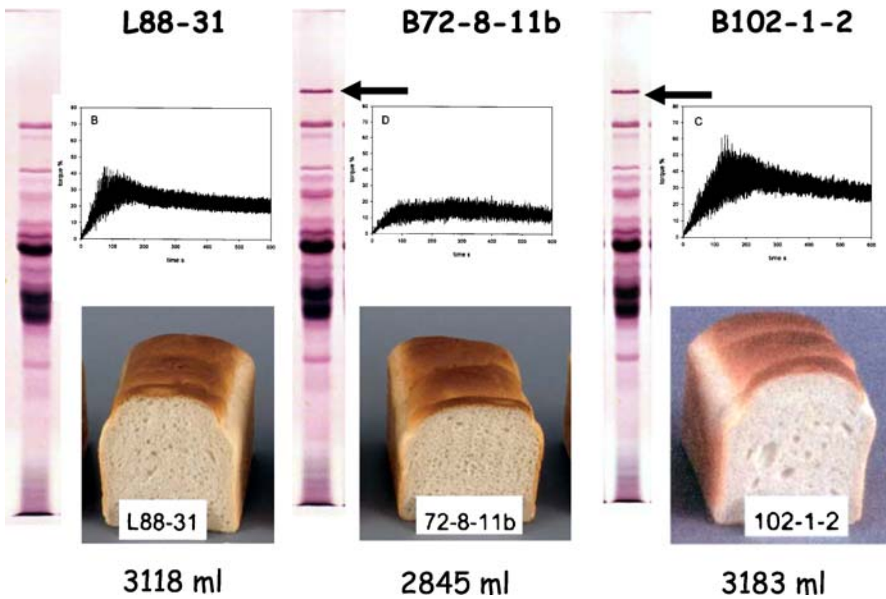

Fig. 3 The transgenes encoding subunits 1Ax 1 and 1Dx 5 have contrasting effects on breadmaking performance. The figure shows SDS-PAGE separation of gluten protein, Mixograph profiles and loaves baked from the control line (L88-3) and transgenic lines B72-8-11b expressing the 1Dx 5 transgene and B102-1-2 expressing the 1Ax 1 transgene, both in the L88-31 background. The subunits encoded by the transgenes are indicated by *arrows* in the SDS-PAGE separations. Compiled from data in [170, 171]

on mixing. Nevertheless, the results achieved by expressing the 1Ax 1 subunit show that genetic modification can be used to develop cultivars with increased dough strength. We have also shown that similar improvements can be achieved with modern elite European cultivars, either by direct transformation [173, 174] or by introgression of the transgenes from model lines by crossing [175].

4.5 Content and Bioavailability of Minerals

Plant seeds are potentially important sources of minerals for nutrition of humans and livestock, but a high proportion of the minerals present are unavailable as they are in the form of mixed salts of phytic acid (*myo*-inositol-1,2,3,4,5,6-hexakisphosphate). Thus, phytate accounts for over 70% of the total phosphorus as well as substantial amounts of Mg^{2+}, K^{+}, Fe^{3+}, Zn^{2+}, Ca^{2+} and Cu^{2+}. Phytates act as storage reserves in the seed and are degraded during germination. For example, phytin granules are abundant in the embryo and aleurone of cereal grains. However, animals cannot digest phytate and consequently it is excreted. The excretion of phosphorus can lead to eutroph-

ication of natural waters adjacent to farmland used for intensive livestock production. The low availability of calcium, iron and zinc in cereals and other plant foods can also contribute to nutritional deficiency in humans, particularly women and children in developing countries.

Genetic engineering can be used to digest the phytin and increase the mineral availability in seeds, by expression of genes encoding phytase. Genes from *Aspergillus* species have been used for this as they express extracellular phytase enzymes and have been produced commercially. The expression of phytase has been reported in a range of crop species, i.e. soybean, oilseed rape, rice and wheat [5, 176–180], using the *phyA* gene from *Aspergillus niger*. Feeding studies with transgenic soybean showed a 50% reduction in phosphate excretion by broiler chickens [176], while feeding transgenic canola to piglets and broilers showed similar positive effects on growth to those achieved by supplementation with exogenous phytase enzyme [178]. Drakakaki et al. [181] also expressed the *Aspergillus niger* phytase in maize using the rice glutelin promoter, using a CaCo-2 cell model to show increased availability and uptake of iron. Work in progress is focused on expression of heat stable forms of phytase (from *A. fumigatus*, *Selenomonas ruminatium* and *E. coli*), to prevent loss of activity during food and feed preparation [5, 182, 183].

Iron deficiency is the most widespread mineral deficiency in humans which has been estimated to affect up to 30% of the total world population [184], and improving the release from phytates is one possible strategy to increase iron availability. An alternative, or complementary, approach is to increase the amounts of other iron-binding compounds in the seed. Ferritin is an iron-binding protein which provides a storage reserve of iron in plants, bacteria and animals [185]. The expression of ferritin genes from soybean [186, 187] and *Phaseolus* [5] in developing seeds of rice has been shown to result in two- to threefold increases in the iron content of the grain, demonstrating the feasibility of using genetic engineering to increase iron availability. Qu et al. [188] showed that a similar increase in iron accumulation occurred when the soybean ferritin was expressed in rice grain, but that this was associated with decreased iron in the leaves. Hence, accumulation may ultimately be limited by iron uptake and transport. Finally, Drakakaki et al. [181] showed that combined expression of soybean ferritin and fungal phytase in rice resulted in a 20–70% increase in the iron content of maize seeds, and increased availability of iron to CaCo-2 cells.

4.6 Oil Composition

A drawback for farmers who grow oilseed rape (canola) is that rape oil is one of the cheapest edible oils on the market. The value of the crop is, therefore, relatively low and there is a lot of interest in increasing it. This has been achieved through genetic modification by introducing a gene from the Cali-

fornia Bay plant that causes an accumulation of lauric acid to approximately 40% of the total oil content, compared with 0.1% in unmodified oilseed rape [189]. Lauric acid is a component of soaps that is traditionally derived from high-value coconut or palm oils.

A different modification has been made to the oil of soybean. In this case, the GM variety accumulates oleic acid to approximately 80% of its total oil content, compared with approximately 20% in non-GM varieties [190]. This was achieved by suppression of a gene which encodes an enzyme that converts oleic acid to linoleic acid. Oleic acid is very stable at high temperatures and, at present, the oil from the GM soybeans is used for industrial purposes. Relatively small amounts of these specialised GM oilseed rape and soybean varieties are grown to contract, but those farmers who are able to grow them benefit from a premium price for their crop.

More specialised industrial oils could also be produced by expressing fatty acid biosynthesis enzymes from more exotic sources. For example, genes from *Crepis palaestina* and *Crepis alpine* have been used to synthesise vernolic acid and crepenynic acid, respectively, in seed of *Arabidopsis* [191], while genes from *Morordica charantia* and *Impatiens balsamina* directed the synthesis of α-eleostearic acid and α-parinaric acid, respectively, in soybean embryos [192].

Several edible oils have pharmaceutical or neutraceutical properties, resulting from the presence of long-chain polyunsaturated fatty acids (PUFAs). PUFAs are defined as containing three or more double bonds with a fatty acid chain of 18 or more carbons [193]. They are further classified into two families, commonly called omega-3 and omega-6, depending on the position of the last double bond in relation to the methyl end of the fatty acid chain. The precursors of these compounds are made by plants but not animals and are therefore essential components of the human diet. PUFAs that have attracted the interest of biotechnologists include γ-linolenic acid (GLA), which is found in borage and evening primrose oils and is used in the treatment of skin conditions such as atopic eczema, and arachidonic acid (AA), which is only found in a few mosses and fungi. AA is a constituent of breast milk and is important for brain and eye development in infants. Two omega-3 PUFAs from fish oil, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have anti-inflammatory properties and are believed to be efficacious for heart and circulatory system health, brain development and function, mood and child behaviour.

Unfortunately, these fatty acids can only be sourced from plants that are poorly adapted to cultivation with low yields or from fish oil, which is a diminishing resource. The aim of biotechnologists is to take the genes that encode the enzymes responsible for their biosynthesis and engineer them into crop plants. The recent development of an *Agrobacterium*-mediated transformation procedure for Rigel, a commercial cultivar of evening primrose, allowed the over-expression of a cDNA encoding a Δ^6-desaturase from

borage under the control of a CaMV 35S promoter. Analysis of the transformed plants demonstrated an altered profile of PUFAs with an increase in GLA and octadecatetraenoic acid in leaf tissues when compared with control lines [194]. A successful example in the model plant, *Arabidopsis*, was also reported recently [195]. AA and EPA were produced in substantial quantities after the introduction of genes encoding a Δ^9-specific elongase from the alga *Isochrysis galbana*, a Δ^8-desaturase from the protist *Euglena gracilis* and a Δ^5-desaturase from the fungus *Mortierella alpina*.

However, further work is required to optimise the production of PUFAs in crop plants, including modification of the enzymes that mediate acyl lipid exchange to increase their selectivity for PUFAs. This is discussed in an excellent recent review [196].

4.7 Isoprenoids, Phytosterols and Vitamins

Another group of compounds with neutraceutical properties are the isoprenoids. These are a large family of compounds based on a five-carbon structure called isopentenyl diphosphate (IPP), which is the precursor of all isoprenoids in eukaryotes. In plants, they include phytosterols (Fig. 4), the plant hormones gibberellins and abscisic acid, components of photosynthetic pigments, phytoalexins and a variety of other specialised compounds. Many of these compounds may be of interest to biotechnologists, but the phytosterols have attracted particular attention because the predominant naturally occurring phytosterols are structurally related to cholesterol. These compounds, which are known as 4-desmethylsterols because of the absence of methylation at the fourth carbon in the chain and include β-sitosterol, campesterol and stigmasterol, competitively inhibit the uptake of cholesterol from the small intestine in humans [197]. They are purified from plant sources such as soybean and added to margarines and other products designed to reduce serum cholesterol levels. Increasing their levels in oilseeds would make them cheaper to purify; alternatively, oils with enhanced phytosterol levels could be used to make these products without supplementation.

Although there are two routes for the biosynthesis of isoprenoids in plants, phytosterols are produced from IPP that is derived from acetyl-CoA via a biosynthetic pathway in which mevalonic acid is an intermediate. The NADPH-dependent reduction of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) to mevalonic acid, catalysed by the enzyme HMG-CoA reductase, is an important regulatory step for this pathway. A 3.2-fold increase in seed sterol levels has been achieved in tobacco (as a model) by expressing a truncated rubber tree (*Hevea brasiliensis*) HMG-CoA reductase [198]. However, this has not yet been achieved in a crop plant.

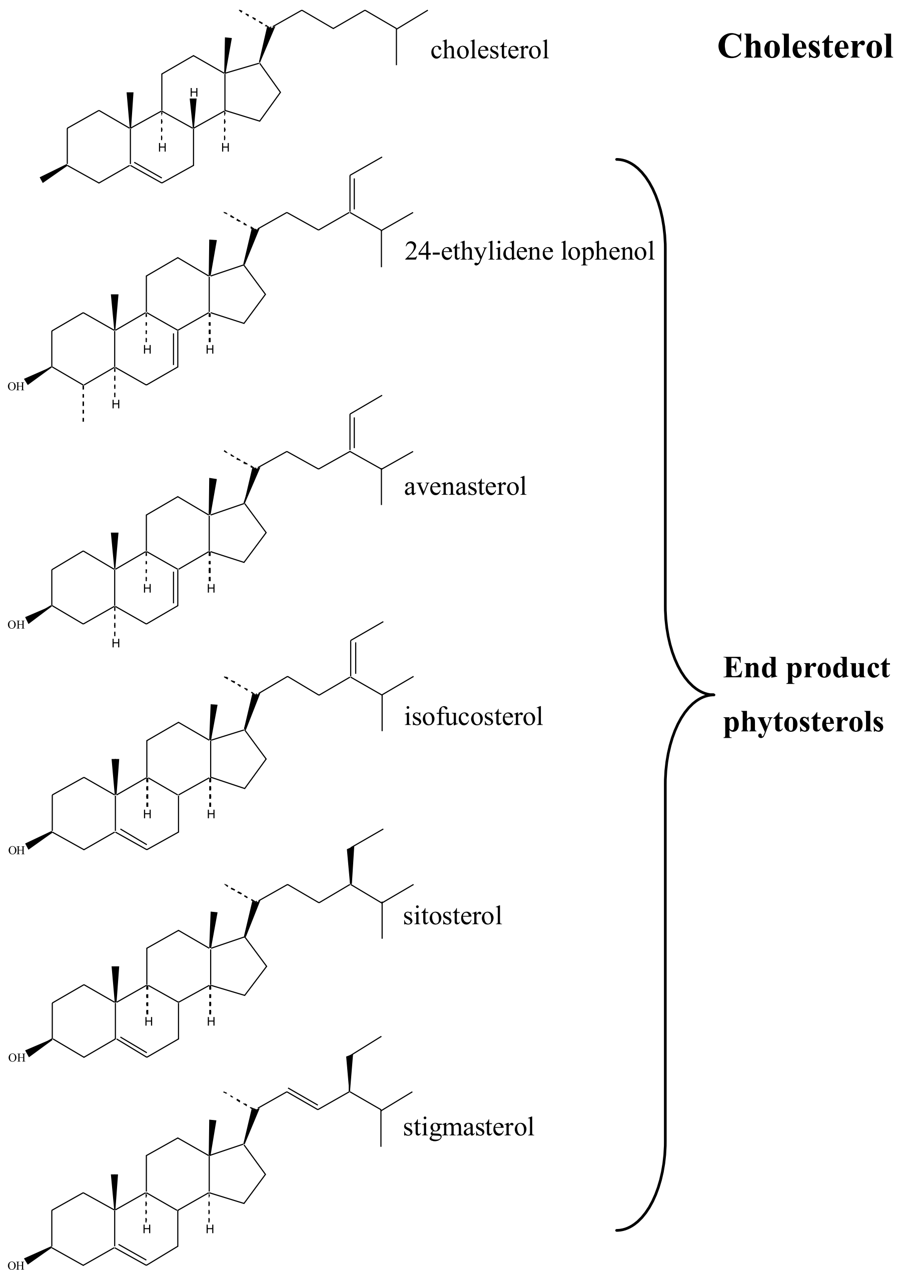

Fig. 4 Schematic diagram showing the structure of cholesterol and the predominant naturally occurring phytosterols. This close structural relationship is responsible for the efficacy of phytosterols in the competitive inhibition of cholesterol uptake from the small intestine in humans [197]. (Figure kindly provided by Dr. Sandra Hey, Rothamsted Research)

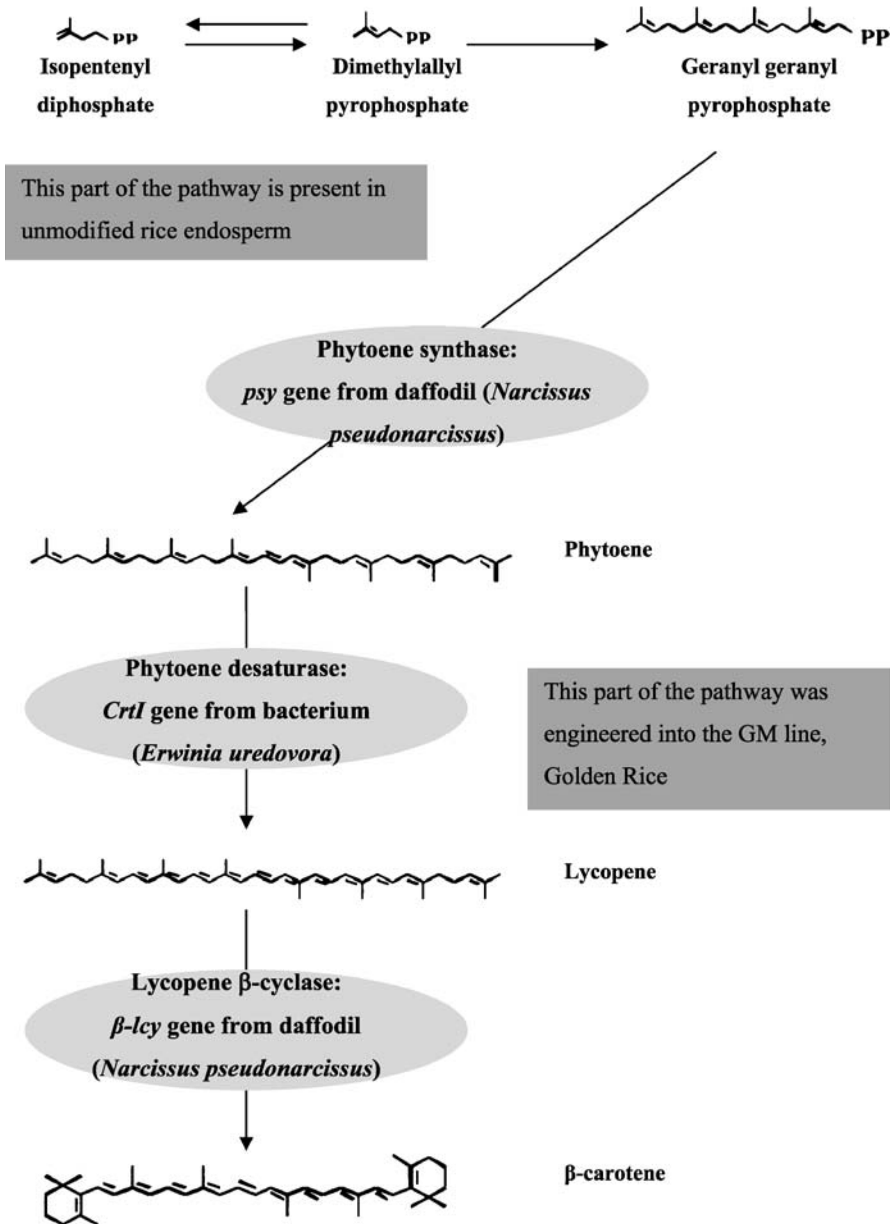

Fig. 5 Biosynthetic pathway for the production of the vitamin A precursor, β-carotene, in Golden Rice [4], with diagrammatic representatives of the compound structures

The isoprenoid family includes the fat-soluble vitamins E and K, deficiencies in which are associated with arterial disease and, in the case of vitamin K, post-menopausal osteoporosis. Strategies for increasing the levels of some of

these nutrients are described in [199]. The real need for increased vitamin levels in foods is, of course, in developing countries. An example of a severe but avoidable health problem in poor countries is night and total blindness brought about by vitamin A deficiency. This is associated in particular with a reliance on rice as a staple food, and it is estimated that a quarter of a million children go blind each year because of vitamin A deficiency in Southeast Asia alone.

One possible solution to this problem is to address the low levels of vitamin A in rice grain, which does contain vitamin A but only in the husk, which is discarded because it rapidly goes rancid during storage. This has been achieved in an experimental GM rice line called Golden Rice (the name deriving from the colour of the grain) [4]. Three genes were used: phytoene synthase (*psy*) and lycopene β-cyclase genes from daffodil (*Narcissus pseudonarcissus*), and a phytoene desaturase (*crtI*) gene from the bacterium *Erwinia uredovora* (Fig. 5). The enzymes encoded by these genes convert geranylgeranyl diphosphate, which is present in rice endosperm, into β-carotene, a precursor that humans can process into vitamin A. Golden Rice also contains a gene that encodes phytase, which is an enzyme that breaks down phytate, a compound that prevents iron absorption (see above). However, Golden Rice is an experimental line, not a commercial variety, and the trait is now being crossed into commercial breeding lines at the International Rice Research Institute (IRRI) in the Philippines and by plant breeders in India.

5 Conclusions

Genetic modification offers opportunities to improve the production and composition of crops with benefits to the environment and consumers, with particular significance for production in less developed countries where crop yields are limited by biotic and abiotic stresses and restricted diets may lead to nutritional deficiencies. Despite the current hostility to using genetic modification technology in western Europe it has been rapidly adopted in the Americas and Asia, and recognised as an important adjunct to classical plant breeding.

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Production of Secondary Metabolites Using Plant Cell Cultures

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Abstract Plant cell cultures represent a potential source of valuable secondary metabolites which can be used as food additives, nutraceuticals, and pharmaceuticals. The synthesis of phytochemicals by the cell cultures in contrast to these in plants is independent of environmental conditions and quality fluctuations. In many cases, the chemical synthesis of metabolites is not possible or economically feasible. Moreover, the natural food additives are better accepted by consumers in contrast to those which are artificially produced.

In this chapter, the process for obtaining the secondary metabolites from plant cell cultures is represented as a multi-stage strategy, and each link should be described

according to specifications of cell cultures or products. For the establishing of high-producing and fast-growing cell lines, the parent plants should be selected. The expression of synthetic pathways can be influenced by environmental conditions, the supply of precursors, and the application of elicitors, and it can be altered by special treatments such as biotransformation and immobilization. The efficiency of bioprocessing can be increased by the simplification of methods for product recovery, based on the principle of continuous product release into the cultivation media. This can be induced through influencing membrane permeability by chemical or physical factors, e.g., high electric field pulses.

The combined research in the fields of establishment of in vitro cultures, targeting of metabolite synthesis, and development of technologies for product recovery can exploit the potential of plant cells as sources of secondary metabolites.

Keywords Biotransformation · Elicitors · Exudation · Food additives · Immobilization · Membrane permeabilisation · Plant cell culture

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
BA	Benzyladenine
DMSO	Dimethylsulfoxide
DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid
FW	Fresh weight
IAA	Indole-3-acetic acid
NAA	1-Naphthalene acetic acid

1 Introduction

Plant cell culture systems represent a potential source of valuable secondary metabolites which can be used as food additives (flavors, fragrances, and colorants), nutraceuticals, and pharmaceuticals [65]. The problems related to obtaining of secondary metabolites from plants include environmental factors, political and labor instabilities in the producing countries, uncontrollable variations in the crop quality, inability of authorities to prevent crop adulteration, and losses in storage and handling. In many cases, the chemical synthesis of these is either extremely difficult or economically infeasible [42].

The production of useful and valuable secondary metabolites from cell cultures is an attractive proposal. Cell culture technology was developed as a possible tool to both study and produce plant secondary metabolites. The evolving importance of the secondary metabolites has resulted in a high level of interest in the possibility of altering their production through improving cultivation technology [65]. During the past four decades, research has concentrated on the use of plant cell cultures, particularly in Japan, Germany,

and the USA, for the commercial production of a wide range of secondary metabolites, in the same way as bacteria and fungi have been used for antibiotic or amino-acid production [40]. For example, there has been tremendous success in the production of shikonin from cell cultures of *Lithospermum erythrorhizon*, berberine from *Coptis japonica* [36], and sanguinarine from *Papaver somniferum* [11].

This chapter reviews the recent advances in the optimization of environmental factors for metabolite production by plant cell culture, new developments in plant cell bioprocesses, and emerging research on phytochemical recovery.

2
Plant Cell Culture Technique

2.1
Plant Secondary Metabolites Produced by Cell Cultures

Plants form an important part of our everyday diet, and their nutritional values have been intensively studied for decades. Over 80% of the approximately 30 000 known natural products are of plant origin [45], which is estimated to be nearly fourfold greater than that in the microbial kingdom.

For centuries, humans has been used plants as a source of carbohydrates, proteins, and fats for food and shelter. In addition to essential primary metabolites, higher plants synthesize a wide variety of secondary metabolites.

Plant secondary metabolites can be defined as compounds that have no recognized role in the maintenance of fundamental life processes in plants, but they do have an important role in the interaction of the plant with its environment. They mostly have an ecological role as attractants of pollinating insects or in defence mechanisms against predators. The distribution of secondary metabolites in plants is far more restricted than that of primary metabolites; a compound is often only found in a few species, or even within a few varieties within a species. The production of these compounds is often low (less than 1% DW), and it depends greatly on plant species and plant's physiological and developmental stage [42]. Moreover, secondary metabolites often accumulate in the plant in specialized cells or organs.

2.2
Application of Plant Cell Cultures

Many plants containing high-value compounds are difficult to cultivate [49]. At the same time, the chemical synthesis of plant-derived compounds is often not economically feasible because of their highly complex structures and specific stereo-chemical characteristics. The production of valuable secondary

metabolites in plant cell cultures is an attractive alternative to the extraction of the whole plant material.

Plant cell cultures were first established in the late 1930s. However, it was only in 1956 that Pfizer Inc. filed the first patent for the production of metabolites by cell cultures [50]. Larger quantities of visnagin and diosgenin were isolated from cell cultures than from the whole plant [5]. In 1978, Zenk (1978) demonstrated the outstanding metabolic capacities of plant cells and highlighted the spontaneous variability of plant cell biosynthetic capacity. This natural variability is exploited to identify high-yielding cultures for use on an industrial scale [4]. Since the late 1970s, research and development in this area has seen a high increase in the number of filed patent applications. In 1983, shikonin was produced by plant cell cultures on an industrial scale for the first time by Mitsui Petrochemical Industries Ltd. [20].

Currently, plant cell culture has direct commercial applications as well as value in basic research into cell biology, genetics, and biochemistry.

The application of plant cell culture has three main aspects [65]:

1. **breeding and genetics:**
   - **micropropagation** – using meristem and shoot culture to produce large numbers of identical individuals;
   - **selection** – screening of cells, rather than plants, for advantageous characters;
   - crossing distantly related species by **protoplast fusion** and regeneration of the novel hybrid;
   - production of dihaploid plants from **haploid cultures** to achieve homozygous lines more rapidly in breeding programs;
   - **transformation**, followed by either short-term testing of genetic constructs or regeneration of transgenic plants;
   - **removal of viruses** by propagation from meristematic tissues;
2. **model system** for study of plant cell genetics, physiology, biochemistry, and pathology;
3. **production of secondary metabolites** – growth in liquid culture as a source of products.

This chapter reviews the recent advances in metabolite production by plant cell cultures.

Application for Production of Secondary Metabolites

When compared with the intact plant, cultured plant cells often produce different quantities with different profiles of secondary metabolites and these quantitative and qualitative features may change with time [59].

As shown in Table 1, some metabolites in plant cell cultures can be accumulated with a higher titer compared with those in the parent plants,

Table 1 Product yield from plant cell cultures compared with the parent plants

Product	Plant	Yield (% DW) Culture	Yield (% DW) Plant	Culture/ plant	Refs.
Ajmalicine	*Catharanthus roseus*	1.0	0.3	3.3	Lee and Shuler 2000
Anthraquinones	*Morinda citrifolia*	18	2.2	8	Zenk 1977
Berberine	*Coptis japonica*	13	2	3.3	Fujita and Tabata 1987
Caffeic acid	*Vanilla planifolia*	0.02	0.05	4	Knorr et al. 1993
Ginsenoside	*Panax ginseng*	27	4.5	6	Matsubara et al. 1989
Nicotine	*Nicotiana tabacum*	3.4	2.0	1.7	Mantell et al. 1983
Rosmarinic acid	*Coleus blumei*	27	3	9	Petersen and Simmond 2003
Shikonin	*Lithospermum erythrorhizon*	20	1.5	13.5	Kim and Chang 1990
Ubiquinone-10	*Nicotiana tabacum*	0.036	0.003	12	Fujita and Tabata 1987

suggesting that the production of plant-specific metabolites by plant cell culture instead of whole plant cultivation possesses definite potential [65].

Kim and Chang (1990) showed that shikonin by *Lithospermum erythrorhizon* was accumulated in higher levels in cultured cells than in the intact plants. Similar results were shown by Petersen and Simmonds (2003) in the production of rosmarinic acid by *Coleus bluemei*. Higher quantities of berberine have been obtained from growing cells of *Coptis japonica* [20]. This plant accumulates significant amounts of berberine in its roots in four to six years; similar concentrations could be obtained in four weeks using tissue culture. Hara et al. have isolated a cell line of *Coptis japonica* that contained 13% DW of berberine. This culture produced about 1500 mg l^{-1} of this antibacterial alkaloid in 14 days. There are a number of examples of cultured cells producing metabolites not observed in the plant. Thus, *Lithospermum erythrorhizon* cultures have been observed to synthesize rosmarinic acid [57].

2.2.1 Food Additives from the Plant Cell Cultures

The reason for the use of metabolites synthesized by the plant cell cultures as food additives is not only that they are difficult or impossible to synthesize chemically, but consumers also more easily accept a natural product than an artificially produced one [50]. Food additives contribute to making food-

stuffs palatable and attractive by enhancing or improving their flavor, color, and texture. Food technologies try to respond to these criteria especially with regard to the texture, taste, and aroma of the foodstuff. The need to have the same taste and aroma in order to suit the consumer tastes makes it compulsory to use additional natural or artificial aromas.

Since the late 1950s, many food additives have been questioned mainly by national and international regulatory authorities about their safety for long-term use and consumption. At the same time, the consumer associations, aware of the inclusion of additives in foodstuffs, have been exerting pressure on governmental bodies to have chemical or artificial additives replaced by natural additives from plant tissues, or additives synthesized by plant cell cultures [52]. The most valuable food additives that can be obtained from the plant cell cultures are food colorants (anthocyanins and betalaines), flavors (saffron and vanillin), sweeteners (steviosides), pungent food additives (capsaicin), and anti-bacterial food preservatives (thio-

Table 2 Food additives from plant cell cultures

Product type	Plant species	Refs.
Colours		
Anthocyanins	*Vitis vinifera*	Curtin et al. 2003
	Aralia cordata	Sakamoto et al. 1994
	Perilla frutescens	Zhong 2001
Betalaines	*Beta vulgaris*	Trejo-Tapia et al. 2007
	Chenopodium rubrum	Knorr et al. 1993
Crocin	*Crocus sativus*	Chen et al. 2003
Carotenoids	*Lycopersicon esculentum*	Rhodes et al. 1991
Anthraquinones	*Cinchon. ledgeriana*	Rhodes et al. 1991
	Morinda citrifolia	Zenk 1977
Naphthoquinones	*Lithospermum erythrorhizon*	Kim and Chang 1990
Flavours		
Vanillin	*Vanilla planifolia*	Dornenburg and Knorr 1996
Garlic	*Allium sativum*	Rhodes et al. 1991
Onion	*Allium cepa*	Rhodes et al. 1991
Coffee flavour	*Coffea arabica*	Kurata et al. 1998
Cocoa flavour	*Theobromo cacao*	Rao and Ravishankar 1999
Pungent food additive		
Capsaicin	*Capsicum frutescens*	Rhodes et al. 1991
	Capsicum annuum	Johnson and Ravishankar 1996
Sweeteners		
Stevioside	*Stevia rebaudiana*	Rao and Ravishankar 1999
Glycyrrhizin	*Glycyrrhiza glabra*	Rao and Ravishankar 1999
Thaumatin	*Thaumatococcus danielli*	Rao and Ravishankar 1999

phene). Some food additives obtained from plant cell cultures are listed in Table 2.

Aromas and Fragrances

Natural aromas are a mixture of numerous compounds; more than 500 have been identified in roasted coffee beans and 200 in apples. Natural aromas are susceptible to the conservation processes of foodstuffs, such as sterilization, pasteurization, freezing, etc. Some aromas are altered by enzymatic or chemical reactions and usually disappear if stored for a long period. This is why their substitutes have been sought since the end of the 19th century. Artificial aromas used to be manufactured from coal or oil derivatives, and they used to be added in very low concentrations (10^{-6}–10^{-9}). The present trends are either to produce synthetic molecules, which are identical to natural molecules, or to use plant cell cultures [65]. Aromas from the cell cultures have an advantage of a constant composition and are independent on the season. Thus, the characteristic aromas of cocoa and coffee have been produced by cell cultures of *Tlaeobroma cacao* and *Coffea arabica*, respectively [33].

Pigments

The use of additional pigments was strongly criticized by the associations of consumers in the 1970s, because most of the colors are produced by chemical synthesis and they are unrelated to any naturally occurring material. The biotechnological methods used for producing natural food colorants consist of growing higher plant cells [65].

1. **Shikonin compounds**, such as shikonin and its derivatives acetyl and isobutyl shikonin, accumulated in roots of *Lithospermum erythrorhizon*. Because of a shortage of this plant, the mass cultivation of *Lithospermum erythrorhizon* cells to produce shikonin compounds has been successfully established [25].
2. **Anthocyanins** are the large group of water-soluble pigments responsible for many of the bright colors in flowers and fruit. They change color over the pH range due to the existence of four pH-dependent forms: at low pH they are red and at pH over six they turn blue. They are commonly used in acidic solutions in order to impart a red color to soft drinks, sugar confectionary, jams, and bakery toppings. Pure anthocyanins are priced at \$2000 kg^{-1}, but crude materials (grape pomaces and waste from juice and wine industries) are rather inexpensive [10]. Many researchers describe the production of anthocyanins using cultured cells of various plant species; most of them seem to use an anthocyanin-producing cell line as a model system for secondary product production because of their color, which allows production to be easily visualized.

3. **Crocin**, the main pigment of *Crocus sativus* stigma, is used extensively as yellow food colorant. Commercial production of saffron pigment is restricted by its high price and limited availability. As a geophyte, saffron grows slowly and propagates only by vegetative production through the formation of daughter corms. It takes 200 000 flowers and over 400 h of hand labor to produce 1 kg of saffron stigma. A plant tissue culture method offers a great potential for crocin production [8].
4. **Madders** are red colorants from *Rubia tinctorum*, a perennial plant from the coastal regions of the Mediterranean, and its roots have been used as red dyes in Western Europe. The major components in the pigment are alizarin, purpurine, and its glycoside, ruberythric acid. Pure alizarin is an orange crystal and is soluble in boiling water and in other solvents. Alizarin shows a yellow color in acidic to neutral pH and tends to be reddish with increasing pH. It is highly resistant to heat and light, which is favorable to the food industry. Through the selection of high-producing cell lines and elicitor application, yellow-pigment-producing cells of *Rubia tinctorum* were obtained [61].

2.2.2 Pharmaceuticals from Plant Cell Cultures

Higher plants are a rich source of bioactive constituents used in **pharmaceutical industry**. Some of the plant-derived natural products include drugs, such as morphine, codeine, cocaine, quinine, anti-cancer *Catharanthus* alkaloids, belladonna alkaloids, colchicines, phytostigminine, pilocarpine, reserpine, and steroids, such as diosgenin, digoxin, and digitoxin [42].

Table 3 Plant-derived pharmaceuticals of importance

Product	Use	Plant species	Cost US $ kg^{-1}
Ajmalicine	Antihypertensive	*Catharanthus roseus*	37 000
Ajmaline	Antimalarial	*Rauvolfia serpentine*	75 000
Camptothecin	Antitumour	*Camptotheca acuminata*	432 000
Codeine	Sedative	*Papaver somniferum*	17 000
Colchicine	Antitumour	*Colchium autumnale*	35 000
Ellipticine	Antitumour	*Orchrosia elliptica*	240 000
Morphine	Sedative	*Papaver somniferum*	340 000
Shikonin	Antibacterial	*Lithospermum erythrorhizon*	4500
Taxol	Anticancer	*Taxus brevifolia*	600 000
Vinblastine	Antileukemic	*Catharanthus roseus*	1 000 000
Vincristine	Antileukemic	*Catharanthus roseus*	2 000 000

Rao and Ravishankar (2002)

Plant-derived drugs represent a huge market value. According to Rao and Ravishankar (2002), worldwide, 121 clinically useful prescription drugs are derived from plants. Furthermore, 12% of drugs considered as basic and essential by the WHO are exclusively derived from flowering plants [49].

The surveys of plant medicinal usage in the USA have shown an increase from just about 3% of the population in 1991 to over 37% in 1998 [46]. Prescription drugs containing phytochemicals were valued at more than US$30 billion in 2002 in the USA [48]. 75% of the world's population relies on plants for traditional medicine. Some plant-derived pharmaceuticals are listed in Table 3.

An example of a high-value drug produced from plant cell cultures is paclitaxel, an anti-cancer drug originally extracted from the bark of 50-year-old Pacific yew trees, *Taxus brevifolia* [58].

2.2.3 Advantages and Disadvantages of Plant Cell Cultures

The advantages of plant cell cultures over the conventional production are as follows:

1. it is independent of geographical and seasonal variations and environmental factors – the synthesis of bioactive secondary metabolites runs in controlled environments and the negative biological influences that affect secondary metabolites production in nature are eliminated (microorganisms and insects);
2. it offers a defined production system which ensures the continuous supply of products, uniform quality, and yield;
3. it is possible to select cell lines with higher production of secondary metabolites;
4. it is possible to produce novel compounds that are not normally found in parent plant;
5. it allows the efficient downstream production;
6. plant cell can perform stereo- and regio-specific biotransformations for the production of novel compounds from cheap precursors;
7. with automatization of cell growth control and regulation of metabolic processes, cost price can decrease and productivity increase.

There are a number of successfully established and commercialized plant cell cultures producing a high amount of secondary metabolites (Table 4).

However, this technology is still being developed and despite the advantages, there is a variety of problems to be overcome before it can be adopted for the production of useful plant secondary metabolites.

Table 4 High yields of secondary products

Product	Plant species	Yield (% DW)	Refs.
Anthocyanins	*Perilla frutescens*	8.9	Zhong 2001
Anthraquinones	*Morinda citrifolia*	18.0	Zenk 1977
	Coleus blumei	21.4	Petersen and Simmond 2003
Sanguinarine	*Papaver somniferum*	2.5	Dicosmo and Misawa 1995
Serpentine	*Catharanthus roseus*	2.2	Moreno et al. 1995
Shikonin	*Lithospermum erythrorhizon*	13.5	Kim and Chang 1990

Obstacles for the Cell Cultures

In theory, it is anticipated that cell cultures will be suitable for industrial production of useful plant chemicals in a manner similar to that of microbial fermentation. Nevertheless, there are some significant differences between microbial and plant cell cultures that must be considered when attempting to apply plant cell cultures to the available technology.

Generally, the problems with the plant cell cultures can be classified as biological (slow growth rate, physiological heterogeneity, genetic instability, low metabolite content, product secretion) and operational (wall adhesion, light requirement, mixing, shear sensitivity, aseptic condition) [65].

Table 5 shows a comparison of some of the characteristics of plant and microbial cultures of relevance to fermentation. In particular, it serves to demonstrate some of the problems that can be encountered with plant cell

Table 5 Characteristics of microbial and plant cell relevant to fermentation

Characteristics	Plant cell	Microoorgnism
Size		
Diameter (μm)	40–200	1–10
Volume (μm^3)	$> 10^5$	1–50
Inoculum	5–10	≤ 1
Growth	aggregates	single cells
Cultivation time	2–3 weeks	2–10 days
Duplication time, hrs	15–120	0.3–6
Oxygen consumption, $O\,h^{-1}\,g^{-1}$	≤ 5	50
Water content (%)	> 90	80
Product accumulation	mostly intracellular (vacuole)	mostly extracellular (medium)
Requirements for acepticity	high	low

Dörnerburg and Knorr 1997

cultures. The large size of the plant cell contributes to its comparatively high doubling time, which thus prolongs the time required for a successful fermentation run. The vacuole is the major site of product accumulation and, since product secretion is uncommon, the high metabolite yields seen in microorganisms that secrete product cannot be expected. There is some ongoing research on membrane permeabilization of plant cells, which may serve to relieve the constraints of product inhibition by facilitation of leakage into the extracellular medium (See Sect. 4.3 "Membrane Permeabilisation") [17]. Thus, there are some considerable hurdles which need to be overcome at the biochemical level.

3 Strategies to Increase Secondary Metabolite Production

The objective of the food industry is to develop techniques to allow production of secondary products from the plant cell culture which would be less expensive than extraction of the whole plant grown under natural conditions and less expensive than the synthesis of the product. Confronted with having to increase the amount of secondary metabolites in plant cell cultures, the need for biochemical and molecular research on the secondary metabolism of plants has been frequently emphasized [12]. The research in this area could lead to the successful manipulation of secondary metabolism and could significantly increase the amounts of the compounds. It should be possible to achieve the synthesis of a wide range of compounds, such as alkaloids, flavonoids, terpenes, steroids, glycosides, etc., using plant-cell-culture technology.

The strategy for obtaining the secondary metabolites from the plant cell cultures can be represented as a multi-stage process (Fig. 1). Each link may be optimized separately or in combination with other processes or treatments.

1. The initial step of this technology includes the selection of **parent plant** according to its molecular and biochemical characteristics, particularly regarding the high contents of the desired metabolites. In theory, any part obtained from any plant species can be employed to induce callus tissue; however, successful production of callus depends upon plant species and their qualities. Dicotyledons are rather amenable for callus tissue induction as, compared to monocotyledons, the calluses of woody plants generally grow slowly. Stems, leaves, roots, flowers, seeds, and any other parts of plants are used, but younger and fresh explants are preferable explant materials.
2. Afterwards, the **selection of cell line** becomes important. It includes the establishment of **high-producing** and **fast-growing** in-vitro cultures. It is possible to identify cell lines that can produce amounts of compounds equal or even higher than in the plant from which they derive [11]. More-

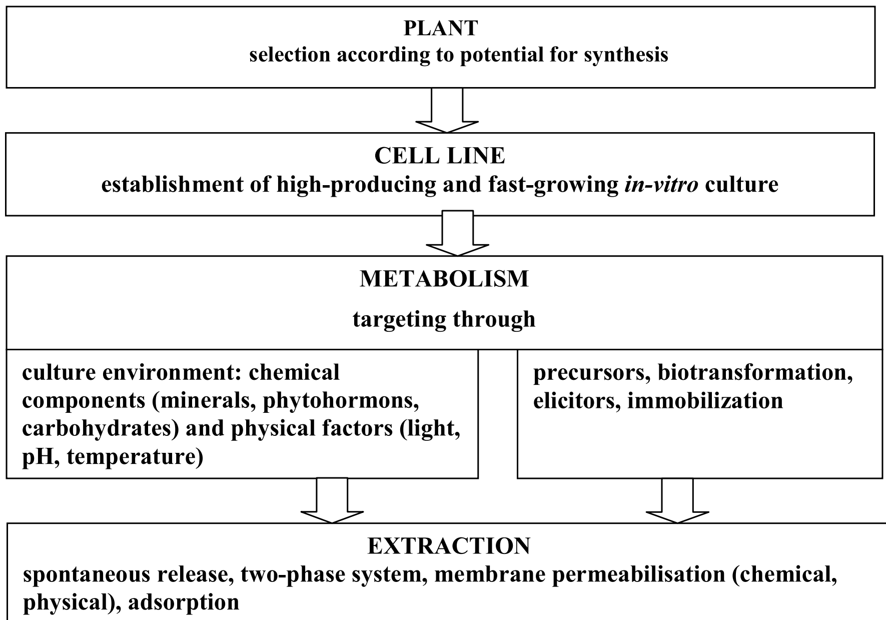

Fig. 1 Scheme of plant secondary metabolite production in cell cultures

over, increase of metabolite levels using **mutants** is possible, and selection of suitable analogues for this purpose could be an important factor in order to produce a variety of products. Maximization of the production and accumulation of secondary metabolites by plant cultured cells requires production of new genotypes through **protoplast fusion** or **genetic engineering**; however, this presupposes the identification of the genes encoding key enzymes of secondary metabolic pathways and their expression. Use of mutagens increases the variability which already exists in living cells. Furthermore, new molecules, which have previously not been found in plants, can be produced by cell cultures.

3. **Targeting metabolism**. A number of chemical and physical factors strongly affect the production of secondary metabolites. The expression of many secondary metabolite pathways is easily altered by external factors such as environmental conditions (chemical and physical) and special treatments (precursors, elicitors).
   (a) Plant cell **culture medium** includes inorganic components, organics, and phytohormones. Changing of medium components (concentration, proportion, and form) is a very powerful way of enhancing the culture efficiency of plant cell cultures. Thus, high auxin level stimulates cell growth, but often negatively influences secondary metabolite production [63].
   Physical conditions, such as light, temperature, and medium pH, have

also been examined for their effect upon secondary metabolite accumulation in many types of cultures.

(b) **Special treatments** include feeding with precursors, application of elicitors, biotransformation, and immobilization.

The concept of feeding with **precursors** is based upon the idea that supply with compounds which are intermediate or at the beginning of biosynthetic route gives a good chance of increasing the yield of the final product.

The production of the desired metabolites is often limited by the lack of particular precursors; **biotransformation** using an exogenous supply of biosynthetic precursors may improve the accumulation of compounds. Biotransformation is a process through which the functional groups of compounds are modified by cell cultures to chemically different product [24]. Plant cells can transform natural or artificial compounds introduced into the cultures through a variety of reactions, such as hydrogenation, dehydrogenation, isomerisation, glycosylation, and hydroxylation.

Plants and plant cells in vitro show physiological and morphological responses to microbial, physical, or chemical factors, which are known as **elicitors**. Since the secondary metabolites protect plants from the environmental changes, the way to induce their synthesis is to apply unfavorable factors, i.e., simulate pathogen attack, herbivores, heavy metals, etc. Elicitation is a process of induced or enhanced synthesis of secondary metabolites by the plants to ensure their survival, persistence, and competitiveness. Biotic and abiotic elicitors are used to stimulate secondary metabolite product formation in plant cell cultures.

Cell **immobilization** can result in much higher concentrations of the plant cells because of the certain grade of cell specialization while hundreds or thousands of them are immobilized in one aggregate. Most of the research in this area has utilized hydrocolloidal gels, such as alginate and carrageenan, which were used to entrap the plant cells into a gel matrix while allowing easy access of substrates.

4. Perhaps the most efficient bioprocessing concepts for the production of phytochemicals result in **spontaneous release into medium** where they can be more easily recovered. One of the most fruitful areas of research for the production of lower-value products may be the study of methods to induce product leakage from cells that normally accumulate the product. A study of the intracellular compartments in which synthesis of chemicals occurs may also be necessary, since the substances are transported to the vacuole for accumulation. Thus, an alternative consideration is prevention of vacuolar accumulation and, consequently, enhancement of substances released into the medium.

Plants often have sites of synthesis and storage of the secondary metabolites in separate cells or organs. Inhibition of metabolic enzymes as well as inhibition of membrane transport can be eliminated by the accumulation of synthesized products in a second phase introduced into the aqueous medium or **two-stage** system.

3.1 Selection According to Molecular and Biochemical Characteristics

Screening and selection of plant species and cultivars rich in useful metabolites are the strategies for enhancement of secondary metabolite content in plant cell cultures. Plants with high contents of the desired products should be used for callus induction to obtain high-producing cell lines.

3.1.1 Plant Genotype and Cultivar

Genetic potential is one of the most important factors influencing the biochemical status of plants and plant cell cultures. Environmental and physiological factors may modify the expression of genes participating in phytochemical synthesis, but the genetic background is the major determinant. The diversity of genetic potential toward secondary metabolite profile can be shown on the example of the plants from the family *Brassicaceae*. It comprises roughly 350 genera and 3500 species and includes vegetables, ornamental species, and oil cultures. The glucosinolate profiles and levels vary extensively and are usually represented by six to ten individual glucosinolates (Table 6).

The comparison of the profiles of main glucosinolates in *Brassica* vegetables shows that the general content and distribution of sub-classes of glucosinolates is unique for each species. For example, the main glucosinolate in *Brassica juncea* seed is dominated by progoitrin, *Brassica oleracea* seeds

Table 6 Distribution profile of glucosinolates in *Brassicaceae*

Species	Glucosinolates	% of total glucosinolates		
	(mg 100 g^{-1} FW)	Aliphatic	Aromatic	Indole
Brassica rapa L. var. *rapa*	21–340	42	30	18
Brassica rapa L. var. *rapa teltoviensis*	790–890	29	46	25
Brassica oleracea L. var. *capitata alba*	26–275	47	33	20
Brassica oleracea L. var. *italica*	40–340	47	9	44
Brassica oleracea L. var. *botrytis*	14–208	47	14	39

Schreiner M, 2005

contain mainly gluconapoleiferin, whereas *Brassica napus* seeds contain gluconapoleiferin, gluconapin, and glucobrassicanapin [55].

3.1.2
Obtaining of Fast-Growing and High-Productive Cell Lines

Plant cell cultures are sometimes characterized with inherent genetic and epigenetic instability. Variability between cells often leads to gradual reduction in productivity and can be attributed to genetic changes by mutation in the culture, or epigenetic changes caused by physiological conditions. These undesirable changes can be reversed by the screening for a desired cell population from the heterogeneous ones, typically presented in plant cell cultures [17].

Cell cloning methods provide a promising way of selecting cell lines yielding increased levels of product. The physiological characteristics of individual plant cells are not always uniform. For example, pigment-producing cell aggregates typically consist of producing cells and non-producing cells. The heterogeneity in the biochemical activity existing within a population of cells has been exploited to obtain highly productive cell lines. This is similar to monocolony isolation of bacteria.

For example, by cell cloning using cell aggregates of *Coptis japonica*, Matsubara et al. (1989) obtained strain that grew faster and produced a higher amount of berberin. During the three weeks of cultivation, the selected cell line of *Coptis japonica* produced sixfold higher amount of berberine, particularly 1.2 $g\,l^{-1}$, as primary callus. The selected strain was very stable, producing a high level of berberin even after 27 generations. As shown in Table 7, a strain of *Euphorbia milli* accumulated about sevenfold the level of anthocyanins produced by the parent culture after 24 selections [40].

In cultures of *Lithospermum erythrorhizon*, extensive screening of a number of clones resulted in a 13-fold to 20-fold increase in shikonin produc-

Table 7 Influence of cell cloning on productivity of plant cell cultures

Products	Plants	Factors (increase of production)	Refs.
Anthocyanins	*Vitis vinifera*	2.3–4	Curtin et al. 2003
	Euphorbia milli	7	Mulabagal and Tsay 2004
Berberine	*Coptis japonica*	2–6	Matsubara et al. 1989
Biotin	*Lavendula vera*	9–10	Misawa 1985
Shikonin	*Lithospermum erythrorhizon*	7–20	Kim and Chang 1990
Ubiquinone-10	*Nicotiana tabacum*	15–180	Dicosmo and Misawa 1995

tion [25]. *Lavendula vera* cells grown in the light accumulated a high level of free biotin [38]. To select a high-producing cell line, pimelic acid, a precursor of biotin, was used. The level of biotin accumulated by a selected cell line was 0.9 $\mu g\, l^{-1}$, which was ten times the amount found in the leaves.

Japan Tobacco Inc. isolated a number of strains of *Nicotiana tabacum* producing high levels of ubiquinone-10 [11]. After the 13th recloning, a strain was selected from approximately 4000 cell clones tested. When *Nicotiana tabacum* BY-2, a parent strain used for the cloning, was isolated, the titer for ubiquinone-10 was only 0.36 $mg\, g^{-1}$ DW; therefore, the level was increased by selection until 5.2 $mg\, g^{-1}$, which corresponded to 180 times the amount produced by the parent plant.

Cell cloning is a very useful technique to increase the level of secondary metabolites. However, it is not obvious why cultures contain both high- and low-yielding cells. Kim and Chang (1990) indicated that the lack of specific enzymes represents the most important reaction for the inability of plant cell cultures to produce secondary metabolites.

Protoplast fusion. Maximization of the production and accumulation of secondary metabolites by plant cultured cells requires production of new genotypes through protoplast fusion, but this presupposes the identification of the genes encoding key enzymes of secondary metabolic pathways and their expression once introduced in the plant cells. This suggests that use of mutagens to increase the variability already exists in living cells.

Since most cultured cells occur as aggregates, selection of high-producing but aggregated cell lines of *Lithospermum erythrorhizon* is not effective and is labor intensive. The Mitsui group prepared protoplasts from the cultured cells with appropriate enzymes and selected high shikonin-compound-producing protoplasts using a cell sorter [46]. The selected protoplasts were generated to cell lines and cultivated in suspension. From 48 cell lines, they obtained a cell line having 1.8-fold the productivity of the parent line. The cell line showed stable production of shikonin compounds.

Sakamoto et al. (1994) reported about the visual selection of *Euphorbia millii*. This procedure was repeated 28 times and one of the cells was determined to produce 1.32% DW anthocyanins in the cells. The levels of the pigments in flowers and leaves were 0.28% and less than 0.01%, respectively.

Use of mutagens. Mutation strategies have been employed in order to obtain overproducing cell lines [46]. In this method, a large population of cells is exposed to a toxic (or cytotoxic) inhibitor or environmental stress, and only cells that are able to resist the selection procedures will grow. For example, *p*-fluorophenylalanine, an analogue of phenylalanine, was extensively used to select high-yielding cell lines with respect to phenolics. Increased capsaicin in *p*-fluorophenylalanine cell lines of *Capsicum annuum* was reported.

In the fermentation industry, induction of genetic mutant strains of microorganisms is used extensively to produce a variety of products, includ-

ing amino acids, nucleotides, and antibiotics. However, mutagenesis has limited applicability to plant cell cultures, because of their diploid genetics: the chance of obtaining a double mutation in a target gene is less than 10^{-6} [63]. Although, in principle, haploid plants can be produced from another cultures, in practice, haploid cell cultures tend to revert to the diploid state. This makes the chance of isolating over-producing cells from mutagen treatment of haploid cells very low. Furthermore, biosynthetic pathways of many secondary metabolites and their regulation mechanisms in higher plants are not always precisely understood; therefore, it is also difficult to know what kind of mutants should be induced in order to increase product synthesis.

However, Berlin et al. (1981) induced *p*-fluorophenylalanine-resistant cell lines of tobacco cell cultures and found that, out of 31 resistant cell lines, five lines of *Nicotiana tabacum* and five lines of *Nicotiana glauca* accumulated higher levels of phenolics. The resistant strain of *Nicotiana tabacum* produced six to ten times higher levels of cinnamoyl putrescine than that of the parent strain.

Generally, plant cells accumulate their metabolites intracellularly, which is disadvantageous in commercial production because the amount of released compounds is usually low. Induction of a mutant having altered permeability could be important. *Thuja occidentalis* excreted monoterpenoids, but the levels in the medium were only 5% of those in the plant. However, *Macleya microcarpa* cells excreted nearly all the alkaloids detectable in the culture flask [65]. After three days of cultivation of *Tinospora rumphii* cells, 0.57 mg (5.3% DW) of isoquinoline alkaloids have been found in cells and 0.50 mg in culture filtrate, and, after seven days, 0.50 mg of the alkaloids were accumulated in the cells and 1.02 mg in filtrate.

3.2
Targeting Metabolism

A number of chemical and physical factors, such as media components' pH, temperature, and light, affect production of secondary metabolites in plant cell cultures [40]. Manipulation of growing conditions is one of the most fundamental approaches for optimization of culture productivity.

3.2.1
Culture Environment

3.2.1.1
Chemical Parameters

Plant cell culture media include inorganic components (macroelements and microelements), organics (sacharose), and phytohormones.

To cultivate the callus and cells in suspension, various kinds of media (inorganic salt media) have been designed. Agar or its substitutes are added to the media to prepare solid medium.

Many media have been developed and modified and nutrient compositions of some typical media are described in Table 8.

One of the most commonly used media for plant tissue cultures is that developed by Murashige and Skoog (MS) [14]. The significant feature of the MS medium is its very high concentration of nitrate (NO_3^-), potassium (K^+), and ammonia (NH_4^+) (see Table 8). Many researchers are also using the B5 medium established by Gamborg. The levels of inorganic nutrients in the B5 medium are lower than in the MS medium.

The effects of the medium employed in various processes have been reported, e.g., effects of calcium and phosphate in the cultivation of *Coffea arabica* suspended cells, phosphate effects on sapogenin steroid production in suspension cultures of *Agave amanuensis* [56], and phosphate and sucrose in nicotine production by *Nicotiana tabacum* cell cultures [35].

Inorganic Components

Zenk et al. (1978) tested various basal media for the production of serpentine, indole alkaloids by *Catharanthus roseus* suspension, as summarized in Table 9. The results indicate that the amount of serpentine depends on the composition of the basal medium used. Among them, Murashige–Skoog's (MS) formulation was recognized to be the most suitable for the production of this particular alkaloid.

Nitrogen. Plant tissue culture media, such as MS, LS, or B5, have both nitrate (NO_3^-) and ammonium (NH_4^+) as sources of nitrogen. For example, nitrogen source is very important for plant suspension cultures of *Holarrhena antidysenterica* for accumulation of alkaloids [65], in cell suspensions of *Vitis vinifera* for anthocyanin formation, and in shikonin production by *Lithospermum erythrorhizon* cell cultures [25].

The ratio of NH_4^+/NO_3^- and overall levels of total nitrogen have been shown to markedly affect the production of secondary plant products. The reduced levels of NH_4^+ and increased levels of NO_3^- promoted the production of shikonin and betacyanins, whereas higher ratios of NH_4^+ to NO_3^- increased the production of berberine and ubiquinone [14]. Reduced levels of total nitrogen improved the production of capsaicin in *Capsicum frutescens*, anthraquinones in *Morinda citrifolia*, and anthocyanins in *Vitis* species [54, 64].

Phosphate concentration in the medium has a great effect on the production of secondary metabolites in plant cell cultures. Higher level of phosphate enhanced the cell growth, whereas it had a negative influence on secondary product accumulation.

Medium limited in phosphate either induces or stimulates both the product and the levels of key enzymes leading to the product. Reduced phosphate

Table 8 Media for plant cell cultures ($mg\,l^{-1}$)

Components	Murashige–Skoog	White	Gamborg	Nitsch	Schenk–Hildebrandt	Knop
$(NH_4)_2SO_4$	–	–	134	–	–	–
$MgSO_4 \times 7H_2O$	370	720	500	250	400	250
Na_2SO_4	–	200	–	–	–	–
KCl	–	65	–	1500	–	–
$CaCl_2 \times 2H_2O$	440	–	150	25	200	–
$NaNO_3$	–	–	–	–	–	–
KNO_3	1900	80	3000	2000	2500	250
$Ca(NO_3)_2 \times 4H_2O$	–	300	–	–	–	1000
NH_4NO_3	1650	–	–	–	–	–
$NaH_2PO_4 \times H_2O$	–	16.5	150	250	–	–
$NH_4H_2PO_4$	–	–	–	–	300	–
KH_2PO_4	170	–	–	–	–	250
$FeSO_4 \times 7H_2$	27.8	–	27.8	–	15	–
Na_2EDTA	37.3	–	37.3	–	20	–
$MnSO_4 \times 4H_2O$	22.3	7	10	3	10	–
$ZnSO_4 \times 7H_2O$	8.6	3	2	0.5	0.1	–
$CuSO_4 \times 5H_2O$	0.025	–	0.025	0.025	0.2	–
H_2SO_4	–	–	–	0.5	–	–
$Fe_2(SO_4)_3$	–	2.5	–	–	–	–
$NiCl_2 \times 6H_2O$	–	–	–	–	–	–
$CoCl_2 \times 6H_2O$	0.025	–	0.025	–	0.1	–
$AlCl_3$	–	–	–	–	–	–
$FeCl_3 \times 6H_2O$	–	–	–	–	–	–
$FeC_6O_5H_7 \times 5H_2O$	–	–	–	10	–	–
KI	0.83	0.75	0.75	0.5	1.0	–
H_3BO_3	6.2	1.5	3	0.5	5	–
$Na_2M_0O_4 \times 2H_2O$	0.25	–	0.25	0.25	0.1	–
Sucrose	30 000	20 000	20 000	50 000–	30 000	–
Glucose	–	–		36 000	–	–
Myo-inositol	100	–	100	–	1000	–
Nicotinic acid	0.5	0.5	1.0	–	0.5	–
Pyridoxine HCl	0.5	0.1	1.0	–	0.5	–
Thiamine HCl	0.1–1	0.1	10	1	5	–
Ca–pantothenate	–	1	–	–	–	–
Biotin	–	–	–	–	–	–
Glycine	2	3	–	–	–	–
Cysteine HCl	–	1	–	10	–	–
Folic acid	–	–	–	–	–	–
Glutamine	–	–	–	–	–	–

Gamborg and Phillips, 1995

Table 9 Effects of different media on growth and serpentine production in cell suspension cultures of *Catharanthus roseus*

Basal medium	Cell yield g DW l^{-1}	Serpentine mg l^{-1}	Serpentine, % DW
Blaydes	7.6	4.4	0.06
Gamborg – B5; + 2,4-D (1 mg l^{-1})	4.6	0.5	0.01
Gamborg + 2,4 D (2 mg l^{-1})	5.2	0	0
Gamborg + NAA (1.86 mg l^{-1})	7.6	1.2	0.02
Gamborg	5.1	0	0
Heller + IAA (0.175 mg l^{-1}); BA (1.13 mg l^{-1})	5.4	6.6	0.12
Linsmaier and Skoog	9.3	0	0
Murashige and Skoog	8.9	10.4	0.12
Nitsch and Nitsch	2.3	2.0	0.09
Velicky and Martin	5.0	0	0
White	0.8	0	0

Zenk, 1978

levels induced the production of ajmalicine and phenolics in *Catharanthus roseus* and nicotin in *Nicotiana tabacum* [35]. In contrast, increased phosphate was shown to stimulate synthesis of digitoxin in *Digitalis purpurea* and betacyanin in *Chenopodium rubrum* [5].

Potassium ion (K^+) serves as a major contributor to osmotic potential, a specific requirement for protein synthesis, and an activator for particular enzyme systems [65]. Higher K^+ concentration caused slower cell growth. More of soluble sugar was stored within the cells under K^+ deficiency.

Microelements are required in trace amounts for plant growth and development, and have many diverse roles [21]. Manganese, iodine, copper, cobalt, boron, molybdenum, iron, and zinc usually comprise the microelements, although other elements, such as nickel and aluminum, are frequently found in some formulations. Iron is usually added as iron sulphate, although iron citrate can also be used. Ethylenediaminetetraacetic acid (EDTA) is used in conjunction with the iron sulphate. EDTA complexes allow slow and continuous release of iron into the medium. Uncomplexed iron can precipitate out of the medium as ferric oxide.

Plant cell cultures are usually grown heterotrophically using simple sugars as **carbon source** and inorganic supply of other nutrients. The level of sucrose affected the productivity of secondary metabolites in cultures [35]. **Sucrose or glucose** at 2 to 4% are suitable carbon sources, which are added to the basal medium. Fructose, maltose, and other sugars also support the growth of various plant cells. The choice of the most suitable carbon source and its optimal concentration depend on the plant species and products.

In many cases, the concentration of the carbon source affects cell growth and yield of secondary metabolites. Sucrose concentrations of 2.5% and 7.5%

in *Coleus blumei* media resulted in rosmarinic acid yields of 0.8 and 3.3 $g\,l^{-1}$, respectively [38].

Carbon source was also found to be a significant factor in plant cell metabolism, which affected the accumulation of alkaloids by suspension cultures of *Holarrhena antidysenterica* and of shikonin by *Lithospermum erythrorhizon* cell cultures [65]. For indole alkaloid accumulation in cell culture as of *Catharanthus roseus*, 8% (w/v) sucrose was found to be optimal in the tested concentration range of 4–12% [27]. Yields of benzophenanthridine alkaloids from suspension cultures of *Eschscholtzia californica* were increased tenfold to 150 $mg\,l^{-1}$ by increasing the sucrose concentration to 8% [5]. The osmotic stress created by sucrose alone and with other osmotic agents was found to regulate anthocyanin production in *Vitis vinifera* [13] and nicotine accumulation in suspension cultures of *Nicotiana tabacum* [35]. However, higher concentrations of sucrose at 5% reduced the anthocyanin production in cell suspension cultures of *Aralia cordata*, where 3% favored the anthocyanin accumulation [54].

Vitamins, such as thiamine (vitamin B1) and myo-inositol, are considered essential for the culture of plant cells in vitro. However, other vitamins are often added to plant cell culture media. Thus, MS medium includes myo-inositol, nicotinic acid, pyridoxine HCl, and thiamine HCl [14].

Amino acids are also commonly included in the organic supplement. The most frequent are glycine (arginine, asparagine, aspartic acid, alanine, glutamic acid, glutamine, and proline are also used), but in many cases their inclusion is not essential [21]. Amino acids provide a source of NO_3^- and, like NH_4^+, uptake causes acidification of the medium. Other supplements include casamino acid, peptone, yeast extracts, malt extracts, and coconut milk. Coconut milk is also known as a supplier of growth regulators.

Gelling agents. Media for plant cell culture in vitro can be used in either liquid or solid forms, depending on the type of culture being grown. For any culture types that require to be grown on the surface of the medium, it must be solidified or gelled. Agar, produced from seaweed, is the most common type of gelling agent, and is ideal for routine applications. However, because it is a natural product, the agar quality can vary from supplier to supplier and from batch to batch.

Growth regulators concentration is often a crucial factor in secondary product accumulation [63]. Phytohormones or growth regulators are required to induce callus tissues and to promote the growth of many cell lines. Since each plant species requires different kinds and levels of phytohormones for callus induction, its growth, and metabolites production, it is important to select the most appropriate growth regulators and to determine their optimal concentrations.

Auxins and cytokinins have shown the most remarkable effects on growth and productivity of plant metabolites. The type and concentration of auxin or

cytokinin or the auxin/cytokinin ratio dramatically alter both the growth and the product formation in cultured plant cells.

1. **Auxins** are generally used in plant cell culture at a concentration range between 0.1 to 50 μM. An increase of auxin levels in the medium stimulates dedifferentiation of the cells, cell division, and callus formation and growth. They are reported to diminish the level of secondary metabolites. That is the reason why auxins are commonly added to the medium for callus induction. However, for production of metabolites, they are added at a low concentration. As an auxin, 2,4-D or NAA is frequently used. The growth regulator 2,4-D has been shown to inhibit the production of secondary metabolites in a large number of cases. The elimination of 2,4-D or its replacement by NAA or IAA enhanced the production of anthocyanins in suspension of *Daucus carota*, nicotine in *Nicotiana tabacum* [35], shikonin in *Lithospermum erythrorhizon*, and of anthraquinones in *Morinda citrifolia* [58, 64]. However, stimulation by 2,4-D has been observed in carotenoid biosynthesis, in suspensions of *Daucus carota* [35], and in anthocyanin production in *Oxalis linearis* [37].
2. **Cytokinins** are used in plant cell culture at a concentration range of 0.1 to 10 μM. They promote cell division and modulate callus initiation and growth. Cytokinins have different effects depending on the type of metabolite and species concerned. Kinetin stimulated the production of anthocyanin in *Haplopappus gracilus*, but it inhibited the formation of anthocyanins in *Populus* cell cultures [46].
3. **Gibberellins** are represented by over 90 forms, but gibberelic acid is the most commonly used for plant cell cultures. Dicosmo and Misawa (1995) reported that the growth of *Taxus cuspidata* callus was significantly promoted by the addition of gibberellic acid into the solid medium. However, gibberellic acid suppresses production of anthocyanins in a number of cultures [54].

3.2.1.2 Physical Factors

Physical factors, such as light, temperature, and medium pH, effect secondary metabolite accumulation in many types of cultures.

Temperature. A temperature range of 17–25 °C is normally used for the induction of callus tissues and growth of cultured cell suspension [46]. However, each plant species as well as its cell culture may favor a different temperature. When the temperature was maintained at 19 °C, biotransformation of digitoxin to digoxin was favored, whereas 32 °C was optimal for the purpureaglycoside-A formation in *Digitalis lanata* cell cultures. A higher yield of ubiquinone in *Nicotiana tabacum* cell cultures has been observed at 32 °C when compared to 24 °C. Courtois and Guren (1980) reported a 12-fold

higher production of alkaloids in cell cultures of *Catharanthus roseus* at 16 °C as compared to the normal 27 °C.

Light. The spectral quality, intensity, and period of light irradiation may affect plant cell cultures. Sakamoto et al. (1994) demonstrated the stimulatory effect of light irradiation on the formation of compounds such as anthocyanins, vindoline, catharanthine, and caffeine in cell suspension cultures. Thus, the accumulation of anthocyanin was strongly stimulated by light in cell cultures of *Daucus carota* and *Vitis vinifera* [10]. Mulder-Krieger et al. (1988) found that illumination affected the composition of sesquiterpenes in callus cultures of *Marticaria chamomilla*. Illumination of *Coffea arabica* cell suspensions enhanced caffeine biosynthesis by a factor of ten [33].

Medium pH. The medium pH is usually adjusted between five and six, and pH extremes are avoided. In medium, hydrogen ion concentration changes during the culture growth. The medium pH decreases during ammonia assimilation and increases during nitrate uptake [46].

Osmotic pressure. Accumulation of anthocyanins was enhanced by a high osmotic potential in *Vitis vinifera* cell suspension cultures [14]. Addition of sucrose or mannitol in the medium enhanced the osmotic pressure and the level of anthocyanins accumulated in *Vitis vinifera* culture was increased to 1.5 times and reached 55 μg cell^{-1}.

3.2.2 Treatments

3.2.2.1 Precursor Feeding

Precursor feeding has been an obvious and popular approach to increasing secondary metabolite production in plant cell cultures. The concept is based upon the idea that any compound, which is an intermediate, in or at the beginning of a secondary metabolite biosynthetic route, stands a good chance of increasing the yield of the final product. Attempts to induce or increase the production of plant secondary metabolites, by supplying precursor or intermediate compounds, have been effective in many cases. Feeding ferulic acid to cultures of *Vanilla planifolia* resulted in an increase in vanillin accumulation [53]. Similarly, anthocyanin synthesis in *Daucus carota* was restored by the addition of a dihydroquarcetin (naringen). Furthermore, addition of geraniol to *Catharanthus roseus* cell cultures led to accumulation of nerol and citronellol [34]. Fontanel and Tabata (1987) reported that an addition of 500 mM tropic acid to the medium of *Scopolia japonica* increased the amount of alkaloids by up to 14 times.

In many cases, amino acids have been used as inexpensive precursors of secondary metabolites. Amino acids have been added to cell suspension culture media for production of tropane alkaloids, indole alkaloids, etc. Phenyl-

alanine is one of the biosynthetic precursors of rosmarinic acid [44], and its addition to *Salvia officialis* suspension cultures stimulated the production of rosmarinic acid and it shortened the production time as well.

Use of the distant precursor, phenylalanine, and a near precursor, such as isocapric acid, resulted in enhanced capsaicin content in cell cultures of *Capsicum frutescens* [24]. The addition of leucine led to enhancement of volatile monoterpenes in cultures of *Perilla frutescens* [34].

3.2.2.2 Elicitation

Secondary metabolites represent the adaptations of plants to environmental stress, or they may serve as defensive, protective, or offensive chemicals against microorganisms, insects, and higher herbivorous predators. When infected by pathogenic microorganism, plants respond with rapid activation of various spatially and temporally regulated defense reactions. These responses include oxidative cross-linking of cell wall proteins, production of phytoalexins, hydrolytic enzymes, and incrustation of cell wall proteins with phenolics, and, finally, hypersensitive death of plant cell. Microbial invasion of plants induce the synthesis of anti-microbial secondary metabolites in the same way as stress factors, such as UV-irradiation, osmotic shock, fatty acids, inorganic salts, and heavy metal ions, induce the synthesis of secondary metabolites in plants. Plant cells in vitro show physiological and morphological responses to microbial, physical, or chemical factors, which are known as elicitors.

Elicitor may be defined as a substance which, when introduced in small concentrations to a living cell system, initiates or improves the biosynthesis of specific compounds. Elicitation is the induced or enhanced biosynthesis of metabolites due to the addition of trace amounts of elicitors [42].

Production of many valuable secondary metabolites using various elicitors was reported [40]. In many cases elicitors used in cell culture are methyl jasmonate, salicylic acid, chitosan, and heavy metals.

Classification of Elicitors

The type and structure of elicitors varies greatly. Depending of their origin, they are classified as biotic or abiotic.

Biotic stress can be caused by bacterial, viral, or fungal attack, as well as by **biotic elicitors**. They include:

- Enzymes, cell wall fragments of microorganisms, polysaccharides derived from microorganisms (chitin or glucans), and glycoproteins;
- phytochemicals produced by plants in response to physical damage, fungi or bacteria attack, polysaccharides derived from plant cell walls (pectin

or cellulose), fragments of pectin formed by action of microorganisms on plant cell wall [66];
- chitosan, glucans, salicylic acid, methyl jasmonate (formed by the action of plant on microbial cell walls) [17].

Abiotic elicitors are the substances of non-biological origin. The causes of the abiotic stress can be of chemical or physical nature; among them are:

- Chemicals such as inorganic salts, heavy metals, some chemicals that disturb membrane integrity,
- physical factors like mechanical wounding, ultraviolet irradiation, high salinity, high or low osmolarity, extreme temperature (freezing, thawing), high pressure.

Elicitation and Production of Secondary Metabolite by Plant Cell Cultures

Table 10 illustrates different plant species producing various secondary metabolites on elicitation.

For example, sodium orthovanadate and vanadyl sulphate induced the accumulation of isoflavone glucosides in *Vigna angularis* cultures and indole alkaloid accumulation in *Catharanthus roseus* cultures, respectively ([42].

Most of the strategies employing fungal elicitors utilize undefined mixtures, such as autoclaved fungal homogenate or fungal culture filtrates. With the consideration of several parameters, such as elicitor specificity and concentration, duration of contact, and quality of cell wall materials, substantial enhancement of product accumulation has been reported.

Microbial infections of intact plants often elicit the synthesis of specific secondary metabolites. The best understood systems are those of fungal pathogens, in which case the regulatory molecules have been identified as glucan polymers, glycoproteins, and low molecular weight organic acids (Table 11).

Table 10 Abiotic elicitors and production of secondary metabolites

Abiotic elicitor	Product	Cell culture	Refs.
High electric field pulses	Amaranthin	*Chenopodium ruburum*	Knorr et al. 1993
High hydrostatic pressure	Amaranthin	*Chenopodium ruburum*	Knorr et al. 1993
	Anthraquinones	*Morinda citrifolia*	Döernenburg and Knorr 1997
Metal ions: Cu^{2+}, Cd^{2+}, Al^{3+}, Zn^{2+}, Cu^{2+}, Va^{2+}	Isoflavonoids	*Vigna algularis*	Namdeo 2007
Ultrasound	Saponins	*Panax ginseng*	Hu et al. 2003
	Anthraquinones	*Morinda citrifolia*	Dörnerburg and Knorr 1997

Table 11 Biotic elicitors and production of secondary metabolites

Biotic elicitor	Product	Cell culture	Refs.
Agaropectin	Shikonin	*Lithospermum erythrorhizon*	Namdeo 2007
Chitosan	Anthraquinones	*Rubia tinctorum*	Vasconsuelo et al. 2004
	Antraquinones	*Morinda citrifolia*	Dornenburg and Knorr 1997
Fungal elicitor	Acridone expoxide	*Ruta gravelones*	Namdeo 2007
	Antraquinones	*Morinda citrifolia*	Dornenburg and Knorr 1997
	Codeine, morphine	*Papaver somniferum*	Dicosmo and Misawa 1995
	Taxol	*Taxus* sp.	Wang et al. 2003
	Rosmarinic acid	*Coleus blumei*	Szabo et al. 1999
	Sanquinarine	*Papaver somniferum*	Dicosmo and Misawa 1995
Jasmonic acid	Anthocyans	*Viti vinifera*	Curtin et al. 2003
Methyl jasmonate	Capsidiol, nicotine	*Nicotiana tabacum*	Namdeo 2007
	Rosmarinic acid	*Coleus blumei*	Szabo et al. 1999
	Taxol	*Taxus* sp.	Tabata 2006
Salicylic acid	Azadirachtin	*Azadirachta indica*	Namdeo 2007
Yeast elicitor	Antraquinones	*Morinda citrifolia*	Dornenburg and Knorr 1997
	Rosmarinic acid	*Coleus blumei*	Petersen and Simmond 2003

Dicosmo and Misawa (1995) described that a cell line of *Papaver somniferm* synthesized and accumulated sanguinarine, a quaternary benzophenanthridine alkaloid when exposed to a homogenate of the fungas *Botrytis*. A portion of the sanguinarine was released into the culture medium. Treatment of *Papaver somniferum* cell suspensions with a homogenate of *Botrytis mycelium* resulted in accumulation of 3% DW of sanguinarine.

The enhancement of production of secondary metabolites after elicitation is compared with that of the control, as shown in Table 12.

In addition, the content of rosmarinic acid in cultured cells of *Lithospermum erythrorhizon* increased after addition of yeast extract: a maximum was reached in 24 h [44]. When the plant cells were treated with yeast extract, on the 6th day of the cultivation, the level of rosmarinic acid increased 2.5 times.

However, the use of microbial elicitors may not be economical since an elicitor-producing microorganism should be cultivated separately from culti-

Table 12 Comparison of production of secondary metabolite after elicitation

Cells culture	Elicitor	Products	Product concentration			Refs.
			Control	Elicitation		
Catharanthus roseus	*Pythium* sp.	Ajmalicine	0	400	$\mu g\,l^{-1}$	Asada and Shuler 1989
Morinda citrifolia	Chitin	Anthra-quinones	3	7	$\mu g\,g^{-1}$ FW	Dornenburg and Knorr 1997
Rubia tinctorum	Chitosan Sp-cAMPS Forskolin	Anthra-quinone	58	128 69.3 56.9	$\mu mol\,g^{-1}$ FW	Vasconsuelo et al. 2004
Papaver bracteatum	Dendryphion	Sanguin-arine	50	450	$\mu g\,g^{-1}$ FW	Dicosmo and Misawa 1995
Vitis vinifera	Jasmonic acid	Antho-cyanins	9.2	20.7	$mg\,g^{-1}$ DW	Curtin et al. 2003

vation of plant cells. The fermentation cost for an elicitor-producing microorganism is not always low.

Characteristics of Elicitors

Several parameters, such as elicitor concentration and selectivity, duration of elicitor exposure, age of culture, cell line, growth regulation, nutrient composition, quality of cell wall materials, and substantial enhancement of product accumulation have been reported.

Elicitor concentration. Namdeo (2007) reported higher accumulation of ajmalicine in *Catharanthus* roseus cultures when treated with different concentrations of elicitor extracts of *Trichoderma viride*, *Aspergillum niger*, and *Fusarium moniliforme*. Ajmalicine accumulation was higher in cells elicited with higher concentration (5.0%) of elicitor extracts compared to lower concentration (0.5%). However, further increasing of the concentration up to 10.0% adversely affected the accumulation of ajmalicine. High dosage of elicitor has been reported to induce hypersensitive response leading to cell death, whereas an optimum level was required for induction.

Duration of elicitor exposure. Cells of *Catharanthus* roseus exposed with elicitor extracts of *Trichoderma viride* for 24, 48, 72, and 96 h were examined. About threefold increase in ajmalicine production by *Catharanthus* roseus cells elicited with extracts of *Trichoderma viride* for 48 h [2]. However, further increasing exposure time resulted in decrease in ajmalicine content.

Age of culture. *Catharanthus* roseus cells of 20-day-old cultures showed higher yields of ajmalicine on elicitation. Highest ajmalicine (166 μg^{-1} DW)

was accumulated in 20-day-old cells elicited with extracts of *Trichoderma viride* [42].

Apart from these characteristics, the efficiency of elicitation also depends on elicitor specificity, cell line or clones of microbial elicitor used, presence of growth regulators, and the environmental conditions.

3.2.3 Immobilization and Application of Immobilized Cells

Immobilization has been characterized as a technique that confines a catalytically active enzyme or cell and prevents its entry into the mobile phase, which carries the substrate and product [30]. Immobilization of plant cells has distinct advantages as biocatalyst over the immobilized enzyme system. Immobilizing cells in a gel, which is permeable to the molecules of the nutrient medium or on polymers (with a view to preserving their metabolic capacity and to using them several times), has the advantage of extending the production time of cells (over six months) and of making the cells catalyse the same reaction almost indefinitely.

The use of immobilized cells should bypass the direct extraction of the compounds from the biomass as the products arise in the medium itself. Immobilized cells can carry out multi-enzyme operations; by selecting highly biosynthetic cells, catalytic activity can be enhanced; there is no need to provide co-factors since cells produce them themselves.

Immobilized plant cells can be used for single and multi-step biotransformations of precursors to desired products as well as for the de-novo biosynthesis of secondary metabolites (see Sect. 3.2.4 "Biotransformation").

Immobilization of plant cells is considered to be of importance in research and development in plant cell cultures, because of the potential benefits that could be provided [30]:

- The extended viability of cells in the stationary (and producing) stage, enabling maintenance of biomass over a prolonged time period;
- simplified downstream processing (if products are secreted);
- promotion of differentiation, linked with enhanced secondary metabolism;
- reducing the risk of contamination;
- reduced shear sensitivity (especially with entrapped cells);
- promotion of secondary metabolite secretion, in some cases;
- minimization of fluid viscosity increase, which in cell suspension causes mixing and aeration problems.

An immobilization system, which could maintain viable cells over an extended period of time and release the bulk of the product into the extracellular medium in a stable form, could dramatically reduce the costs of phytochemicals production in plant cell culture. However, an immobilized system also has the problems described below:

- Immobilization is normally limited to cases where production is decoupled from cell growth;
- initial biomass must be grown in suspension;
- secretion of product into the extracellularly medium is imperative;
- where secretion occurs, there may be problems of extracellular degradation of the products;
- when gel entrapment is used, the gel matrix introduces an additional diffusion barrier.

Biocatalysts can be immobilized by confinement within a porous membrane (gel entrapment, membrane reactor, interfacial membrane) or by attachment to a solid surface (inner surface of a porous structure, outer surface of a carrier by adsorption or covalent bond) [30].

Physical entrapment in a porous matrix is the most flexible and most popular approach employed for whole cell immobilization. A process designed for the efficient entrapment of whole cells should allow for the following:

- High retention of cell viability (biological activity of the entrapped cells should not be impaired by the immobilization conditions);
- porosity of the formed gel should be uniform and controllable (free exchanges of substrates, products, co-factors, and gases is essential for efficient performance of the immobilized cells);
- gel should retain good mechanical, chemical, and biological stability (it should not be easily degraded by enzymes, solvents, pressure changes, or shearing forces);
- gel should be composed of reasonably priced components.

Various immobilization methods have been developed (entrapment, adsorption, and covalent coupling). The most widely used technique involves the entrapment of cells in some kind of gel or combination of gels which are allowed to polymerize around them. Brodelius and Pedersen (1993) as well as Alfermann and Petersen (1995) described the entrapment of viable cells of *Catharanthus roseus*, *Morinda citrifolia*, and *Digitalis lanata* in calcium alginate gel and this technique has received much attention. Calcium alginate, agar, agarose, gelatin, carrageenan, and polyacrylamide can be used as matrix [25]. However, gels of alginate are most widely used because of their simplicity and relative lack of toxicity. The other alternative supports are polyurethane foam and hollow-fibre membranes. Table 13 gives a number of examples of the systems of immobilization, which have been used with plant cells together with the associated plant species and their products.

Work with *Catharanthus roseus* showed that agar, agarose, and carageenan were all suitable immobilization matrices, suitable for the maintenance of cell viability; but alginate was superior in terms of ajmalicine production [7].

Adsorption immobilization has been successfully used with a number of plant species. *Capsicum frutescens* cells immobilized on polyurethane foam produced 50 times as much capsaicin as suspension cells [24]. In general,

Table 13 Immobilized plant cell systems used for production of secondary metabolites

Immobilization method	Plant species	Substrate/ precursor	Product	Refs.
Biotransformation				
Agarose	*Catharanthus roseus*	Cathenamine	Ajmalicine	Asada and Shuler 1989
Alginate	*Digitalis lanata*	Digitoxin	Digoxin	Alfermann and Petersen 1995
Polyurethane foam	*Papaver somniferum*	Codeinone	Codeine	Dicosmo and Misawa 1995
Synthesis from precursors				
Alginate	*Nicotiana tabacum*	Phenylalanine	Caffeoyl putrescine	Berlin et al. 1981
Alginate, agarose	*Catharanthus roseus*	Tryptamine, secologanin	Ajmalicine	Brodelius and Pedersen 1993
	Lithospermum erythrorhizon		Shikonin	Kim and Chang 1990
Polyurethane foam	*Capsicum frutescens*	Isocapric acid	Capsaicin	Brodelius and Pedersen 1993
De novo synthesis				
Alginate	*Morinda citrifolia*		Anthraquinon	Dornenburg and Knorr 1997
Aginate, agarose	*Catharanthus roseus*		Ajmalicine	Brodelius and Pedersen 1993
Hollow fibres	*Glycine max*		Phenolics	Brodelius and Pedersen 1993
Polyurethane foam	*Capsicum frutescens*		Capsaicin	Johnson and Ravishankar 1996

it appears that mild immobilization either through gel entrapment or surface adsorption enhances productivity and prolongs the viability of cultured cells.

Immobilized cells can also be used as biocatalysts for biotransformations (see Table 14 and Sect. 3.2.4 "Biotransformation"). Such a system compares favorably with the use of freely suspended cells since, in the case of immobilization, the catalyst is theoretically reusable and the product is easily separated from the biomass.

Immobilization can have a dramatic impact on cellular physiology and secondary product formation. The cell culture responses are summarized in Table 14.

Dicosmo and Misawa (1995) found that glass fibres could be used as a carrier of plant cells to produce useful plant metabolites. *Papaver somniforum* cells were immobilized on fabric of loosely woven polyester fibres arranged in a spi-

Table 14 Effects of immobilization on secondary metabolite production in cell cultures

Type of immobilization	Plant species	Product	Fold change	Refs.
Foam	*Capsicum frutescens*	Capsaicin	> 100	Johnson and Ravishankar 1996
Calcium alginate	*Lithospermum erythrorhizon*	Shikonin	2.5	Kim and Chang 1990
Natural glass	*Papaver somniferum*	Saquinarine	2	Dicosmo and Misawa 1995
Gel	*Coffea arabica*	Methylxanthin	13	Brodelius and Pedersen 1993
	Capsicum frutescens	Capsaicin	> 100	Johnson and Ravishankar 1996
	Chenopodium rubrum	Betacyanin		Knorr and Berlin 1987

ral configuration on stainless steel support frame to produce sanguinarine, an antibiotic in oral hygiene. The yield was 3.6 mg g^{-1} FW by immobilized cells and was more than twice as much as by suspension cells.

Polyurethane-immobilized *Capsicum frutescens*-cell-fed capsaicin precursors produced this metabolite at levels of up to 100 times those of non-fed cultures. *Capsicum frutescens* cells immobilized on polyurethane released capsaicin entirely into the medium, although other species immobilized by the same method retained the product intracellularly [24].

Many metabolites still appear to accumulate in the cell vacuoles and it is therefore important to further gain information on how these metabolites may be released into the culture medium. *Chenopodium rubrum* cells, immobilized in alginate beads, secreted the red betacyanin pigment amaranthin into the medium [31]. However, the pigment was subsequently degraded; chitosan and DMSO permitted further product release into the extracellular medium, but this was also accompanied by product degradation.

3.2.4 Biotransformation and its Advantages

Biotransformation can be defined as a process through which organic compounds can be modified by cell cultures resulting in chemically different products. There are two main reasons to choose plant cells for biotransformation purposes: Firstly, these cells are generally able to catalyze the reactions stereospecifically, resulting in chirally pure products. Secondly, they can perform regio-specific modifications that are not easily carried out by chemical synthesis or by microorganisms [46]. These reactions include reduction, oxidation, hydroxylation, acetylation, esterification, glucosylation,

isomerization, methylation, demethylation, epoxidation, etc. [1]. The presence of biotransformation potential in plant cells is a necessary condition for practical application.

Advantages of biotransformation include enhancement in the productivity of the desired compound and the production of novel compounds. Importantly, the studies on biotransformation lead to basic information to elucidate the biosynthetic pathway, and catalysis can be carried out under mild conditions, thus reducing undesired by-products, energy, safety, and costs. The range of flavor metabolites and pharmaceuticals produced by plant cell cultures through biotransformation is shown in Table 15.

The conversion of monoterpenes was studied with *Mentha* species by Dornenburg and Knorr (1997). Suspension cultures of *Mentha canadensis* and *Mentha piperita* were able to synthesize limonene as well as oxygenated, acetylated, or glucosilated monoterpenes. However, the yields of these compounds were low and monoterpene glucosides were accumulated in higher amounts than free monoterpenes. *Mentha* suspension cultures metabolized exogenous monoterpene ketones and monoterpene alcohols within 24 h, and glucosilation occurred. Glucosilation was a detoxification mechanism of phytotoxic compounds by plant cells, and it resulted in accumulation of glucosilated and water-soluble products. Otherwise, the exogenously applied toxic monoterpenes were degraded and metabolized by cell cultures without special accumulation sites. Exogenous terpenes have been shown to be rapidly metabolized by cell suspension cultures to form biotransformation and degradation products. It was concluded that the plant cell culture processes for acceptable product yields can be conceivable if the desired product can be accumulated in a nonpolar organic phase or adsorbed.

For a successful process, the following prerequisites must be met (Knorr 1987): the culture must have the necessary enzymes; the substrate or precursor must not be toxic to the culture; the substrate must reach the cellular compartment of the cell; and the rate of product formation must be faster than its further metabolism.

Table 15 Biotransformation of flavor compounds by plant cell culture systems

Plant species	Substrate	Product	Refs.
Coffea arabica	Vanillin	Vanillin glucosides	Johnson and Ravishankar 1996
	Capsaicin	Capsaicin glucoside	Johnson and Ravishankar 1996
Mentha spp.	Pulegone	Isomenthone	Dornenburg and Knorr 1997
	Menthol	Neomenthol	
Papaver bracteatum	Linalyl acetate	Linalool, geraniol	Dicosmo and Misawa 1995
Vanilla planifolia	Ferulic acid	Vanillin	Romagnoli and Knorr 1988

4 Release and Product Recovery

4.1 Exudation

Many compounds, synthesized by plants, are stored in the vacuole. Enhancing transfer of compounds from the vacuole to the culture medium would be very useful in terms of costs for product recovery. This could include the development of additional chemical or environmental agents to induce such exudation. It may also be possible to recover the substances secreted into the medium. For this method, it could be necessary to examine the physiological mechanisms of metabolite release from the plant cells.

Lee and Shuler (2000) showed that the accumulation of indole alkaloids in *Catharantus roseus* vacuoles has been attributed to an ion-trap mechanism, whereby the basic indole alkaloids are trapped in the acidic vacuole due to their positive charge at low pH, preventing diffusion across the tonoplast. Kim et al. (2004) reported that almost all of the taxol produced by *Taxus brevifolia* cell cultures was detected in the culture filtrate.

4.2 Two-Stage Systems

The sites of synthesis and storage of secondary compounds in plant cells often take place in separated compartments. The accumulation of secondary metabolites in cell cultures is most likely associated with the presence of highly specialized structures containing secretory and accumulatory elements, such as oil glands, glandular trichomes, or a glandular epidermis [17]. Encapsulation of cytotoxic compounds also serves as a self-protection mechanism of intact plants. In undifferentiated callus or suspension cultures, these accumulation sites are missing. This is probably the reason for the low yields of such compounds reached in these plant cell cultures.

A low accumulation of secondary compounds in cell cultures in a number of cases may not be due to a lack of key biosynthetic enzymes, but rather due to feedback inhibition, enzymatic or non-enzymatic degradation of the product in the medium, or volatility of substances produced. In such cases, it should be possible to increase the net production by the addition of an artificial site for product accumulation, for example, by use of second solid or liquid phase introduced into the aqueous medium.

The use of in situ product removal of metabolites has a number of key potential advantages beyond promoting secretion. The removal and sequestering of the product in a non-biological compartment may increase its total production [46].

The addition of an artificial site for the accumulation of secondary metabolites can be an effective tool for increasing biosynthetic pathways in plant cell cultures. If the formation of a product is subject to feedback inhibition or intracellular degradation, the removal and sequestering of the product in an artificial compartment may increase total metabolite production. Table 16 summarizes several examples of two-phase or adsorption cultures.

Robbins and Rhodes (1986) reported that the addition of amberlite XAD-7 resin to *Chinchona ledgerina* cells stimulated the production of anthraquinones by 15 times, which was $539\,\mathrm{mg\,l^{-1}}$, compared to a medium without adsorbent. The yields of ajmalicine and serpentine produced by *Catharantus roseus* were also increased by the addition of XAD-7 and the ratio between both alkaloids produced was changed [34]. It is of interest that production of these alkaloids, which accumulate inside cells, was affected by

Table 16 Adsorbents used for two-phase plant cell cultivation systems

Adsorbents	Cell cultures	Refs.
Activated charcoal	*Marticaria chamomilla*, *Nicotiana tabacum*, *Vanilla fragrans*	Dornenburg and Knorr 1997
β-Cyclodextrin	*Mucuna pruriens*, *Mentha canadensis*	Dornenburg and Knorr 1997
Miglyol	*Matricaria chamomilla*	Rao and Ravishankar 2002
	Mentha canadensis, *Thuja occidentalis*, *Valeriana wallichii*, *Vitis vinifera*	Dornenburg and Knorr 1997
	Pimella anisum	Mulder-Krieger et al. 1988
Polyethylenglycol	*Nicotiana tabacum*	Knorr et al. 1987
Polydimethylsiloxan	*Eschscholizia californica*	Dornenburg and Knorr 1997
RP-8	*Marticaria chamomilla*, *Mentha piperita*, *Pimella anisum*, *Valeriana wallichü*	Dornenburg and Knorr 1997
XAD-2	*Galium vernum*, *Thuja occidentalis*	Dornenburg and Knorr 1997
XAD-4	*Nicotiana rustica*, *Thuja occidenialis*	Dornenburg and Knorr 1997
	Vanilla fragrans	Knorr et al. 1985
XAD-7	*Catharanthus roseus*	Lee and Shuler 2000
	Chinchona ledgerina	Robbins and Rhodes 1986
	Vanilla fragrans	Dornenburg and Knorr 1997
Wofatite	*Galium vernum*	Dornenburg and Knorr 1997
Zeolith	*Nicotiana tabacum*	Dornenburg and Knorr 1997

the presence of resin. The addition of XAD-4 increased the vanilla flavor production in *Vanilla fragrans* suspension cultures [28]. A similar approach was conducted by the addition of charcoal: it led to up to 60-fold improvements in yields of coniferyl alcohol in *Marticaria chamomilla* culture and the addition of Miglyol or silica gel RP-8 stimulated ethanol production in cell cultures of *Pimpinella anisum* [41].

Two-phase systems even accumulate traces of secondary metabolites from the culture medium, thus avoiding any type of feedback inhibition. Another effect may be the enhancement of secondary metabolite release from the cultures or the initiation of a release of compounds normally stored within the cells. Secreted secondary metabolites may be protected from degradation in the culture medium because of excreted catabolic enzymes and acids. Evaporation of the product into the gas phase can be reduced by trapping flavor compounds in artificial accumulation sites. Desired plant products can then be removed selectively from the culture systems. The product can be concentrated by in situ recovery, and downstream purification may be reduced if product removal from the culture medium and cells is selective. Consequently, recovery and purification are generally simplified, thus reducing production costs.

4.3 Membrane Permeabilisation

In most cases, products formed by plant cell cultures are stored in vacuoles. In order to release the products from vacuoles of plant cells, two membrane barriers (plasma membrane and tonoplast) have to be penetrated. Cell permeabilization depends on the formation of pores in one or more of the membrane systems of the plant cell, enabling the passage of various molecules into and out of the cell [7]. Attempts have been made to permeabilize the plant cells transiently, to maintain the cell viability, and to have short time periods of increased mass transfer of substrate and metabolites to and from the cell.

Permeabilization of plant membranes for the release of secondary metabolites is often connected with the loss of viability of the plant cells treated with permeabilizing agents and methods. Various methods have been used to initiate product release from cultured plant cells. These methods include chemical treatments (e.g., with solution of high ionic strength, change of external pH, permeabilization with dimethylsulfoxide DMSO, chitosan) and physical treatments (e.g., high electric field pulses, ultrasonics, ultra-high pressure.) [17, 28].

4.3.1 Chemical Permeabilisation

Active uptake mechanisms have also been reported for indole alkaloids in *Catharantus roseus* vacuoles [39]. In terms of product release, it is pertinent

to note that in cell cultures, an efflux of alkaloids was observed under certain conditions, indicating equilibrium between the intracellular and extracellular compartments that could be perturbed by medium acidification with subsequent product release. The release of serpentine by *Catharantus roseus* cells was observed when the cells were filtered and resuspended in fresh or conditioned medium, and it was suggested that temporary membrane uncoupling was responsible for it.

According to Dornenburg and Knorr (1997), *Chenopodium rubrum* cells could be permeabilized by treatment with chitosan. This polycationic polysaccharide induces pore formation only in the plasmalemma of the plant cell cultures. The leakage caused by chitosan can be considered as leakage from cytosol. Long-term permeabilization with chitosan showed a time-dependent amaranthin release from Chenopodium rubrum cells into the culture medium. Brodelius and Pedersen (1993) tested five permeabilizing agents on three different species, and although product release was achieved, cell viability dropped in most cases. The exceptions were DMSO and Triton X-100, applied to *Catharantus roseus* cells.

Trejo-Tapia et al. (2007) reported that treatment of *Beta vulgaris* cell culture for 15 min with 0.7 mM Triton X-100 induced the release of 30% of betacyanines without loss of cell viability (70%). After this permeabilization treatment, *Beta vulgaris* cultures regrew normally, reaching a maximum biomass concentration of 48% higher than non-permeabilized cultures after 14 days of culture. In addition, maximum betacyanines concentration was only 25% lower than that of non-permeabilized cultures.

4.3.2 Physical Permeabilisation

Physical factors causing membrane permeabilisation include high electric field pulses, high hydrostatic pressure, ultrasound, etc.

Application of the high electric field pulses is based on the principle of development of membrane pores under external electric fields. Depending on electric field strength or pulse number, the pore formation can be reversible or irreversible. Application of high electric field pulses [32] led to high levels of cell permeabilization in cultures of *Chenopodium rubrum*, but at field strengths beyond 0.75 kV/cm and constant amount of ten pulses, cell viability approached zero values.

Knorr et al. (1993) have shown that treatment with high hydrostatic pressure of 50 MPa increased the production of amarantin and antraquinones in cell cultures of *Chenopodium ruburum* and *Morinda citrifolia*. It was found that pressure higher than 250 MPa causes the loss of cells viability, most likely because of permeabilisation of tonoplast.

It has been assumed that the pressure-dependent destruction of the tonoplast, the loss of compartmentation, and subsequent release of the content of

the vacuoles may have caused the pH change in the medium and the resultant cell death.

Knorr et al. (1993) concluded that application of electric field pulses or high pressure has only limited potential for cell permeabilization with concurrent retention of cell viability. However, both procedures could become effective tools for product recovery from plant cells and tissues with minimum effects on product composition.

5
Industrial Production of Useful Biochemicals by Plant Cell Cultures

With technological advancement in the future, plant cell culture will have a greater contribution to the market. For example, the current world market of raw materials of ginseng is about one billion US$ [65]; although cell-cultured *Panax ginseng* occupies less than 1% of the market, its share will increase greatly with enhancement of the culture productivity.

Mitsui Petrochemical Industries Ltd. provides the industrial-scale production of shikonin by *Lithospermurn erythrorhizon* cell cultures. The process involved two stages: plant cells are first grown in a 200-l bioreactor and the resulting biomass is then transferred into a second bioreactor, in which the composition of the culture medium favors the synthesis of shikonin. The productivity of cell cultures reaches $60\,mg\,g^{-1}$ per week, which is 1000 times higher than that of the plant roots, which required a longer time of five to seven years [50]. The success of shikonin production can be regarded to the selection of a cell line, which accumulates tenfold higher level of shikonin than roots of the mature plant. This achievement also results from selection of optimal growth conditions and production media. Thus, cell cultures have now become the major commercial source of shikonin.

The Bio-organic Division of the Bhabha Atomic Research Centre (India) is carrying out research on mass cultivation of selected cell lines of *Rauwolfia serpentina* (ajmaline, reserpine), *Papaver somniferum* (thebaine, codeine, and morphine), *Artemisia annua* (artemisinin), and other plant species [11].

6
Conclusions and Outlook

In recent years, the market for plant products has rapidly expanded, and this trend will continue because more and more people prefer to use natural products. It is widely recognized that cultured plant cells represent a potential source of valuable phytochemicals, but only a few cell cultures are commercially used as a stable and productive source of the secondary metabolites. Over the last few decades, many strategies, such as media manipulation,

phytohormone regulation, precursor feeding, plant cell immobilization, biotransformation, and bioconversion, have been applied for optimization of the synthesis of the desired products from the plant cell cultures in appreciable quantity and at competitive economic value. For plant cell culture techniques to become economically viable, it is important to develop methods that would allow for consistent generation of high yields of products from cultured cells.

The increased use of genetic tools and an emerging picture of the structure and regulation of pathways for secondary metabolism will provide the basis for the production of commercially acceptable levels of products.

Selection of productive cell lines can result in accumulation of products in higher levels in cell cultures as compared to plant tissues. In order to obtain yields in high concentrations for commercial exploitation, efforts are focused on the stimulation of biosynthetic activities of cultured cells using various methods. The introduction of the techniques of molecular biology and production of transgenic cultures can affect the expression and regulation of biosynthetic pathways.

Knowledge of biosynthetic pathways of desired compounds in plants and cell cultures opens new possibilities to regulate the production of phytochemicals by feeding with precursors, application of elicitors, etc.

Because of the complex and incompletely understood nature of plant cells in in-vitro cultures, case-by-case studies have been used to explain the problems occurring in the production of secondary metabolites from cultured plant cells. Production of secondary metabolites by plant cell cultures must be competitive with other conventional means of production, such as extraction from the field-grown plants, alternative chemical synthesis, microbial fermentation, or improvement of the plant itself through somaclonal variation, and genetic engineering.

The significant advance in optimization of plant cell cultures can be achieved, although at present there are two main obstacles: the lack of an adequate process monitoring and control system for plant cells and the heterogeneity and instability of the cells.

The combined efforts of experts of plant science, food technology, pharmacognosy, biochemistry, molecular biology, and fermentation technology can exploit the potential of plant cells for the production of plant secondary metabolites.

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Food and Agricultural Biotechnology: A Summary and Analysis of Ethical Concerns

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Abstract The range of social and ethical concerns that have been raised in connection with food and agricultural biotechnology is exceedingly broad. Many of these deal with risks and possible outcomes that are not unique to crops or animals developed using recombinant DNA. Food safety, animal welfare, socio-economic and environmental impacts, as well as shifts in power relations or access to technology raise concerns that might be generalized to many technologies. These aspects of the controversy over biotechnology are analyzed below as elements of general technological ethics, and key norms or values pertinent to each of these categories are specified in some detail. However, a number of special concerns unique to the use of rDNA in manipulating plant and animal genomes have been raised, and these are reviewed as well. The chapter concludes by reviewing two broad policy strategies for responding to the issues, one involving labels and consumer consent, the other applying the precautionary principle.

Keywords Animal welfare · Environmental risk · Farm structure · Food safety · Libertarianism · Precautionary principle · Utilitarianism

Modifying the genetic structure of plants, animals, and microbes through recombinant DNA and adult cell cloning of mammals is a controversy that has multiple dimensions, many of which can be usefully addressed through the lens of ethics. Many in the public have been concerned with biotechnology in food and agriculture [48]. Public opinion surveys document the fact that popular worries about biotechnology blend the possibility of unknown and unacceptable risks with apprehension about ethics [48, 49].

Simply defining "ethics" in this context may be one of the most difficult tasks. On one hand, the term indicates almost universally recognized norms. Norms can be standards of conduct found implicitly within everyday social interaction or explicitly articulated as legal or professional codes of practice, and within religious texts, folktales, literature, and philosophy. On the other hand, the subject matter of ethics is equally often presumed to be inherently personal, introspective, and unsuited to public discourse. As such, the ethics of food and agricultural biotechnology will traverse personal reactions of individuals (some of which may be wholly unique to the individual in question), the traditions and values of particular social groups, and broadly shared social norms. This chapter will summarize and explain a variety of expressed rationales that have been applied to the use of, opposition to, or regulation of agricultural biotechnology. The aim is to summarize and represent the debate, emphasizing those aspects of the debate that have implicitly or explicitly utilized ethics in developing an argument.

One must make choices about how one constructs a summary. Pence, for example, summarizes the arguments of biotechnology's proponents by emphasizing humanitarian goals like ending hunger, while characterizing opponents' views as holding that biotechnology is unnatural, a "mutant harvest" [120]. In doing so, he makes "naturalness" a focus. This was also the main organizing principle for an earlier study by Reiss and Straughan that included medical as well as agricultural biotechnology [132]. Comstock also discusses the ethical significance of holding that biotechnology is unnatural, but his summary emphasizes how he himself came to see the humanitarian rationale for biotechnology as overriding concerns about the social and environmental risks associated with transgenic crops and genetically engineered animal drugs [30]. All of these authors characterize the debate as having two clearly opposed ethical perspectives and all win up on the "pro biotechnology" side of the debate. This way of framing the debate in terms of humanitarian benefit from increasing agricultural productivity, on the one hand, and unnatural or risky technology, on the other, has also been the subject of a book by Lacey, who takes a less favourable view of biotechnology. Lacey believes that the pro-biotech perspective is rooted in an ethical perspective that stresses processes of control and predictability, while the anti-biotech perspective is based on scepticism about the viability and desirability of control [86].

We take a somewhat more complex approach to the debate. Rather than seeing it as a simply two-sided opposition, we interpret controversy over agricultural biotechnology as the convergence of several longstanding struggles over the trajectory of food use and food production on these new technologies. Because food consumption is both a biological necessity and rich in cultural significance, any new way of producing or preparing food is likely to have ethical ramifications. Technology can affect safety and access to food, which raises questions of fairness and equity about the entire system of producing and distributing food. Thus, much of the debate over biotechnology concerns ethical imperatives or problems that are (or could be) associated with any food technology. We summarize these issues under the heading of "Technological Ethics". Yet, as the books by Pence, Comstock, and Reiss and Straughan emphasize, there *are* aspects of biotechnology that cause ethical apprehension unlike anything that arises in connection with chemical, mechanical, and other food technologies. We will refer to these simply as "Special Concerns". Some of them indeed overlap with questions in biomedical applications of genetic technology.

1
Technological Ethics

The German philosopher Hans Jonas (1903–1993) is the modern era's founding father for technological ethics. He believed that technological ethics should combine science-based attempts to anticipate the effects of technological innovation with ethical analysis based the recognition that many who are affected by technology are unknown to those who plan and execute a technological practice. Jonas called his approach "the principle of responsibility (*Prinzip Verantwortung*)" and saw it as a new thing in ethics, which had hitherto been conceived of in terms of face-to-face reciprocity amongst people who were well known to one another, or at least bonded by a common culture and social institutions [70].

The anticipatory approach sketched by Jonas points in a very different direction than the "pro" and "con" weighing of biotechnology undertaken by Pence, Comstock, or Lacey. The *Prinzip Verantwortung* enjoins scientists and engineers to anticipate possible forms of harm. It is, in many respects, a forerunner of what is today called "risk assessment" or "risk governance". One key task implied by Jonas's view is to integrate ethics more fully into what is too often thought of as a purely technical or scientific methodology. However, if risks are analyzed in an ethically sophisticated manner and then deemed acceptable or properly managed, Jonas's approach entails that the developers of technology are at liberty to proceed. The reason is that technological innovations are expected to improve workplace efficiencies and these efficiencies are expected both to make new goods available and to lower consumer

cost. Thus, technological innovation is broadly supported by the belief that it will improve quality of life. Beyond this, there is really no need for a "pro-technology" argument, at least not at the outset. There is only the need for a responsible effort to determine the unintended consequences of technical change.

Jonas's approach to technological ethics provides a structure from which to examine agricultural biotechnology that revolves around five categories: (1) impact on human health (i.e., food safety); (2) impact on the environment; (3) impact on non-human animals; (4) impact on farming communities in the developed and developing world; and (5) shifting power relations (e.g., the rising importance of commercial interests and multinationals). We consider each of these categories in turn.

1.1 Food Safety

If one already believes that eating so-called "genetically modified organisms" (GMOs) could be dangerous, one is also very likely to believe that it is unethical to put people in a position where they might eat them, especially without their knowledge. If one does not believe that GMOs are dangerous, one is not likely to believe that putting people into that position is an ethical issue. What is at issue in this description of the divide between critics and advocates of GMOs is not a question of ethics. Both critics and advocates of GMOs would agree that it would be unethical to expose people to food-borne hazards. Their disagreement is about whether there *are* hazards associated with the human consumption of GMOs, and over the likelihood that any potential hazards will actually manifest themselves in the form of an injury to human health.

Ethical issues do arise out of the uncertainty involved in answering these empirical questions, however. Given that there are disagreements over the nature and extent of food safety risk, what principle should guide future action? One answer is that future actions with respect to GMOs should be based on the best available science. The ethical rationale for this approach relies on a consequence-based justification. If GMOs have demonstrable benefits of some sort, if only the potential to increase the cost-efficiency of crop production and build wealth for farmers and seed companies, then (other things being equal) it would be ethically wrong to prohibit GMOs without some sort of evidence that they pose a hazard to human health. Baseless concerns ought not stifle innovation when technological and economic stultification that is not in the public interest would result. This approach to dealing with the uncertainty surrounding claims of food safety requires criteria for deciding when an alleged hazard is baseless. "The best available science" is supposed to provide these criteria in the risk governance approach utilized in many regulatory agencies [101].

Even under the best circumstances of strong scientific consensus on hazards, mainstream risk governance suffers from problems often associated with the utilitarian or consequentialist form of ethical reasoning with which it is closely allied [147]. Any approach to ethics that rationalizes some chance of a hazardous outcome in terms of benefit to the general public will be vulnerable to criticisms that stress individual rights. Take, for example, the widely discussed risk of allergenicity associated with GMOs. Genes make proteins and any protein is a potential allergen. Therefore, one cannot exclude the possibility that genetic engineering of foods may introduce proteins into foods that will cause allergic reactions – slight or severe – in some portion of the population. Since food allergies are not well understood, and since they may affect very small percentages of the population, there is much uncertainty in the attempt to anticipate or characterize the likelihood of allergic reactions before GMOs are released for public consumption. The approach to food safety described above presumes the acceptability of some small probability of serious health effects on these few in exchange for overwhelming economic and technological benefits to the many.

Here, the utilitarian and libertarian foundations of technological ethics come into conflict with one another. Libertarian ethical approaches assert that individuals have inviolable rights shielding them from harm caused by others. In this case, they have a right not to be harmed by inadvertently consuming a protein that they could not have known they were allergic to. Uncertain risks present a key problem in operationalizing the libertarian approach in this case. Is an individual's right violated even when the risk is purely hypothetical, or when the probability that a hazard will materialize is reasonably believed to be fairly low? One way to arrive at an affirmative answer to this question is to draw an analogy with cases where a very small proportion of the general population experiences a known harm in exchange for significant benefits to the rest. If, for example, a drug is known to cause death for one person in one million, it is possible to characterize the probability of death for anyone taking the drug as 0.000001. Simply accepting the cost of one sure death in a million users of the drug would certainly be ethically controversial. Since uncertainties associated with hypothetical hazards or weak data can also be expressed as probabilities, it is possible to see these cases as similar.

If we judge such costs as "unacceptable", it seems to be a clear case of allowing the rights of the few to outweigh the interests of the many. Some opponents of biotechnology take this position. One response to this extreme individual rights view is to place each individual in a position to look after their own interests where food safety is concerned. This approach follows the ethical logic of informed consent: people should be free to take whatever risks they choose, but they should not be put in a position of risk without adequate notification and an opportunity to choose otherwise [67, 165]. This approach, however, has its own challenges. Comstock [31], for example, discusses em-

pirical research showing how detailed food information can distort personal decision making. It is possible to provide information that allows one person to make an informed choice while simultaneously putting another person in a position where they will make an uninformed choice. As such, some argue that governments should be judicious and sparing in the information that they require to be supplied to consumers. This argument effectively brings us back to the "best scientific evidence" approach to dealing with uncertainty described already.

1.2 Ethical Significance of the Environment

Environmental risk has emerged as a key category for social and political controversy in industrialized societies. Unlike food safety risks, which can be addressed conceptually in terms of individual choices and individual rights, environmental risks cannot typically be addressed through policies that allow individuals to apply their own values as to whether a risk is acceptable or not. Environmental risks necessarily involve political decisions [94, 124]. Complex and well-developed constituencies contest a wide array of issues along environmental lines, and sociological perspectives on environmentalism and environmental movements suggest a number of ways in which environmental concerns might be interpreted with respect to political values and interest group politics [37, 133]. This political context exacerbates the fundamental uncertainty associated with sorting out the scientific evidence. This augmented uncertainty can be seen to raise even more ethical questions. For example, one central and abiding ethical question unifies a host of approaches with the hazard identification phase of mainstream risk assessment: What counts as an ethically significant environmental impact?

Answers to this question can raise three different kinds of ethical concern: human health effects accruing from environmental exposure, such as air or water borne pathogens (as opposed to ingestion through food); catastrophic impacts that would disrupt ecosystem processes in ways that threaten to destabilize human society; and, finally, effects that are felt less by humans than by the broader environment. The category of catastrophic impacts includes dwindling energy supplies, human population growth, and global warming; The final category may be classified as ecocentric (or non-anthropocentric) impacts. Interpreting each of these three types of environmental impact as having ethical significance involves distinct ethical concepts and values, some of which are widely endorsed and others less so.

Environmental philosophers have stressed two general approaches for developing an environmental ethic: duties to posterity (our obligations to consider and lessen the negative impacts of our actions on future generations) and ecocentric ethical values (our obligations to care for nature for its own sake) [60]. Environmental impacts in the first two categories manifest them-

selves as impacts on human beings. Environmental exposure to disease risks include cancer induced by chemical pollution, emphysema and lung diseases from air pollution, poisonings and non-fatal diseases such as allergies and reduced fertility speculatively associated with hormone disrupting chemicals in the environment. The ethical imperative to limit these risks is very clear. Ethical issues arise because it is not clear how to resolve uncertainties that arise in assigning a probability to the unwanted impact and because there are different ways to think about the social acceptability of environmental exposure to human health risks [61]. Critics of biotechnology have noted that transgenic crops are also being developed to produce drugs and industrial products, and that these products must be contained in order to limit environmental exposure to human health hazards [2].

In the 1980s, the environmental risks associated with agricultural biotechnology were thought to be their potentially catastrophic ecological consequences. In contrast to environmental exposures that might lead to human health hazards, the science that would be used to predict and measure the likelihood of ecological catastrophe is less well developed. Ecologists raised the possibility of widespread disruption of atmospheric processes associated with ice-nucleating bacteria early in the development of agricultural biotechnology (see [172] for an overview). The speculation that biotechnology would contribute to a narrowing of the genetic diversity in major food crops was also an early concern [35]. During the 1990s, the potential environmental impacts foreseen were less sweeping. Particular attention has been given to the potential for escape of herbicide tolerant genes into weedy relatives of crop plants, and to the possibility that insect pests will acquire resistance to *Bacillus thuringiensis* (Bt) [84, 137]. Though such events are not in themselves catastrophic, their ethical significance derives from interpreting them as contributing to a broad destabilization of the global food system.

Although questions of uncertainty and risk acceptability might also arise in connection with impacts on wildlife or ecosystems, here there is more debate over why such impacts might be thought to have ethical significance [126]. Prior to 1999, crop biotechnology was not widely associated with environmental impacts on wilderness or endangered species. In that year, news reports that Bt-crops could affect monarch butterflies enlivened the prospect of unintended impact on non-target species for the first time (see www.news.cornell.edu/releases/May99/Butterflies.bpf.html). This has awakened public recognition of the way that agricultural biotechnology could have an impact on wild species, and provides an example of how ecocentric environmental impacts could be brought about by genetic agricultural technologies. In Canada, genetically engineered canola could outcross with wild rape [28]. Research on genetically engineered fish has long been associated with the potential for negative impact on wild populations [104]. There are also less well-known products, such as recombinant vaccines, that could also

have negative impact on wild habitat [175]. As experience and experimental studies accumulate, the list of possible hazards is expanding, and scientists' ability to quantify the likelihood that such hazards will materialize is increasing [192].

An additional type of environmental impact requires one to see a farmer's field as having a kind of ecological standing or integrity of its own. Biotechnology might be understood as threatening in virtue of the possibility that transgenic plants may appear in a field in which a non-transgenic crop is growing, either by pollen drift, contamination of the seed supply, or when volunteer transgenics survive over the winter to reappear in a field sown to non-transgenics in the succeeding year [14, 95]. The key ethical question is: Why does this matter? Some answers to this question are based on economics. A farmer may lose the ability to gain a price premium for a non-transgenic crop or, in the worst case, lose the ability to sell the crop in some international markets altogether. Here, an ecological or environmental mechanism contributes to an impact that is better classified as "socioeconomic" than "environmental". Other answers relate to consumer preferences of the sort discussed in connection to food safety (above). Still other answers may foreshadow the discussion of purity and unnaturalness that is taken up in the section on special concerns.

Ironically, public opinion surveys suggest that Canadians and Americans have not historically associated ecological risks of agricultural biotechnology with ethical concern, though there may be a greater tendency to do so in recent years [38, 129]. Ecological impacts of agricultural biotechnology elicit more ethical concern globally than in North America (see [36, 50]). Attentiveness to potentially catastrophic risk and to preservation of farmland has created a groundswell of environmentally based concern about agricultural biotechnology in Europe. The difference between North American and European attitudes may reflect cultural and philosophical norms about the place of agriculture within nature, with Europeans seeing agriculture as part of nature and North Americans associating nature with wild or unmanaged ecosystems. Alternatively, it may reflect different ways in which environmental issues are capable of mobilizing individuals into effective forms of political action, a difference that may be rooted in respective national histories or in the structures of political organization [51].

1.3
Moral Status of Animals

The impact of human action on non-human animals is controversial because some people deny that animals can be harmed at all. The belief that animals are non-sentient machines who feel no pain is often attributed to René Descartes (1596–1650), and, without question, it has been influential in the use of animal experimentation within the medical sciences [142]. Immanuel

Kant (1724–1894) believed that animals could not be harmed because they lacked reason, and argued that the moral wrong associated with animal abuse owed not to any harm suffered by the animal, but solely to the harm that a perpetrator inflicts upon himself in acquiring a habit of poor character ([74], pp 239–241). Long before these pivotal figures in European philosophy, philosophers of the ancient world, such as Aristotle, had defended the view that animals lack the mental faculties that would make human conduct toward them morally significant [162].

The philosophers Singer and Regan have jointly campaigned to support the view that animals do count morally, arguing that past attitudes caused untold animal suffering in medical research, product testing, and animal agriculture. However, Singer and Regan oppose one another in their accounts of *why* animal suffering is morally significant. Singer argues from the perspective of utilitarian philosophy, and quotes Jeremy Bentham (1748–1832), who wrote of animals, "[T]he question is not, Can they *reason*? nor, Can they *talk*? but, Can they *suffer*?" (Bentham 1948, p 311 [5]). Here, the ethical principles regarding non-human animals follow from the utilitarian mandate to act in ways that maximize the ratio between pleasure (or satisfaction) and pain or suffering. If animals experience pain (and Singer supports the common sense belief that they do with substantial scientific evidence), then we are morally obligated to take their pain into account when evaluating our actions in ethical terms [159, 160]. Regan presents his argument for animal rights by challenging the utilitarian approach that Singer adopts. Regan believes in an approach based on rights that guard the interests of moral subjects. Here, the key philosophical question concerns whether or not animals possess the traits that characterize moral subjectivity. Regan specifies these traits in terms of conscious experiences that support a feeling of personal identity over time. Regan concludes that vertebrate animals, at least, do experience a continuing sense of themselves, and that they are, in his terminology, "subjects-of-a-life", or bearers of a cognitive unity requiring our moral respect [130, 131].

Public interest groups advocating humane treatment of animals monitor developments in animal agriculture closely, and it is in this connection that they have taken an interest in biotechnology, though there has also been debate on transgenic mice developed for biomedical research [97]. More recently, genetic transformation and cloning of livestock has moved past experimental stages [167]. The U.S. Food and Drug Administration (FDA) has concluded that food from cloned animals is safe to eat [42]. Consequently, estimates now have food from cloned animals being available to consumers as early as 2010 [167]. Early survey research indicated that animal biotechnology is strongly associated with ethical concern among members of the public [39, 47, 163]. For some groups these concerns remain strong (see www.centerforfoodsafety.org). There are also a number of authors associated with social movements to protect animals who have decried food and agricultural biotechnology [44–46, 88, 143]. However, other authors who

have argued strongly for recognition of animal interests have not found gene technology to be especially problematic [138, 139, 181]. Clearly, some of those who find animal genetic engineering problematic are among those who see gene technology as intrinsically wrong, and this topic is treated as a special concern discussed below. Gene technology applied to animals raises two additional issues that might also be applied to animal breeding and that thus belong in the category of general technological ethics.

The first issue is that gene technologies have the potential to produce suffering in animals. Some of the first genetically engineered animals were very dysfunctional [138], and there continue to be questions about the health of cloned animals [194]. The ethical issue is whether the purposes to which animals are being put justifies any pain and suffering they experience. Rollin has argued for an ethical principle that would proscribe applications of biotechnology where animals experience more pain and suffering as a result of the modification than comparable, non-transgenic animals might in a similar situation [138]. If followed, Rollin's principle would entail that animal applications of biotechnology should be acceptable to the extent that existing practices within livestock production are ethically acceptable. But existing practices are sharply criticised by animal advocates, and applications that conform to Rollin's principle have already been controversial. For example, recombinant bovine somatotropin (rBST), a genetically engineered animal drug used in dairy production, has been controversial because all cows with higher rates of milk production are also at a higher risk for health problems. The use of rBST puts more cows into that group, but the FDA chose to interpret the animal health risk from rBST as consistent with that of other approved ways to boost milk production. Critics saw the same data as evidence that rBST increases the risk of health problems in animals on which it is used ([175]; see [127] for a discussion of the Canadian debate on rBST). A similar situation may be emerging with cloning, as critics see birth defects as associated with cloning, while the FDA has associated them with other approved procedures (such as in vitro fertilization and embryo transfer) which may be used in conjunction with cloning [176].

The second set of ethical issues surfaced when Rollin suggested that genetic engineering should be utilized to render animals being used in medical experiments "decerebrate" – physically incapable of experiencing pain [138]. A less drastic version of the idea that one uses genetic technology to address pain by making animals incapable of feel pain might be applied to livestock. Gene technology (including both transgenics and classical breeding) could be used to produce animals that are more tolerant of production diseases that create welfare problems in existing animal production systems [146]. If animal suffering is the predominant ethical concern, there may be a compelling ethical argument for doing this. Yet, many animal advocates find this to be an abhorrent suggestion, though it has proved difficult to articulate reasons that do not revert back to the claim that animals have a form of *telos*, or in-

tended (inherent) design. This notion of *telos* has been cited by a number of critics who find genetic engineering of animals to be intrinsically wrong, and these arguments are discussed below as a form of concern special to biotechnology [117, 185].

1.4 Socio-Economic Impact and Social Justice

As noted already, the social logic of technological innovation presumes that increasing the efficiency of production practices is generally, if not inherently, beneficial to society. Nevertheless, technology is a concern for social justice when general processes and specific products affect the distribution of economic rewards (and penalties) throughout society, or when less tangible social goods, such as social cohesion or social legitimacy, are damaged. These social impacts have been persistently associated with agricultural biotechnology (see [125] for an overview), and the focus here will be to discuss a sample of these criticisms with a focus toward understanding the norms and principles at work in these arguments.

Issues of social justice have been based on concerns that involve many different ethical claims. Some have a history that extends back to the origins of the industrial revolution; while others exemplify social concerns uniquely characteristic of the late twentieth century or of biotechnology itself. Here, it will be useful to divide socio-economic impact into two subcategories and to offer an extended discussion of each. First, there has been a longstanding debate over the effect and justifiability of yield-enhancing agricultural technology, in one sense, a focused rejection of the implied social logic of technological innovation itself. Seen in light of this debate, biotechnology is just the latest example of a technologically caused social phenomenon that has been the debated in agriculture for at least 100 years. Second, there is a related but, nonetheless, distinct debate that associates biotechnology with relatively recent trends in shifting power relations, globalization, the rise of international corporations, and the transformation of national sovereignties. Because so few people are now intimately associated with or knowledgeable about agricultural industries, it is easy to mistake the old debate for the new one. In the interest of disentangling these threads here, we treat these ethical issues as more distinct from one another than they may be in the minds of many who level these criticisms.

1.4.1 Impacts on Farms and Farm Communities

Agricultural production technology affects economies of scale in farming or food distribution, as well as the control that different persons or groups maintain with respect to the overall food system. Mechanized harvesters are an

obvious example of the former phenomenon, as they are economical to operate only when they will be used on a sufficiently large acreage. There have been numerous studies of this phenomenon in the Californian produce sector [49, 150]. The latter problem is especially associated with technological innovations that shift decision making from farmers to agricultural supply companies, be they equipment, fertilizer, or seed supply firms. The phenomenon was originally noticed by Karl Kautsky (1899) and has been the focus of several studies on biotechnology [55, 82]. Perhaps more than any of the other ethical concerns discussed in this chapter, food and agricultural biotechnology represent nothing more than a case study for the ethical questions associated with these phenomena.

In assessing long-running historical arguments, it is helpful to trace the way that agricultural technologies have played a key role throughout history. It is then plausible to see late twentieth century themes that link opposition to science and technology and movements of social liberation as building on these long-running historical arguments, but, in considering food and agricultural biotechnology, it is also important to have a firm grasp of the agrarian context in which these arguments originated. Some of the foundational arguments for contemporary discussions of social justice received influential formulations during seventeenth and eighteenth century debates over agricultural land reform. Developments in transport technology and infrastructure made it feasible for farmers and landowners to seek competitive prices for grain. This practice sparked additional innovations (such as enclosure and increased use of draft animals) that increased yields. It also disrupted the system of tithes and shares that had been the foundation of feudal and village economies [170].

On one side of the political dispute that emerged from this technological change were those who developed arguments in favour of the changes which fell along two strands of ethical argument that have already been mentioned throughout the chapter:

1. *The Libertarian Approach*: People who invest labour in the production of goods have the right to seek the most favourable price for their goods; and
2. *The Utilitarian Approach*: The increased efficiency of technological innovation served all in the long run – technological innovations promote the greatest good for the greatest number.

On the other side were those who argued that these transformations destroyed the integrity of village communities. They argued that the older system of exchange, in which every person in the village was entitled to a share of the local crop, better satisfied the ethical demands of social justice [103, 161, 170].

The ethical issues associated with early transformation of rural areas in Europe were generalized and evolved into very broad and sweeping views on social justice during the nineteenth and twentieth century. Arguments

that favoured agricultural technology eventually took shape as the neo-liberal principles endorsing the social efficiency of unregulated markets, on the one hand, and the sanctity of private property, on the other. Arguments opposing technological improvement of agricultural production and rural infrastructure evolved into socialist and communitarian conceptions of social justice. The anti-technology dimension of these arguments was gradually muted, particularly in strong leftist and Marxist interpretations of social justice. Karl Marx (1818–1883) believed strongly in the power of technological development as a force of liberation. Thus, there is a sense that some of the broadest concepts of social justice have their roots in disputes over agricultural technology. Disputes over agriculture and rural development continued throughout the twentieth century, but participants in these debates were not necessarily mindful of their historical origins. It is useful to consider disputes that make general claims about agricultural technology and rural development from those that focus specifically on the developing world.

First, new agricultural technology had its greatest effect on rural communities in industrial societies during the twentieth century and, especially after World War II, a century-long debate over the ethical and political wisdom of allowing industrial principles to shape agricultural production ensued [80]. The ethical dimension of the debate consists in values that would decide between two claims. On one side, technological innovations adopted by profit-seeking farmers, processors, and food retailers reduce overall food costs, resulting in consumer benefits that are thought to *outweigh* the financial and psychological costs of those who suffer economic reverses when their farming operations decline or become bankrupt as a result of their relatively high cost of production. On the other side, preserving the economic opportunity represented by family farms and the small businesses that arise to support them is seen as an essential component of social justice, one that *cannot* be outweighed by diffuse economic benefits. Critics of innovation may also claim that small-scale rural communities promote participatory local governance and are, therefore, most consistent with the ethical principle that social justice depends upon: consent of the governed. It was virtually inevitable that any new agricultural technology developed in the last quarter of the twentieth century would be subsumed by this debate. Some of the first social science publications on food and agricultural biotechnology framed it in precisely the terms of the century-long debate over the structure of agriculture and the ethical importance of the family farm [73, 81, 151]. Thompson provides a discussion of the literature on biotechnology in the context of ethical issues involving social justice [175].

A second strand of ethical concern over social justice examined the impact of food and agricultural biotechnology in developing countries. Here, too, there was an ongoing debate over the "Green Revolution" agricultural development policies being pursued by organizations such as the World Bank, FAO, the Consultative Group on International Agricultural Research, the Rocke-

feller Foundation, and the international development agencies of industrialized nations. Like the first strand of debate, critics of the Green Revolution have argued that increases in agricultural productivity have been gained at the expense of rural ways of life, a repeat of failures and tragedies that have faded from the memory of people in the industrialized world. Here, too, it was inevitable that biotechnology would be subsumed by the existing debate [113, 114]. On the part of those who support the actions of the official development organizations, it is argued that developing countries must follow the lead of the developed world in adopting yield enhancing agricultural technology. As above, it is argued that the benefits of increased food production outweigh any short run reverses suffered by individual farmers. Indeed, given the threat of famine, it is argued that the social demand for more food production is compelling [9, 122, 133, 189].

Those holding an opposing view raise factual questions about the success of the Green Revolution. The ethical dimension of their viewpoint notes that the infusion of technology and capital into peasant economies and traditional agricultural production systems causes an upheaval in the existing social relations. In addition to claiming that this upheaval destroys the culture and way of life in traditional societies, critics of Green Revolution-style development note that the poorest of the poor are the most vulnerable when such massive transformations of social structure occur. They counter the argument that food needs in the developing world override concern for cultural integrity with an argument that appeals to the basic rights of individuals whose lands, jobs, and ways of life are destroyed in the wake of development projects [34]. These general criticisms have been extended to biotechnology in a series of critical discussions dating back to the mid-1980s [21, 22, 77, 81, 82, 121].

1.5 Shifting Power Relations and Intellectual Property

In addition to the above noted effects on farming communities, there have been several other concerns that have been associated with the dominance of hierarchical decision making styles and linked to the growing power of multinational companies. Critics of food and agricultural biotechnology claim that policy making has been dominated by men who exhibit a decision making style that has been the target of, among others, the feminist social movement. They note the prevalence of a viewpoint that characterizes critical attitudes as emotional or irrational, and equates rational decision making with an emphasis on economics and cost-benefit style comparison of decision options. They also believe that decision makers see nature as an object of human domination. Consistent with much of the literature in feminism, they see the domination of nature and the domination of women as themes with a common historical, intellectual, and cultural origin. Hence, they argue that opposition to biotechnology and the overthrow of the existing decision-making elite for

biotechnology follows from an ethical commitment to feminist philosophies of social justice. Shiva is particularly known for linking feminist ethics to the critique of the Green Revolution noted above [155–158]. The argument has been made as a more general postmodern critique of both agricultural and medical biotechnology by social critics, such as Tokar [177], Heller [63], and Bowring [11].

A more general set of concerns has been raised in connection with industry's impact on publicly funded science. *Biotechnology's Bitter Harvest* [54] was one of the most influential publications to make a forceful ethical critique of food and biotechnology in a clear way. Although the report included a critique of biotechnology on environmental grounds, its primary argument was that U.S. agricultural universities were abandoning an ethical commitment to serve farmers, turning instead to the development of technology that would primarily benefit agribusiness and agricultural input firms. This argument can be seen as a direct outgrowth of the issues concerning farming communities discussed above. Yet, in directing the brunt of its criticism at the planning and conduct of publicly funded agricultural research, the authors of this report made claims with a substantially different ethical importance. Their argument connects with that of social critics who have been expressing concerns that commercial interests were having a growing influence on the conduct of science [19, 20, 83, 128].

A third strain of argument focuses again on issues relating to international development. Much of world's most valuable plant genetic resources lie in the territory of developing countries and much of it is found in land races. Land races are crop varieties that have been grown by indigenous farmers who have selected for valuable traits by a process of trial and error. Developed country plant breeders have made many advances by extracting these valuable traits from the seeds of land races. In the past, neither the indigenous farmers who grow land races nor the governments of their countries have been compensated for the use of these genetic resources. Critics have claimed that a double form of injustice occurs when these genetic resources are first taken without compensation, and then sold back to developed countries in the form of seeds protected by patents or under plant breeders' rights [89, 153]. This argument is also tied to the concern that biotechnology might hurt small farmers, but here the injury being done to them is in the form of property rights, and arguably quite different from the traditional critique of social impacts due to the increasing size of farms and their industrial organization.

Ethical concerns about smallholder control over seeds predate the debate over biotechnology. Social critics have noted this issue with respect to the collection of germplasm for conventional plant breeding [43, 71]. Biotechnology has brought this set of concerns to the forefront of public attention in conjunction with legal debates over the patentability of genes and genetic sequences [87] and over the status of patents and other forms of intellectual property in the TRIPS Agreement, which established basic principles

for adjudicating intellectual property disputes in the World Trade Organization [193]. Defining and defending any given configuration of property rights is an inherently moral and philosophical exercise, hence these technically complex legal debates generally presume some sort of ethical framework in which arguments about what should and should not be recognized as property are mounted [175]. Broadly, the case for recognizing the patentability of genes and gene sequence is a derivative of the case for intellectual property in general, and it is couched in utilitarian terms: In a setting of competitive markets, innovators benefit from their inventions only if they are kept secret and no competitors are able to use them. However, the public benefits if the inventions are made public and everyone can use them. Thus, inventions (intellectual property) should be made public, but if they are made public too soon, inventors lose all incentive to innovate. Hence, the rationale for intellectual property rights, including patents and copyrights, is to give inventors an exclusive right to use or license the use of their invention, but only for a limited time, after which this right ceases to exist, thus maximising public benefit [12].

This basic argument has been challenged on many fronts. Some critics accept the basic utilitarian rationale for patents, but question whether patents in biotechnology are really beneficial [64]. Others see the utilitarian view of patents simply as a subterfuge to allow the growth of capitalist social relations and corporate power [18, 65]. Still others stress the view (noted above) that indigenous people who discover uses for plants and who develop germplasm through generations of trial and error have a prior claim that vitiates this utilitarian rationale [168]. These arguments are linked with concerns about intellectual property rights in the domain of human medicine, where patenting of genes and gene processes are sometimes said to violate human dignity [11]. The ETC Group, a non-governmental organization that has been active in opposing biotechnology, often links their criticisms of gene patents to the so-called Terminator gene, a biologically based means of protecting intellectual property by rendering seed infertile. Although intellectual property arguments can involve exacting technical detail when considered in a legal setting, it has proven relatively easy for critics of biotechnology to link the spread of intellectual property rights in biotechnology with the worst aspects of globalization.

These ethical issues associated with the shifting balance of power in society should be seen as distinct from concerns about the impact of technical change on farming communities. Someone who supports food and agricultural biotechnologies that lower food costs for consumers in the belief that doing so addresses world hunger, might perhaps find erosion of rural communities to be an acceptable cost; but the same person might still fault the way that the science agenda is being established in the era of biotechnology. One concern is that pursuit of profit or receipt of funding from industry might influence the results of research intended to review the safety of prod-

ucts. When added together, dramatic shifts in the role and nature of scientific enquiry, in the structure of international institutions, and in traditional ways of understanding ownership ground a pervasive concern about the general drift of social relations. Critics such as Shiva, Bowring, or Tokar unify a broad array of medical, food-related and legal trends to create a picture of biotechnology as a monolith that must be met with widespread popular resistance. At this point, concerns emerging out of a fairly straightforward need to anticipate unwanted social consequences of biotechnology seem to blend together. To the extent that biotechnology comes to be seen as a force unto itself, reshaping the texture of social life, these arguments over shifting power relations start to resemble what we are calling "special arguments" that move beyond general technological ethics to a focused concern on biotechnology itself.

2
Special Arguments Pertaining to the Use of rDNA Technology

The most sweeping ethical argument against biotechnology would be one that finds the manipulation of genes or cells to be either categorically forbidden or presumptively wrong, so that compelling arguments would need to be adduced in its favour. Empirical research indicates that many members of the lay public who find food or agricultural biotechnology ethically objectionable base their judgment on the view that it is *unnatural* [50, 188]. Philosophers have called these objections to biotechnology "intrinsic objections", meaning that it is the activity of genetic manipulation itself that is wrong, not its consequences [148, 166].

Statements to the effect that biotechnology is unnatural convey a judgment of disapproval, but do little to articulate the basis for that judgment. In one sense, all of agriculture is an unnatural activity, but we should not infer that all of agriculture is therefore of ethical concern. How would one spell out the belief that biotechnology is unnatural in a way that would form the basis for an argument against its use to develop agricultural crops or animals? How would one articulate an intrinsic objection to gene transfer that would cover its use in plants and animals, as well as human beings? A few strategies that have been attempted in the literature can be summarized.

1. *Genes and essences*. Since antiquity, people have thought of living things as having "essences" that constitute their essential being. Nelkin and Lindee note a general cultural tendency to interpret genes as bearers of the traditional notions of essence and purpose that would achieve moral significance in some teleological conceptions of nature [105]. One view of biotechnology may see it as tampering with these essences [8]. Criticisms voiced by Rifkin [134, 135] suggest such a judgment, and it is particularly

associated with those who have suggested that genetic engineering violates a species' *telos* [44–46, 182, 183]. The term "telos" is derived from the philosophy of Aristotle, where it was used to indicate a thing's guiding or final purpose, realized in the case of living organisms through the processes of growth, development, and reproduction that are characteristic of their species. It is associated with *teleology*, a philosophy of nature that seeks to explain biological processes in terms of function, purpose, and design. Although teleology does not necessarily prescribe particular ethical norms, versions of teleology that find a predetermined design in nature move quickly to the ethical judgment that humans deviate from the preordained purposes of this plan at their physical and spiritual peril.

2. *Emotional repugnance*. Genetic modification of foods causes an immediate reaction of repugnance among many. The most sophisticated philosophical statement of the ethical significance that should be associated with that reaction was made in brief article by Kass commenting on the announcement of Dolly, the sheep cloned by the Roslyn Institute in 1997 [76]. Kass's central argument is that mammalian cloning elicits a repulsive reaction from many, and that this repugnance is sufficient ground to regard cloning as intrinsically wrong. In making this case, Kass relies on a conservative tradition in ethics that harks back to the philosophical writings of David Hume, Adam Smith, and Edmund Burke. These philosophers believed that morality was based on sentiments of sympathy with others, and that emotional attachments were a key component in any moral judgement. Although they lived and wrote in a pre-Darwinian culture, they also believed that emotional reactions like repugnance reflect a deep-seated and culturally ingrained wisdom. Societal stability is the result of respecting these emotional reactions, and departure from them entails the risk of upheaval and dissolution. Kass's argument has since formed the basis for a similar argument against applications of recombinant technology to foods [26, 99].
3. *Religious arguments*. Many people clearly attach religious significance to species boundaries and question the wisdom of genetic engineering [17]. Furthermore, many of the world's religions endorse specific injunctions against crossing species boundaries, interfering in reproductive processes, and consuming proscribed foods. Some of the most plausible ways of understanding the view that biotechnology is unnatural or that it tampers with the natural order against the demands of morality involve appeals to divine authority. Furthermore, worldviews that construe nature as bearing specific forms of moral significance may also be considered as resting on religious foundations, especially when they involve beliefs that are not amenable to scientific characterization and measurement. However, documents prepared by religious bodies, such as the Working Group on Genetic Engineering of the World Council of Churches Peace, Justice and Creation Team, tend to mix such explicitly religious arguments with con-

siderations that have been analyzed above under the heading of general technological ethics [190].

3 Evaluating Special Arguments

For the most part, professional philosophers have not been kind to the objection that biotechnology is unnatural. Straughan (1995) and Comstock (1998) review a series of ways to extend the claim that gene technology is unnatural into a more substantive ethical argument for regulating or restricting crop biotechnology. In each case, they find either that the substantive issues do not pertain specifically to the use of rDNA techniques for gene transfer, or that the characterization of naturalness is too vague and fails to exclude many well-accepted uses of technology. Rollin (1995) offers a similar analysis and characterizes arguments that appeal to the unnaturalness of gene transfer as "bad ethics". Sagoff (2001) has replied to the suggestion that biotechnology is unnatural by reviewing the four ways in which John Stuart Mill found that something could be said to be natural, arguing that for the most part, no judgment against biotechnology can be maintained without also tarnishing ordinary plant breeding, if not agriculture itself.

Philosopher Gifford (2000) has shown how conceptions of the gene as a carrier of human essence fail to correspond with the conception of genes that is operative in contemporary molecular biology. Scientific authors do not characterize the processes of cloning or genetic transformation in terms that would support the judgment that essences and *telos* are being affected. As such, there is a gap between the ethical understanding of nature implicit in philosophies that attribute essential or teleological significance to genes or gene processes, and the dominant scientific interpretation of the practices that constitute food and agricultural biotechnology. On the one hand, those who believe that genes have the ethical status of essence or *telos* have not shown how the idea can be made compatible with the scientific understanding of genes as sequences of DNA. One might argue that this line of criticism has reached a dead end until such an argument is forthcoming [175]. On the other hand, one might argue that until scientists and practitioners of biotechnology bear the burden of defending biology against traditional notions of purpose and essence that may still be very active in the worldview of non-scientists, it is entirely appropriate to oppose biotechnology on the ground that it is intrinsically wrong [166].

Sagoff's evaluation of the "naturalness" of biotechnology is relevant to repugnance arguments offered by Kass, Midgeley, and Chadwick. Sagoff (2001) agrees that there is one sense of what it means to be natural that allows us to

sort GM crops and animals into the unnatural basket while leaving traditional foods in the natural one: things can be “unnatural” in the sense of being inauthentic, not true to themselves. Here, Sagoff admits that a cultural tradition might find biotechnology to be unnatural in the sense of being inconsistent with a community’s aesthetic sensibilities. He argues that we should allow ourselves free reign to indulge our aesthetic tastes, but only under the condition that we recognize the full implications of doing so. But Sagoff also believes that the human and environmental costs of rejecting biotechnology would be significant. In spite of his own evaluation of the likely benefits of biotechnology, Sagoff would thus be forced to concede that biotechnology is “out” if indeed an informed public agreed with the repugnance claims made by Kass, Midgeley, and Chadwick. Streiffer and Hedemann (2005) believe that opinion research supporting the demand for labelling suggests that a majority of people have already found biotechnology to be intrinsically unacceptable, and argue that political decisions made in defiance of this finding are illegitimate.

The ethical significance of religious views can be pursued in two ways. First, one may examine the theological or doctrinal basis for this judgment, given the sacred texts, sectarian juridical processes, and doctrinal traditions of specific religions. Clearly, religious deliberations represent an important source of insight with respect to the application of cloning, genetic engineering, and other forms of gene technology to human beings [15, 106, 123, 190]. Second, one may simply acknowledge that the principle of religious tolerance affords people with wide latitude for deriving faith-based opinions on food and agricultural biotechnology, and inquire how these intrinsically personal ethical judgments entail social norms. Worldviews and ethical or spiritual beliefs about nature and natural order must be regarded as protected by principles of religious tolerance even if they do not derive from recognized religious traditions, churches, or theological traditions, and even if they do not involve belief in a supernatural power [166].

4
Responding to the Challenges of Agricultural and Food Biotechnology

The categories discussed so far illuminate possible impacts from the development of food or agricultural biotechnology. Further ethical issues arise when actions are taken to address or ameliorate these impacts. This section of the chapter will review three competing philosophical approaches to the management of such impacts. First, there is what might be called *mainstream risk governance*, an approach advocated by leading scientific organizations and utilized within United States and Canadian regulatory agencies. The thinking behind mainstream risk governance has been opposed by calls to implement the precautionary principle or a corresponding *precautionary approach* and

to require *labelling* of products of biotechnology. The point here is not to provide exhaustive discussions of these approaches, but to describe ethical philosophies implicit within each.

4.1 Mainstream Risk Governance

Products of biotechnology were first introduced in the United States and Canada, and the regulatory agencies and administrative law of these two countries have established a general philosophy of risk management through precedents established by a series of specific decisions made within the respective regulatory agencies. The principles of this philosophy have been articulated in a few early conceptual papers on the risks of agricultural biotechnology [1, 7], a series of U.S. National Research Council Reports [108, 109, 111], and in documents prepared by the Food and Agricultural Organization of the United Nations [41] and the Organization for Economic Cooperation and Development [115]. Advocacy for this approach has often adopted rhetoric characterizing it as "risk-based" or "science-based", implying that alternative perspectives lack scientific grounding [66, 102]. But the terminology can be confusing and inconsistent. For example, Indur Goklany provides an overview of mainstream risk governance in a 2000 white paper for the Center for the Study of American Business under the title "Applying the Precautionary Principle to Genetically Modified Crops" [53].

Utilitarian ethical theory proposes an approach in which decision makers attempt to characterize the likely consequences of a given course of action, and compute an "expectation" that reflects both the relative costs and benefits associated with each consequence as well as the likelihood that they will actually materialize. The ethically correct action is the one having the highest overall yield of expected benefit, happiness, or satisfaction, once expectations of cost, dissatisfaction, or harm have been subtracted. Three key values specify how this utilitarian framework has been applied in evaluating agricultural biotechnology. The mainstream approach is *outcome oriented*, *data driven*, and *comparative*. Arguably it is the last of these values that is most decisive for the relative strengths and weaknesses of mainstream risk governance.

The mainstream approach is outcome oriented in that it evaluates agricultural biotechnology strictly in terms of the expected costs and benefits of its use. This has the effect of excluding most of the issues described above as "special concerns" from the decision making process altogether. If the wrong doing in genetic engineering consists in simply doing it, rather than in some effect that it has on humans, animals, or the environment, the outcome-orientation of classical utilitarian thinking has no way to incorporate this wrong into its general framework. The mainstream approach is data driven in that strong preference is given to empirical studies that have measured

risks, as compared to speculative arguments that hypothesize risk. Analyses by Magnus and Caplan (2002), Streiffer and Hedemann (2005), and by Lacey (2005) argue that, while defenders of mainstream risk governance seem to equate outcome-oriented and data-driven decision making with rationality itself, adopting this approach to decision itself needs an explicitly articulated rationale.

But mainstream risk governance is also comparative in that expected risks and benefits from biotechnology are compared the known outcomes for the status quo. When known and suspected health and environmental effects from chemical-intensive agriculture are included in the expected outcome of conventionally bred crops, the comparative risks of transgenic crops may seem attractive [174]. When food deficits associated with low yields from traditional land races are calculated as part of their risk, the comparative risks of transgenic crops may seem attractive even in settings where chemicals and industrial production methods are little used [27]. In the absence of a comparative framework, it might seem silly to accept the risks that are being increasingly documented for transgenic crops [191]. It is thus the practice of applying roughly consistent standards of comparison both to transgenic and to traditional agricultural production methods that has been central to the relatively favourable regulatory decisions in mainstream risk governance.

At the same time, there are important gaps in the regulatory framework that governs risks from traditional agricultural technology. When the same framework is applied to transgenics, there may be hazards or exposures that are simply not addressed [91, 169]. What is more, socio-economic consequences associated with agricultural technology have never been taken into account in government regulatory decision making in the United States and Canada. This has arguably led to biased policy decisions, lingering resentment regarding the influence of economically powerful actors, and a decline in confidence that outcomes from the introduction of agricultural biotechnology will be appropriately steered [175]. Critics have argued that this gap is particularly significant in the developing world, where the costs (including infrastructure and liability risk) for introducing transgenic crops may be prohibitive [178]. If capitalist markets are the only mechanism for managing socio-economic risks associated with biotechnology, as the current practice of mainstream risk governance continues to imply, the approach may not live up to the principles endorsed in its utilitarian foundations.

4.2 Uncertainty and the Precautionary Principle

The Precautionary Principle is, perhaps, the most visible alternative to the mainstream approach in evaluating agricultural biotechnology. The definitive

statement is taken from the United Nations' Rio Declaration on Environment and Development:

"In order to protect the environment, the precautionary approach shall be widely applied by States according to their capabilities. Where there are threats of serious or irreversible damage, lack of full scientific certainty shall not be used as a reason for postponing cost-effective measures to prevent environmental degradation [179]."

This language implies that precaution is less a single principle or decision rule than a general philosophy which justifies aversion to risk when uncertainty is present. The Precautionary Principle is also often used as a reason to reject practices that have consequences that would be impossible or difficult to reverse or mitigate.

Some authors describe the Precautionary Principle simply as a preference for statistical and evidential burdens of proof that favour public and environmental health interests over commercial and industrial interests in cases where there is little scientific consensus on the levels of risk associated with a practice [32, 118]. Yet, it is also clear that precaution with respect to agricultural biotechnology often involves eschewing the technology at least until uncertainties in current estimates of risk have been substantially reduced [33]. Other authors identify precaution with the integration of ethical concerns into regulatory decision making [6, 116]. Following this line of thinking, others argue that a precautionary approach to uncertainty requires broader public participation in regulatory decision making [24]. The Royal Society of Canada (2001) report *Elements of Precaution* interprets precaution to explicitly endorse the inclusion of intrinsic objections to biotechnology within any consideration of its public acceptability.

Critics of the Precautionary Principle portray it as a decision rule that allows perception of hazard to override documented evidence for hazard in regulation and enforcement of international agreements [57]. This theme has been especially prominent in connection with biotechnology. Critics have described the precautionary approach as "unprincipled" [102], and as mandating contradictory advice concerning transgenic crops [31]. Philosopher Henk van den Belt has written a detailed overview of the debate over the precautionary principle in which he concludes that there is no basis on which any technology, including transgenic technology, could have met the burdens of proof being advanced under the banner of the precautionary approach. Van den Belt's analysis suggests that a distinctive feature of the precautionary approach is that it does not apply comparative or uniform standards in the evaluation of technological alternatives.

There are a number of ethical concerns that are interwoven in debates over the precautionary approach. One is the claim that there is a need to anticipate harm to persons and the environment in advance, and to take action that will forestall this harm. This is a theme that recurs frequently in statements of the Precautionary Principle, but it is not, in fact, a view that would be con-

tested by advocates of the opposing "risk-based" approach. The risk-based approach can be strongly committed to anticipatory action when the evidence warrants. A second concern notes that powerful commercial and industrial interests can influence the assumptions that are deployed in conducting scientific risk assessments. This, too, is a concern that has been voiced repeatedly by those who call not for an abandonment of risk assessment, but for a more objective implementation of risk-based decision making [16, 56, 93]. Thus, it is likely that at least some of the alleged incompatibility between a "risk-based" and a "precautionary" approach is terminological and rhetorical. This is not to minimize the importance of these two ethical concerns; indeed, the fact that they have long been a part of the attempt to develop an adequate approach to technological risk assessment only underscores their importance.

Nevertheless, there are several points on which it is fairly clear that mainstream and precautionary approaches diverge. For one, precautionary approaches do not uniformly, at least, appear to be limited to the outcome-oriented assumptions of mainstream risk analysis. The recognition of intrinsic objections and calls for participation in decision making suggest that non-consequential norms have a clear place in precautionary decision making. Another difference, noted by van den Belt, is that many who advocate a precautionary approach do not consider the comparative risks of transgenic and non-transgenic technology. However, some statements of a precautionary approach suggest that the mainstream approach has not sufficiently applied a comparative norm, arguing that the acceptability of risks from transgenic technology as compared to industrial agriculture begins to fade when the full range of organic and agro-ecological methods that are available for agricultural production are included in the mix [79, 86].

4.3 Consent, Labels, and Choice

While risk governance and precaution have dominated debates over the risks of biotechnology, it is important to note that debates over labels and consumer choice raise a third perspective that frames the acceptability of risk in terms of securing informed consent. The issue of consumer choice was introduced above in connection with food safety, but consumers may desire an alternative to GMOs for reasons that derive from repugnance or religious views, or to express solidarity with political movements focused on animals, ecology, and globalization, or family farms. Some argue that individual consumers must not be put in a position where they are unable to apply their own values in choosing whether to eat the products of biotechnology [67]. Others argue that the use or non-use of biotechnology is immaterial to the underlying values (especially safety and healthfulness) that are the basis of consumer choice [72]. Still others argue that the very act of informing consumers about

GMO foods would mislead consumers into making choices that are not consistent with the underlying purposes that are sought through the purchase and consumption of food [31].

This is a classic example of an ethical debate: one viewpoint stresses individual autonomy and consent, while another stresses rational optimization. The tension between these two ways of approaching ethical norms has been endemic to some of the most protracted ethical debates of the last 200 years. The utilitarian school of philosophical ethics has argued that choice that produces the best consequences is always the best one, while followers of Kant have argued that rational conduct requires respect for the autonomy of others, even when this may not lead to the best consequences, all things considered. While it is not plausible to suggest that ordinary people make systematic commitments to either utilitarian or autonomy-based ethical theory, it is not possible to grasp the ethical and political significance of the labelling debate without some appreciation of the contrast between these two perspectives. Early misrepresentations of the ethical issues involved with consumer consent were arguably the proximate cause for conflicts that remain as lingering problems for food and agricultural biotechnology. The Parliamentary Office Science and Technology (1998) report, the U.S. Congressional Research Service Report [184], and the Nuffield Council on Bioethics Report (1999) are examples of documents that discuss choice issues, but that fail to represent the argument from autonomy in fair terms.

This is not to say that arguments stressing utility neglect choice altogether. According to utilitarian ethical theory, rational individuals seek to maximize personal satisfaction through choice by selecting the course of action that has the best chance of producing an outcome consistent with their personal preferences. If some individuals would prefer so-called GMO-free products, a food system in which this option is available will better serve consumer preferences than one in which this choice is unavailable [107, 154]. This approach to choice puts a consumer's desire for GMO-free foods on an equal footing with other consumer preferences, such as the desire for inexpensive or tasty foods. Indeed, it is possible to use an argument from utility in support of the claim that a food system that precludes purchase of GMO foods is just as bad (from an ethics perspective) as one that denies choice to those who want GMO-free foods.

But this picture does not accurately portray the issues as they would be seen from the perspective of autonomy and consumer consent. Here, the underlying issue is that people should not be prevented from acting on values that are crucial to their personal identity or worldview. A system of choice that constrains a person's ability to act on the basis of religious beliefs, for example, compromises the principle of autonomy in ways that denial of opportunities for inexpensive or tasty food choices does not. The perspective of autonomy and consent demands an argument to show that food choices involve values

that are of deep importance to individuals – importance comparable to that of religious beliefs. But food beliefs are prevalent throughout religion and ethic culture, so this is not a difficult argument to make [26, 136, 165, 195].

5 Conclusion

This chapter has offered a framework for understanding the range of ethical concerns and for appreciating the value judgments that underlie conflicting opinions on the ethical responsibilities associated with food biotechnology. Hopefully, readers can appreciate the multiple bases of ethical concern as well as the extensive range of debate that has already occurred over the ethics of agricultural and food biotechnology. Although this summary discussion may seem to be overly detailed already, the points discussed here represent only a fraction of the voiced opinion and analysis. We conclude with a brief note on the ethics of public trust.

Many authors have noted that public attitudes and distrust of biotechnology or of science in general is the greatest single obstacle to its market acceptance and commercial success [10, 141, 187]. The social science literature on public trust in science builds upon points that have been discussed earlier – environmental impact, uncertainty, animal issues, social justice, and consumer consent. It suggests that the public does not trust the actors that promote food and agricultural biotechnology because they have exhibited ethical failings with respect to one or more of the issues noted [13, 47]. Commercial influence on the conduct of science, discussed above under the heading of "shifting power relations" is also tied to this decline in public trust [92]. Social science research also indicates high variability in the confidence accorded to the messages of activist groups. Non-governmental organizations or NGO's are among the most trusted sources of information for certain subpopulations, but totally untrusted by others [36].

Is there an ethical issue here? Philosophers such as Sandler [145] as well as one author of this chapter [175] have argued that the promoters of biotechnology have displayed an ethics deficit with respect to the virtue of trustworthiness. Trustworthy people display thoughtfulness of purpose and a clear capacity to be mindful of the interests of those by whom they are trusted. We do not trust people who seem to be making reference to their own immediate goals and self-interest at every moment [3]. If these criteria are extended to actors responsible for the development of food and agricultural biotechnology, those who always seem to be engaged in strategic promotion of biotechnology, and never in serious practical discussion, are not trustworthy. This is not a judgment that necessarily reflects on the moral character of the individuals involved. People who are morally trustworthy in their own right may well be involved in groups or associations that are untrustworthy

in virtue of the fact that serious discourse about ethical issues occurs infrequently. As such, the concluding message for this summary of ethical issues is that those involved in the scientific enterprise must earn the public's trust; attending to the full array of ethical issues associated with their science is a prerequisite for doing so.

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