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Hydrocarbon and Lipid Microbiology Protocols

Synthetic and Systems Biology – Applications

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Synthetic and Systems Biology - Applications

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Preface to Hydrocarbon and Lipid Microbiology Protocols[1]

All active cellular systems require water as the principal medium and solvent for their metabolic and ecophysiological activities. Hydrophobic compounds and structures, which tend to exclude water, although providing *inter alia* excellent sources of energy and a means of biological compartmentalization, present problems of cellular handling, poor bioavailability and, in some cases, toxicity. Microbes both synthesize and exploit a vast range of hydrophobic organics, which includes biogenic lipids, oils and volatile compounds, geochemically transformed organics of biological origin (i.e. petroleum and other fossil hydrocarbons) and manufactured industrial organics. The underlying interactions between microbes and hydrophobic compounds have major consequences not only for the lifestyles of the microbes involved but also for biogeochemistry, climate change, environmental pollution, human health and a range of biotechnological applications. The significance of this "greasy microbiology" is reflected in both the scale and breadth of research on the various aspects of the topic. Despite this, there was, as far as we know, no treatise available that covers the subject. In an attempt to capture the essence of greasy microbiology, the *Handbook of Hydrocarbon and Lipid Microbiology* (http://www.springer.com/life+sciences/microbiology/book/978-3-540-77584-3) was published by Springer in 2010 (Timmis 2010). This five-volume handbook is, we believe, unique and of considerable service to the community and its research endeavours, as evidenced by the large number of chapter downloads. Volume 5 of the handbook, unlike volumes 1–4 which summarize current knowledge on hydrocarbon microbiology, consists of a collection of experimental protocols and appendices pertinent to research on the topic.

A second edition of the handbook is now in preparation and a decision was taken to split off the methods section and publish it separately as part of the Springer Protocols program (http://www.springerprotocols.com/). The multi-volume work *Hydrocarbon and Lipid Microbiology Protocols*, while rooted in Volume 5 of the Handbook, has evolved significantly, in terms of range of topics, conceptual structure and protocol format. Research methods, as well as instrumentation and strategic approaches to problems and analyses, are evolving at an unprecedented pace, which can be bewildering for newcomers to the field and to experienced researchers desiring to take new approaches to problems. In attempting to be comprehensive – a one-stop source of protocols for research in greasy microbiology – the protocol volumes inevitably contain both subject-specific and more generic protocols, including sampling in the field, chemical analyses, detection of specific functional groups of microorganisms and community composition, isolation and cultivation of such organisms, biochemical analyses and activity measurements, ultrastructure and imaging methods, genetic and genomic analyses,

[1] Adapted in part from the Preface to *Handbook of Hydrocarbon and Lipid Microbiology*.

systems and synthetic biology tool usage, diverse applications, and the exploitation of bioinformatic, statistical and modelling tools. Thus, while the work is aimed at researchers working on the microbiology of hydrocarbons, lipids and other hydrophobic organics, much of it will be equally applicable to research in environmental microbiology and, indeed, microbiology in general. This, we believe, is a significant strength of these volumes.

We are extremely grateful to the members of our Scientific Advisory Board, who have made invaluable suggestions of topics and authors, as well as contributing protocols themselves, and to generous *ad hoc* advisors like Wei Huang, Manfred Auer and Lars Blank. We also express our appreciation of Jutta Lindenborn of Springer who steered this work with professionalism, patience and good humour.

Colchester, Essex, UK — Terry J. McGenity
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About the Editors

Terry J. McGenity is a Reader at the University of Essex, UK. His Ph.D., investigating the microbial ecology of ancient salt deposits (University of Leicester), was followed by postdoctoral positions at the Japan Marine Science and Technology Centre (JAMSTEC, Yokosuka) and the Postgraduate Research Institute for Sedimentology (University of Reading). His overarching research interest is to understand how microbial communities function and interact to influence major biogeochemical processes. He worked as a postdoc with Ken Timmis at the University of Essex, where he was inspired to investigate microbial interactions with hydrocarbons at multiple scales, from communities to cells, and as both a source of food and stress. He has broad interests in microbial ecology and diversity, particularly with respect to carbon cycling (especially the second most abundantly produced hydrocarbon in the atmosphere, isoprene), and is driven to better understand how microbes cope with, or flourish in hypersaline, desiccated and poly-extreme environments.

Kenneth N. Timmis read microbiology and obtained his Ph.D. at Bristol University, where he became fascinated with the topics of environmental microbiology and microbial pathogenesis, and their interface pathogen ecology. He undertook postdoctoral training at the Ruhr-University Bochum with Uli Winkler, Yale with Don Marvin, and Stanford with Stan Cohen, at the latter two institutions as a Fellow of the Helen Hay Whitney Foundation, where he acquired the tools and strategies of genetic approaches to investigate mechanisms and causal relationships underlying microbial activities. He was subsequently appointed Head of an Independent Research Group at the Max Planck Institute for Molecular Genetics in Berlin, then Professor of Biochemistry in the University of Geneva Faculty of Medicine. Thereafter, he became Director of the Division of Microbiology at the National Research Centre for Biotechnology (GBF)/now the Helmholtz Centre for Infection Research (HZI) and Professor of Microbiology at the Technical University Braunschweig. His group has worked for many years, *inter alia*, on the biodegradation of oil hydrocarbons, especially the genetics and regulation of toluene degradation, pioneered the genetic design and experimental evolution of novel catabolic activities, discovered the new group of marine hydrocarbonoclastic bacteria, and conducted early genome sequencing of bacteria that

became paradigms of microbes that degrade organic compounds (*Pseudomonas putida* and *Alcanivorax borkumensis*). He has had the privilege and pleasure of working with and learning from some of the most talented young scientists in environmental microbiology, a considerable number of which are contributing authors to this series, and in particular Balbina and Terry. He is Fellow of the Royal Society, Member of the EMBO, Recipient of the Erwin Schrödinger Prize, and Fellow of the American Academy of Microbiology and the European Academy of Microbiology. He founded the journals *Environmental Microbiology, Environmental Microbiology Reports* and *Microbial Biotechnology*. Kenneth Timmis is currently Emeritus Professor in the Institute of Microbiology at the Technical University of Braunschweig.

Balbina Nogales is a Lecturer at the University of the Balearic Islands, Spain. Her Ph.D. at the Autonomous University of Barcelona (Spain) investigated antagonistic relationships in anoxygenic sulphur photosynthetic bacteria. This was followed by postdoctoral positions in the research groups of Ken Timmis at the German National Biotechnology Institute (GBF, Braunschweig, Germany) and the University of Essex, where she joined Terry McGenity as postdoctoral scientist. During that time, she worked in different research projects on community diversity analysis of polluted environments. After moving to her current position, her research is focused on understanding microbial communities in chronically hydrocarbon-polluted marine environments, and elucidating the role in the degradation of hydrocarbons of certain groups of marine bacteria not recognized as typical degraders.

Introduction to Systems and Synthetic Biology in Hydrocarbon Microbiology: Applications

Víctor de Lorenzo

Abstract

Contemporary Microbial Biotechnology is experiencing a rapid transition between being a mostly trial-and-error endeavour towards becoming a quantitative and predictable branch of contemporary research. A key ingredient of this shift involves the adoption of Systems and Synthetic Biology approaches for either revisiting typical themes (e.g. bioproduction of added-value molecules) or to develop altogether new ones (such as engineering of sensor/actuator devices). The first wave of goods reaching the biotechnological sector largely comes from metabolic engineering of microorganisms for biofuels, fine chemicals and high-added value molecules. But much more is still to come by applying electric and industrial engineering principles to biological systems as well as by learning from the way natural evolution has solved apparently intractable design problems.

Keywords: Biodegradation, Biofuels, Biosensors, Containment, Logic gates, New materials

1 Environmental Microorganisms Are at the Basis of Modern Biotechnology

The production by Chaim Weizmann of acetone by fermentation of straw and corn stalks with *Clostridium acetobutylicum* [1] is often considered one of the foundational events in the history of modern, scientifically grounded biotechnology. This first wave of bio-based products was enhanced by various orders of magnitude with the later incorporation of recombinant DNA technology in the early 1980s. Instead of relying on extant microbes or their erratically generated mutants restriction-based cloning techniques allowed rationally moving genes between hosts and generate new combinations for the sake of producing added-value molecules. It was during this period of time that the Laboratory of K.N. Timmis made the by then groundbreaking proposition that genetic engineering (GE) could be exploited not only for making bacteria to produce something precious in a bioreactor, but also for releasing them into the environment to get rid of chemical pollution [2, 3]. Even before the official birth of modern genetic engineering with the 1973

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arch-famous paper of Cohen et al. [4], this scenario had been forestalled by Chakrabarty as early as 1972 [5]. What he did was breeding a *Pseudomonas* strain in the Laboratory able to degrade some oil components and therefore applicable to remediate petroleum spills. General Electric filed a patent application on the basis of this work that ignited a long procedure around the legality of claiming intellectual property rights on a living entity. After nearly 10 years, the *Diamond* vs. *Chakrabarty* case resulted in the granting of patent US4259444A, the first of its kind [5]. While this affair anticipated many of the ensuing controversies on the design of GMOs, it contained also some themes on the genetic design of biotechnological agents that reach us to this day. First, it pinpointed environmental bacteria as a fundamental source of strains (and therefore, of genes) empowered with extraordinary activities that could be enhanced in the Laboratory for useful purposes. Second, it identified Molecular Genetics (and later, DNA cloning) as the technology of choice for bringing traditional biotechnology to a new stage of development. And third, it also exposed the limitations of the earlier procedures for generating useful bacteria: the two oil-super degraders *Pseudomonas* strains *P. aeruginosa* NRRL B-5472 and *P. putida* NRRL B-5473 hardly ever made it to actual applications. But despite these downsides, the *zeitgeist* remained that genetic engineering could be adopted as a way to solve not only oil spills, but also virtually any other serious problem facing our industrial society [6].

This was the time when the work of Timmis's Laboratory made a great impact, as it proved for the first time that rational genetic design could indeed be instrumental to both enhance native capabilities of environmental bacteria and generate strains with altogether new traits [2, 3, 7]. Alas, the field soon discovered the difficulties of engineering a whole bacterium – not just a few of its genes, to perform a pre-determined function under working conditions on which there was little or no control (e.g. environmental release for bioremediation). The complexity associated to such a purpose not only involved genetic engineering proper, but also physiology, population ecology and other layers of a complexity pyramid from genomes to landscapes that could not be tackled by the technologies and conceptual frames of the time [8]. Efforts then returned to optimization of typical laboratory bacteria (e.g. *E. coli*) or yeast (*S. cerevisiae*) for producing valuable molecules in a reactor. The >20 years of the *golden time* of what we may call the *traditional* product-oriented GE-based Biotechnology can be arbitrarily set between 1985 and 2006, punctuated by the Lin et al. paper [9] on production of erythropoietin in *E. coli* and the 2006 article from Keasling's Laboratory on production of artemisinic acid in engineered yeast [10]. During this period, other platform strains for biotechnological operations (e.g. *Streptomyces*, *Bacillus*, *Corynebacteria*, *Pseudomonas*) benefited also from

growingly available genetic tools and the access to better cloning and heterologous expression systems. Still, inherent biological complexity imposed a limit to the extent to which live systems could be engineered in a predictable fashion. But this was bound to change with the onset of Systems and Synthetic Biology.

2 Looking at (Micro)Biological Complexity from an Engineering Perspective

Systems Biology is a conceptual umbrella – as well as a collection of technologies that attempt to understand biological systems as wholes rather than as additions of separate components. A more detailed discussion on this paradigm can be found in other chapters of this Protocol series and will not be tackled again here. What is relevant for the sake of this section is the opportunity brought about by Systems Biology to gain a much better comprehension of the relational logic of existing live entities. This means also amazing possibilities to rewire and reshape such systems for improving their properties or for creating altogether new ones, as ambitioned by Synthetic Biology. The last exacerbates the adoption of engineering as an interpretative frame so that it stops being an analogy (like in GE) to become a veritable methodology [11, 12]. In this context, the claim of Synthetic Biology is that engineering principles allow understanding of extant biological objects as well as their rational refactoring for deploying new and predictable qualities. In its more extreme view, Synthetic Biology ambitions to make Biology a branch of Engineering – not in vain the pioneers of the field were not biologists but electric, computational and industrial engineers [12]. But what all this has to do with Environmental Biotechnology?

There are at least three areas where present-day Systems and Synthetic Biology are delivering new perspectives to the field. First, it allows to generate, classify and visualize the big volume of data resulting from all types of *omics* technologies when applied to single microbes, communities and entire microbiomes of the most diverse environmental niches. The perspective is that such data analysis will eventually reflect some patterns that will reveal new knowledge on the object of study. In the meantime, the metagenome is becoming a source of new activities of biotechnological interest that can be mined either through functional screenings or bioinformatic analyses [13]. A second dividend of the most recent Systems and Synthetic Biology is the identification of new candidate species to become genomic and biochemical *chassis* for industrial Biotechnology. One exemplary case is that of *Pseudomonas putida*. This species thrives soil and roots of plants growing in sites with a history of pollution by chemical waste. This has created a selective pressure for naturally evolving a collection of traits that are most desirable to endure the stress typically found in a reactor, including solvent

tolerance [14]. Furthermore, metabolic modelling and measurement of its central carbon fluxes has revealed that the default state of the biochemical network of *P. putida* (and surely many other environmental bacteria) is that of an excess of NAD(P)H [15]. Since this cofactor fuels many of the enzymes that counteract oxidative stress, the species becomes an optimal host for engineering redox biotransformations.

3 Systems and Synthetic Biology Inspire Environmental Biotechnology and Vice Versa

There is still a third outcome of the engineering approach discussed above: we can now revisit many issues that were left unanswered or just abandoned by the late 1990s because their complexity did not allow their proper handling with the technologies of the time. One of them is the lack of predictability of the performance and fate of GE microorganisms once they are released into the environment for, e.g., bioremediation [16]. While public concerns use to be focused on their safety and even security problems, reality is that virtually all attempts to show that such engineered bacteria could actually solve pollution problems have ended up in failure. In many cases, the reasons why they went wrong have been identified, but not really solved yet. However, the new approaches enunciated above permit a fresh look and entertain possible solutions. The key one is that the engineered modules genetically implanted in the environmental strain of choice cause different degrees of metabolic burden that make bacteria less fit once released. While this is not an issue when maintenance of such modules are subject to the selecting pressure of a bioreactor, the challenge becomes serious when GE microbes are placed on a site already colonized by other bacteria and on which physicochemical conditions we have little or no control. This challenge has made *bio-attenuation* (let nature do its job without much interfering) and *bio-stimulation* (promote the overgrowth or activity of specific microbial groups by adding nutrients or fertilizers) to be much more useful thus far than *bio-augmentation* (direct inoculation of a site with a new microbial agent, recombinant or not). But this could change if the field capitalizes on the growing body of conceptual and material tools that stem from Synthetic Biology.

There are quite a few scenarios in which one can foresee a mutual fertilization between the new interpretative frames of Biology argued before and environmental applications. For instance, it is now possible to model the mutual interplay (called *retroactivity*) between a pre-existing genetic and biochemical network and a new engineered circuit on the basis of mutual competition for cell resources [17]. The physiological load of such implants can in fact

be quantified with dedicated reporter systems [18]. But this scenario is not exclusive of artificial setups: retroactivity does occur every time that a mobile genetic element encoding new metabolic capabilities (e.g. a catabolic plasmid) enters a new host in a natural environment. The work on this issue done with the TOL plasmid pWW0 for degradation of *m*-xylene has exposed a complete collection of mechanisms that ease the co-existence between the native and the new biochemical network. This includes regulatory interlocks [19], enzymatic safety valves [20] and even mechanisms to promote genetic diversification at the time of acquiring the incoming genes [21]. Some of these results can have a value for designing artificial modules that are perfectly bearable by the new carrier instead of being detrimental. In the cases where such a co-existence cannot be engineered, Synthetic Biology proposes adoption of *orthogonal* (i.e. autonomous from the host) genetic devices with a minimum interference with the biological carrier [22]. Where can one find suitable parts for assembling such constructs? Many promiscuous mobile genetic elements have in fact evolved to move between different hosts with a minimum or interference with the innate enzymatic and regulatory network. On this basis, many of them have been re-purposed as constituents of a plethora of synthetic circuits [23]. Understanding structural and functional features of genes that are specific of naturally promiscuous plasmids will surely reveal new principles of great utility for engineering new live systems.

Finally, *containment* is yet another area of interest where Synthetic Biology meets Environmental Microbiology. A sector of the public demands the field to keep a close eye on possible risks associated to the accidental or deliberate release of synthetic constructs [24]. On one hand, this is a re-enactment of the same discussions that took place during the birth of Genetic Engineering in the late 1970s [25] and the prospects of releasing GM bacteria for environmental and agricultural uses ten years later [6, 16]. On the other hand, the ease of DNA synthesis that we currently enjoy upgrades former concerns on its possible malicious use and other would-be dangers. This happens at a time when the do-it-yourself-Biology is proposed as a way to democratize research and adopt genetic engineering as a personal endeavour to be done at home – as was the case with personal computers in the not so distant past [26]. The widespreading and simplification of the CRISPR/Cas9 technology of genome editing and its application to virtually all genomes, from bacteria to humans, has once more rung the bells of potential threats of safety and security [27]. This has in turn stimulated the quest for much better firewalls to curb the spread of designed organisms, the ultimate objective being a Certainty of Containment, i.e., zero chances of scape [28]. Approaches to this end include development of alternative genetic codes, essential proteins with non-natural amino acids and replacement of DNA

by xeno-nucleic acids as the information-bearing molecule of living systems. While all these research fronts are very active at the time of writing this article, their actual success when placed in real natural settings remains to be accredited.

4 Outlook

Systems and Synthetic Biology are amazingly dynamic fields of development at the time of writing this article. The Protocols collected in this volume provide but just a small sample of how quantitative approaches to strain design change the way we domesticate environmental bacteria for given biotechnological purposes. Not surprisingly, the low-hanging fruits are to be found in the realm of metabolic engineering, a field that is well familiar with the use of bacteria other than *E. coli* as platforms for synthesis of added-value products. The panel of cases examined below go from biosynthesis of bulk chemicals such as isoprenoids [29], esters [30] and rhamnolipids [31], to biotransformations for adding value to common substrates [32] and finally to produce more sophisticated molecules like lantibiotics [33]. New materials with awesome properties can also be produced by deeply refactored bacteria [34]. All these articles exemplify the benefits of incorporating new tools and approaches to somewhat traditional areas of biotechnological activity. But there is much more that can be done. [35] shows how rewiring and repurposing transcription factors one can generate biosensors for detecting small intracellular molecules. These sensing devices are invaluable for a variety of applications, as they can control expression of biological activities in response to specific endogenous or exogenous stimuli. This has been exploited inter alia for functional screening of metagenomic libraries in which occurrence of specific reactions (and thus of new molecular species) can be translated into detectable/selectable phenotypes caused by a reporter gene [13, 35]. It is very likely that regulatory components of environmental bacteria will soon be upgraded to form logic gates and decision-making devices to be coupled to actuators in the same fashion that has been recently proposed for *Bacteroides thetaiotaomicron* [36]. This will hopefully enable to design smart whole cell catalysts that will detect and react to environmental cues in a pre-programmed fashion. This may ultimately solve many of the problems found in the early days of genetic engineering for environmental applications, as discussed above. The following chapters represent an early token of the developments in this respect that we will witness in full in the not so distant future.

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Designing Bacteria to Produce Esters

Yi-Shu Tai and Kechun Zhang

Abstract

Medium-chain esters such as isobutyl acetate (IBAc) and isoamyl acetate (IAAc) are high-volume solvents, flavors, and fragrances. Compared to long-chain esters, these short-chain esters are more volatile and are flavor components of many fruits. For example, IAAc has banana flavor and is widely used as food or beverage additives. Currently, they are mainly produced from petroleum feedstocks. Alternatively, metabolic engineering enables the total biosynthesis of IBAc and IAAc directly from glucose in *Escherichia coli*. The pathways harnessed the power of natural amino acid biosynthesis. In particular, the native valine and leucine pathways in *E. coli* were utilized to supply precursors. The key enzyme alcohol *O*-acyltransferases (AAT) will then catalyze esterification reactions to produce IBAc and IAAc. In vitro biochemical characterization of AAT can provide rational guidance for future enzyme engineering or identify new enzymes for other target substrates. The below protocol provides the detailed description of expression and purification of AAT, in vitro enzymatic assays, direct biosyntheses of IBAc or IAAc in *E. coli* from glucose, and scale-up production of these valuable products in a benchtop bioreactor.

Keywords: Alcohol acyltransferase (AAT), Biofuel, Esters, Metabolic engineering, Pathway manipulation, Renewable chemicals

1 Introduction

Petroleum is the material basis of modern society that not only fuels our vehicles but also provides precursors for innumerable consumer products. However, concerns such as growing demands, dwindling reserves, and environmental impacts drive the transition from this petroleum-based economy to a green, bio-based economy. Metabolic engineering is a powerful tool that can facilitate the transition [1–3].

Here, medium-chain esters (C6–C10) are selected as the target compounds for biosynthesis in *Escherichia coli* [4]. These volatile chemicals have versatile applications. They can be added in foods and beverages as flavor enhancers. For cosmetic and fragrance industries, these esters are used to create fruity or floral aromas. The global market of flavors and fragrances was $25.3 billion in 2014 (http://www.bccresearch.com/). Moreover, esters can be

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used for coatings, solvents, and potential advanced biofuels. They have high energy content and are fully compatible with existing infrastructure [5]. The industrial production of these esters is currently dominated by Fischer esterification. Fischer esterification is a condensation reaction of a carboxylic acid and an alcohol catalyzed by a strong acid such as sulfuric acid with a reaction temperature between 60 and 110°C. This chemical synthesis process is environmentally unfriendly due to the requirement of petroleum-derived feedstocks, corrosive acid, and high reaction temperature.

Several other processes have been developed, but they all have different limitations. For example, esters extracted from plant materials are often in short supply [6]. Enzymatic synthesis which uses lipase or esterase can catalyze the same condensation reaction as Fischer esterification to produce medium-chain esters. However, the reaction thermodynamically favors hydrolysis of the ester in aqueous solution. Therefore, the production reactions are usually performed in organic solvents such as *n*-hexane to prevent ester hydrolysis [7, 8]. This makes the process environmentally unfriendly. Alternatively, whole-cell biocatalysis is an attractive approach since the reactions are catalyzed under ambient temperature and in aqueous solution. Recently, there are several studies on the production of isoamyl acetate by supplementing 3-methyl-1-butanol (3 MB) to engineered *E. coli* overexpressing alcohol *O*-acyltransferase (AAT) [9–11]. Both pathway and cofactor manipulations were implemented to enhance the production. Similarly, lipase has also been applied by whole-cell biocatalysis to produce isoamyl acetate with supplemented 3 MB [12]. Since 3 MB (a petroleum-derived chemical) was fed to fermentation culture in both cases, these processes are not renewable.

To improve the whole-cell biocatalysis process and make it greener and renewable, here we describe the design and engineering of metabolic pathways for the direct biosynthesis of isobutyl acetate (IBAc) and isoamyl acetate (IAAc) from glucose in *E. coli*. These pathways exploit amino acid pathways to generate 2-keto acids. The metabolic pathways to IBAc and IAAc production were constructed by expanding native valine and leucine biosynthetic pathways in *E. coli* as shown in (Fig. 1) (*see* **Note 1**). The key enzyme to realize the complete pathways is alcohol *O*-acyltransferase (AAT) which catalyzes the condensation of an alcohol with an acetyl-CoA to form esters. The leaving CoA group makes the reaction step thermodynamically favorable, unlike lipase-catalyzed reactions [13].

Based on the metabolic pathways, four plasmids can be constructed as shown in (Fig. 1). Pathway enzymes that fulfill the direct production of IBAc from glucose are expressed in plasmids pIBAc and pZE-kivd-yqhD (Fig. 2a, c) (*see* **Note 2**). Pathway enzymes for IAAc production can be expressed in plasmids pIAAc and pZE-kivd-yqhD (Fig. 2b, c). Plasmid pZE-His-AAT (Fig. 2d)

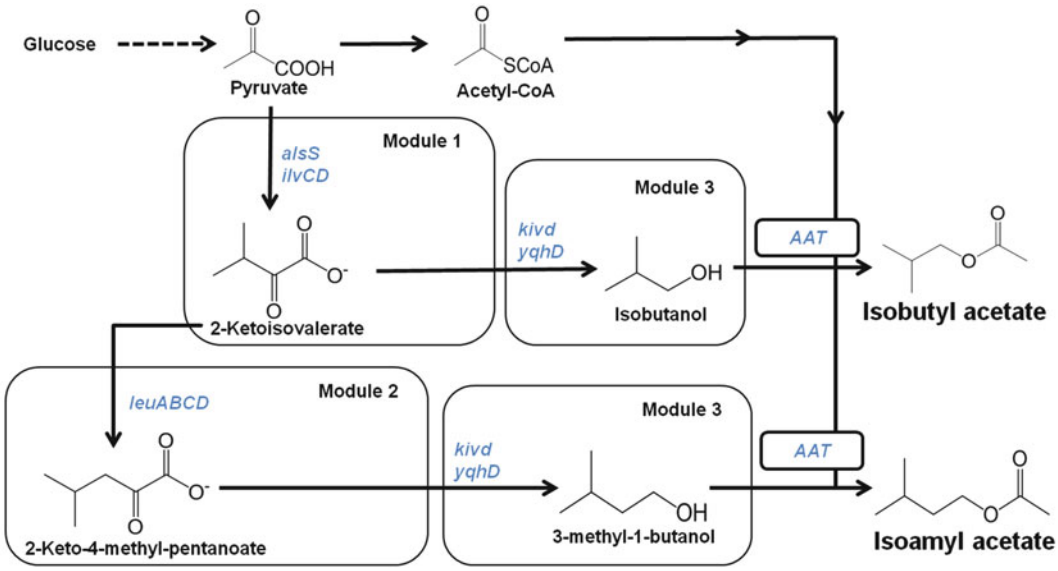

Fig. 1 Metabolic pathways of the target esters, isobutyl acetate (IBAc) and isoamyl acetate (IAAc) from glucose. Module 1 is valine biosynthesis pathway that converts pyruvate into 2-ketoisovalerate with three enzymes AlsS, IlvC, and IlvD. Module 2 is leucine biosynthesis pathway that adds one more carbon to 2-ketoisovalerate and converts it into 2-keto-4-methyl-pentanoate by LeuABCD. Module 3 represents the last two steps in the Ehrlich pathway which convert the 2-keto acids into their corresponding alcohol. Alcohol *O*-acyltransferase (AAT) then catalyzes the condensation of an alcohol and an acetyl-CoA to form IBAc or IAAc

(*see* **Note 3**) is used to produce AAT that can then be purified and used for in vitro enzymatic assays. In this chapter, we describe the expression and purification of AAT, in vitro assays of AAT to determine its kinetic parameters, in vivo production of IBAc and IAAc in shake flasks, and scale-up production of these esters in a 1.3-L benchtop bioreactor.

2 Materials (*see* Note 4)

2.1 Plasmid Construction

1. Vectors: pIBAc or pIAAc previously digested with *Blp*I restriction enzyme. pZE-His-AAT previously digested with *BamH*I and *Xba*I restriction enzymes.
2. Primers: *Blp*I-AAT-For (5′-cgaaagctctctaa**GCTGAGC**aggagaaattaact-AAT sequence-3′) and *Blp*I-AAT-Rev (5′-agcctttcgttttatttgatgcctctaga**GCTCAGC**-AAT sequence-3′) for amplification of AAT. His-AAT-For (5′-gagaggatcgCATCACCATCACCATCAC**GGATCC**-AAT-3′) and His-AAT-Rev (5′-gactgagcctttcgttttatttgatgcc**TCTAGA**-AAT-3′) for amplification of His-AAT.

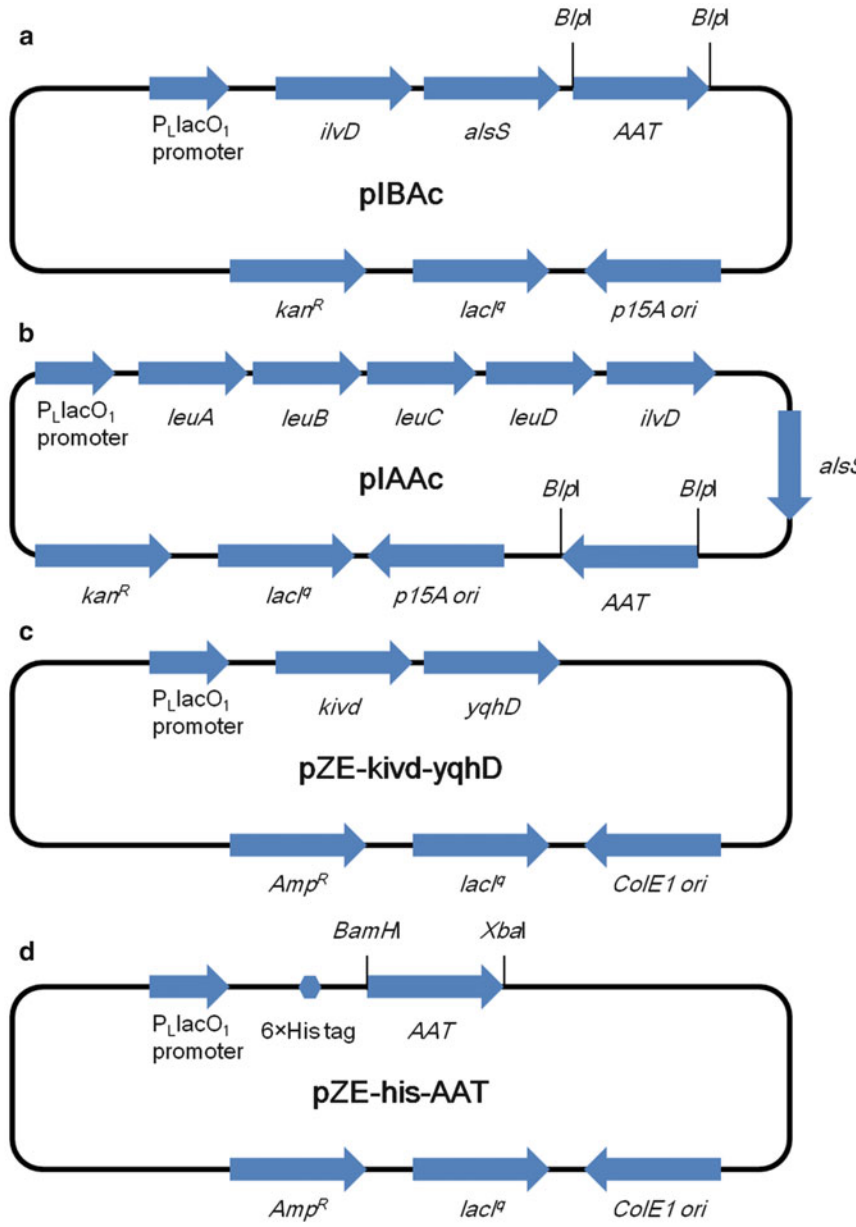

Fig. 2 Maps of the plasmids used in the protocol. Plasmids pIBAc (**a**) and pIAAc (**b**) are kanamycin resistance carrying *p15A* replicon-based medium copy number plasmids. Plasmids pZE-kivd-yqhD (**c**) and pZE-His-AAT (**d**) are ampicillin resistance carrying *ColE1* replicon-based high copy number plasmids

3. PCR templates: *E. coli* K-12 genomic DNA and genomic DNA containing AAT fragment of interest or synthetic AAT fragment codon-optimized for expression in *E. coli*.
4. Phusion® High-Fidelity PCR Kit (New England Biolabs (http://www.neb.com)).

5. Gibson Assembly® Mater Mix (New England Biolabs (http://www.neb.com)).
6. Quick Ligation™ Kit (New England Biolabs (http://www.neb.com)).
7. Restriction enzymes: FastDigest *Blp*I, *BamH*I, and *Xba*I (Thermo Scientific (www.thermoscientific.com/)).
8. Zymoclean™ Gel DNA Recovery Kit (Zymo Research (https://www.zymoresearch.com/)).
9. Zyppy™ Plasmid Miniprep Kit (Zymo Research (https://www.zymoresearch.com/)).
10. Mix & Go *E. coli* Transformation Buffer Set (Zymo Research (https://www.zymoresearch.com/)) is used for making chemically competent cells.
11. Bacterial strains: *E. coli* XL10-Gold strain (Stratagene (http://www.agilent.com/)) is used for cloning.
12. Antibiotics: ampicillin, kanamycin antibiotics solution (*see* Subheading 2.8).
13. Growth media: super optimal broth (SOB), yeast extract tryptone × 2 (2 × YT) (*see* Subheading 2.9).

2.2 Protein Expression

1. Vectors: pZE-His-AAT.
2. Bacteria strain: *E. coli* Rosetta 2(DE3) (EMD Millipore (http://www.merckmillipore.com/)).
3. Antibiotics: ampicillin antibiotic (*see* Subheading 2.8).
4. Growth media: Luria–Bertani (LB) growth media (*see* Subheading 2.9).
5. Isopropyl β-D-1-thiogalactopyranoside (IPTG): use 0.5 mM to induce AAT expression (*see* Subheading 2.8).

2.3 Protein Purification

1. Lysis buffer: 50 mM Tris–HCl, 100 mM NaCl, 10 mM imidazole, 5% glycerol, 1 mM DTT, adjust pH to 7.6.
2. Wash buffer: 50 mM Tris–HCl, 100 mM NaCl, and 25 mM imidazole, adjust pH to 7.6.
3. Elution buffer: 50 mM Tris–HCl, 250 mM NaCl, and 250 mM imidazole, adjust pH to 8.0.
4. Storage buffer: 50 μM Tris–HCl, 2 mM $MgSO_4$, adjust pH to 8.
5. HisPur Ni-NTA resin (Thermo Scientific (www.thermoscientific.com/)). Store at 4°C.
6. Sonication device: Heat Systems Ultrasonics W-225 Sonicator.
7. CrystalCruz™ Chromatography Columns, 1.5 × 15 cm (Santa Cruz Biotechnology, Inc. (http://www.scbt.com/)).

8. Amicon Ultra-15 centrifugal filter devices (Millipore (http://www.emdmillipore.com/)).
9. Quick Start Bradford protein assay kit (Bio-Rad (www.bio-rad.com/)).
10. Glycerol (Sigma (http://www.sigmaaldrich.com)): prepare 87 v% solution; sterilize by autoclave.

2.4 Enzyme Assay

1. Assay buffer: 100 mM KCl, 2 mM $MgCl_2$, 50 mM Tris–HCl in water, adjust pH to 8.0. (All the reagents were purchased from Sigma (http://www.sigmaaldrich.com).)
2. Acetyl coenzyme A sodium salt solution (Sigma (http://www.sigmaaldrich.com)): 10 mM in water. Store at −80°C.
3. Isobutanol (BioUltra, for molecular biology, Sigma (http://www.sigmaaldrich.com)).
4. 3-Methyl-1-butanol (anhydrous, Sigma (http://www.sigmaaldrich.com)).
5. 5,5′-Dithiobis-(2-nitrobenzoic acid) (DTNB) solution (Sigma (http://www.sigmaaldrich.com)): 1 mM in water, make fresh as required.
6. Greiner UV-Star® 96-well plates (Sigma (http://www.sigmaaldrich.com)).

2.5 Shake Flask Fermentation

1. Bacteria strain: Wild-type (WT) *E. coli* indicates strain BW25113.
2. 5 × M9 salts: 64 g Na2HPO4 · H_2O, 15 g KH_2PO_4, 2.5 g NaCl, 5.0 g NH_4Cl/L water. (All the reagents were purchased from Sigma (http://www.sigmaaldrich.com)).
3. Yeast extract solution: 5 g yeast extract/L.
4. Glucose solution: 400 g glucose/L.
5. Antibiotics: ampicillin, kanamycin antibiotics (*see* Subheading 2.8).
6. Thiamine hydrochloride solution (Sigma (http://www.sigmaaldrich.com)): 10 mg/mL in sterile double-distilled (Milli-Q) water, filtered. Store at −20°C.
7. Modified trace metal solution (*see* Subheading 2.8).
8. $CaCO_3$ (Sigma (http://www.sigmaaldrich.com)).
9. Parafilm® M (Sigma (http://www.sigmaaldrich.com)).

2.6 Benchtop Bioreactor Fermentation

1. Glucose feeding solution: 600 g glucose, 7.4 g K_2HPO_4, 1 mL modified trace metal solution/L water, sterilize by autoclave.
2. 28 w/v% NH_4OH solution (Sigma (http://www.sigmaaldrich.com)).

3. Bioreactor media: 7.5 g K_2HPO_4, 2 g $MgSO_4 \cdot 7H_2O$, 2 g citric acid $\cdot H_2O$, 0.3 g ferric ammonium citrate, 20 g yeast extract, 0.8 mL 98% sulfuric acid, 1 mL modified trace metal solution, 1 mL vitamin solution/L water.
4. Eppendorf BioFlo® 115 benchtop bioreactor and fermentor (Eppendorf (http://www.eppendorf.com/)).
5. Growth media: Luria–Bertani (LB) growth media (*see* Subheading 2.9).
6. Modified trace metal solution (*see* Subheading 2.8).
7. Vitamin solution: thiamine 1 g hydrochloride, 1 g D-(+)-biotin, 1 g nicotinic acid, 4 g pyridoxine hydrochloride/L water.
8. Oleyl alcohol (Sigma (http://www.sigmaaldrich.com)).

2.7 Metabolite Detection

1. Mobile phase: 5 mM H_2SO_4 in water (Sigma (http://www.sigmaaldrich.com)).
2. Hexane (Sigma (http://www.sigmaaldrich.com)) used for extraction.
3. Agilent 1260 Infinity HPLC with a differential refractive detector (RID) (Agilent Technologies (http://www.agilent.com)).
4. HPLC column: Aminex HPX 87H column (Bio-Rad (www.bio-rad.com)).
5. Hewlett Packard (HP) 6890 gas chromatograph (GC) equipped with a flame ionization detector (FID) (Agilent Technologies (http://www.agilent.com)).
6. GC column: DB-WAX capillary column (30 m, 0.32 mm inside diameter, 0.50 μm film thickness) (Agilent Technologies (http://www.agilent.com)).

2.8 General Buffers and Reagents

1. Ampicillin (Fisher Scientific (http://www.fishersci.com/)): 100 mg/mL in water. Store at −20°C.
2. Kanamycin (VWR (http://us.vwr.com/)): 50 mg/mL in water. Store at −20°C.
3. Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Fisher Scientific (http://www.fishersci.com/)): 1 M in sterile double-distilled (Milli-Q) water stored in 0.5 mL aliquots at −20°C.
4. Modified trace metal solution: 10 g NaCl, 40 g citric acid, 1 g $ZnSO_4 \cdot 7H_2O$, 30 g $MnSO_4 \cdot H_2O$, 30; 0.1 g $CuSO_4 \cdot 5H_2O$; 0.1 g H_3BO_3, 0.1 g $Na_2MoO_4 \cdot 2H_2O$, 1 g $FeSO_4 \cdot 7H_2O$, 1 g $CoCl_2 \cdot 6H_2O$/L water. Sterilize by autoclave.

2.9 Bacteria Growth Media

1. 2 × YT: 16 g tryptone plus, 10 g yeast extract, 5 g NaCl/L water.
2. LB: 10 g tryptone plus, 5 g yeast extract, 10 g NaCl/L water.

3. SOB: 20 g tryptone plus, 5 g yeast extract, 0.6 g NaCl, 0.186 g KCl, 2.4 g $MgSO_4$/L water, adjust pH to 7; sterilize by autoclave.

To prepare solid media using Petri dishes, agar at the final concentration of 1.5% was added to the LB media. After the autoclave, wait until the media cool down to about 50°C. Then add antibiotics as desired. Final concentrations of the antibiotics used in this study are as follows: ampicillin 100 μg/mL and kanamycin 50 μg/mL.

3 Methods

3.1 Plasmid Construction

1. The AAT of interest should be amplified by PCR using *Blp*I-AAT-For and *Blp*I-AAT-Rev primers (*see* Subheading 2.1). His-AAT is amplified by PCR using primers His-AAT-For and His-AAT-Rev (*see* Subheading 2.1). The PCR reaction conditions are 98°C for 30 s, 30 cycles of 98°C for 10 s and 72°C for 1 min, and a final extension of 72°C for 5 min. The reaction volume is 50 μL.
2. Purify the resulted PCR product using a gel DNA recovery kit (*see* **Note 5**).
3. Clone the AAT insert into previously *Blp*I-digested pIBAc or pIAAc vector by Gibson assembly. Clone the His-AAT insert into previously *BamH*I- and *Xba*I-digested pZE-His-AAT vector by Gibson assembly.
4. Transform the assembly product into XL10-Gold competent cells prepared with SOB and Mix & Go *E. coli* Transformation Buffer Set.

3.2 Protein Expression

1. Transform Rosetta 2 (DE3) competent cells with pZE-HisAAT vectors and plate the transformed cells on LB-agar plates containing 100 mg/L ampicillin.
2. Leave the plates at 37°C overnight (about 12–16 h) until colonies of transformed bacteria are clearly visible.
3. Overnight cultures with colonies streaked off the plates are inoculated 1% in 200 mL 2 × YT containing 100 mg/L ampicillin in a 500 mL baffled Erlenmeyer flask.
4. Grow cells to OD_{600} = 0.6–1.0 (about 3–4 h) at 37°C in a rotary shaker (250 rpm).
5. Add IPTG to the culture to 0.5 m final concentration to induce protein expression.
6. Continue protein production in a rotary shaker (250 rpm) at 30°C for 4 h.

7. Pellet the cells by centrifugation at 3,220 rcf for 15 min. For centrifugation, we use 5810 R refrigerated centrifuge (Eppendorf (http://www.eppendorf.com/)).
8. Cell pellet can be stored at −80°C for several weeks or you can immediately proceed to the cell lysis in protein purification.

3.3 Protein Purification

1. All the following steps are carried out on ice or at 4°C to prevent protein degradation.
2. Resuspend the cell pellet with 15 mL lysis buffer (*see* Subheading 2.3).
3. Lyse cells by sonication. Sonicate each sample with six 1 min cycles with intermittent 1 min rest on ice.
4. Centrifuge at 10,733 rcf for 15 min at 4°C to remove the insoluble part of the cell lysate. For centrifugation, we use 5810 R refrigerated centrifuge (Eppendorf (http://www.eppendorf.com/)).
5. Transfer the soluble fraction to a clean 50 mL conical tube and keep it on ice.
6. Prepare the gravity chromatography column by loading 4 mL HisPur Ni-NTA resin slurry.
7. Allow 20% ethanol (used to make the 50% slurry of Ni-NTA resin) to pass through by gravity to get a 2 mL final resin bed volume.
8. Equilibrate the beads with 10 mL lysis buffer. Allow buffer to drain from the column.
9. Add the prepared protein extract to the resin and collect the flow-through in a 15 mL conical tube.
10. Reapply the flow-through to the resin bed once to maximize binding.
11. Wash the column twice with 20 mL of wash buffer.
12. Elute the bound AAT protein with 12 mL elution buffer. Add the eluted protein in an Amicon Ultra centrifugal filter.
13. Centrifuge at 5,000 rcf for 50 min or more at 4°C until the eluted protein is concentrated down to 0.5 mL or lower.
14. Add storage buffer to 15 mL. Centrifuge at 10,733 rcf for 50 min or more at 4°C until the eluted protein is concentrated down to 0.5 mL.
15. Add 87% glycerol to the purified protein and make it final 20% glycerol. Divide the purified protein into 100 μL aliquots into PCR tubes.
16. Flash frozen with dry ice and methanol mixture and store at −80°C.
17. Analyze the purified AAT by Bradford protein assay and SDS-PAGE.

3.4 Enzyme Assay

1. Prepare ten reaction mixtures with assay buffer (*see* Subheading 2.4), 10 μL of 10 mM acetyl-CoA solution, 10 μL of the purified AAT solution at a concentration of 1 μM, and 0, 0.5, 1, 2, 3, 5, 10, 20, 50, or 100 mM target alcohol (isobutanol or 3-methyl-1-butanol) to a total volume of 100 μL each (*see* **Note 6**).
2. Place the reaction mixtures at 30°C for 30 min (*see* **Note 7**).
3. Stop the reaction by adding 0.06 g NaCl solid powder.
4. Add 0.2 mL 1 mM DTNB solution. Mix well and take 100 μL of the mixture and put it into a 96-well plate.
5. Quantify the yellow 5-thio-2-nitrobenzoate anion product by measuring light absorbance at 412 nm.
6. Adjust the values for unspecific hydrolysis by deducting the absorbance of the controlled sample with no addition of alcohols.
7. Apply a molar extinction coefficient of 13,600 M^{-1} cm^{-1} to calculate the reaction rate.
8. Use the nlinfit function in Matlab to calculate the K_m and k_{cat} values of the AAT.

3.5 Shake Flask Fermentation

1. Transform WT competent cells with pZE-*kivd-yqhD* and pIBAc vector set (for production of IBAc) or with pZE-*kivd-yqhD* and pIAAc vector set (for production of IAAc) and plate the transformed cell on an LB plate containing: hundred 100 μg/mL ampicillin and 50 μg/mL kanamycin. Leave at 37°C overnight (12–16 h) until colonies are clearly visible.
2. Streak off three independent colonies from the plate with freshly transformed cells. Inoculate each colony in 2 mL 2 × YT + 100 μg/mL ampicillin and 50 μg/mL kanamycin in a test tube. Grow shaking (250 rpm) at 37°C overnight (12–16 h).
3. Prepare 125 mL screw cap conical flasks for fermentation by adding 0.3 g $CaCO_3$ into each flask and sterilize them with 121°C for 25 min.
4. Transfer 200 μL of the overnight cultures into the sterilized conical flasks containing 2 mL 5 × M9 salts, 0.5 mL glucose solution, 7.5 mL yeast extract solution, 10 μg/mL thiamine, 0.1 mM IPTG, 100 μg/mL ampicillin, and 50 μg/mL kanamycin.
5. Seal the caps with Parafilm. Place the flasks in a 30°C incubator, gently shaking at 250 rpm for 48 h (*see* **Note 8**).
6. Collect 1 mL samples and spin down the cells by centrifugation at 20,000 rcf for 15 min and take cell supernatant for analyses. For centrifugation we use Eppendorf Centrifuge 5424 (Eppendorf (http://www.eppendorf.com/)).

3.6 Benchtop Bioreactor Fermentation

1. Calibrate pH meter with pH = 7.0 and 10.0 standard buffer. Add 0.5 L bioreactor media into a 1.3 L benchtop bioreactor. Place the caps on the dO_2 and pH meters and bearing housing. Sterilize by autoclave (*see* **Note 9**).
2. After the medium cool down, adjust the temperature to 37°C by heat blanket and pH to 6.8 by 26% NH_4OH solution. Then, add 12.5 mL 40 wt% glucose solution to make a starting 10 g/L glucose concentration. Maintain the airflow rate at 0.5 vvm throughout the fermentation process.
3. Connect the exhaust from the bioreactor to three traps for the ester production as follows: the first one contains 1,800 mL of water cooled with ice–salt mixture, and the second trap is the same as the first one, and the third trap contains 500 mL of oleyl alcohol cooled with tap water.
4. Inoculate transformed production strain in 2 mL 2 × YT + 100 μg/mL ampicillin and 50 μg/mL kanamycin in a test tube. Grow shaking (250 rpm) at 37°C overnight (12–16 h).
5. Transfer 0.5 mL of the overnight pre-culture into 50 mL LB medium + 100 μg/mL ampicillin and 50 μg/mL kanamycin in a 250 mL shake flask.
6. Grow shaking (250 rpm) at 37°C to an OD_{600} = 1.0–1.2. Add the 50 mL inoculums into the bioreactor. Grow the cells at 37°C and 20% dO_2 level to OD_{600} reach 6.
7. Reduce the temperature to 30°C and dO_2 level to 10% (by decreasing the agitation rate). Add 150 μL of 1 M IPTG solution to induce protein expression.
8. Maintain the dO_2 level at 10% by adjusting agitation rate between 300 and 800 rpm.
9. Run the fermentation in batch mode until the glucose is totally consumed, and then manually feed glucose feeding solution to meet metabolic demands. Collect samples from the bioreactor and the three traps every 6–12 h and analyze the production of the target ester and other byproducts (*see* **Note 10**).

3.7 Metabolite Detection

1. Analyze the concentrations of glucose and other byproducts using HPLC-RID. Use 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 mL/min for 40 min. Keep the column temperature at 35°C and RID detector temperature at 50°C. The injection volume is 20 μL.
2. Analyze the concentration of the target ester product (IBAc or IAAc) using GC-FID. Use 2.5 mL hexane to extract from 5 mL cell culture. Mix the mixture for 1 min using vortex, then centrifuge the mixture at 20,000 rcf for 5 min. Take 0.5–1 mL hexane extract for GC analyses.

3. Inject 2 μL of the hexane extract at a 15:1 split ratio. Hold GC oven temperature at 70°C for 2 min and increase it to 120°C with a gradient of 30°C/min. Then increase the oven temperature to 140°C with a gradient of 10°C/min and to 200°C with a 30°C/min gradient. Hold at 200°C for 5 min to clean any remaining chemicals in the column. Hold the temperature of the injector and the FID detector at 250°C.

4 Notes

1. Information about the genes of pathway enzymes are as follows: acetolactate synthase (*alsS*, from *Bacillus subtilis*) [14], 2,3-dihydroxy isovalerate oxidoreductase (*ilvC*, from *E. coli*), 2,3-dihydroxy isovalerate dehydratase (*ilvD*, from *E. coli*) [15], 2-keto-acid decarboxylases (*kivd*, from *Lactococcus lactis*) [16], alcohol dehydrogenases (*yqhD*, from *E. coli*) [17], 2-isopropylmalate synthase (*leuA*, from *E. coli*), isopropylmalate isomerase complex (*leuC* and *leuD*, from *E. coli*), and 3-isopropylmalate dehydrogenase (*leuB*, from *E. coli*).
2. Based on our previous experience, endogenous activity of IlvC is good enough for the production of isobutanol; therefore, we did not overexpress IlvC in the plasmid. Similarly, acetyl-CoA is ubiquitous and is not the limiting substrate for IBAc and IAAc production. Thus, we did not overexpress any acetyl-CoA production enzyme.
3. The DNA sequence of the 6 × His tag is 5′-catcaccatcaccatcac-3′.
4. Names of vendors are provided in the list of Materials (Subheading 2) from which we currently purchase chemicals. However, it does not mean that we endorse these particular vendors.
5. Unless mentioned otherwise, standard protocols of the commercially obtained kit should be followed during cloning and purification processes.
6. Use a multi-channel pipette to add the acetyl-CoA solution at last to start all the reactions at the same time for a more accurate measurement. We performed the reaction in 1.5 mL centrifuge tubes.
7. The appropriate reaction time to characterize enzyme kinetics depends on the activity of AAT. Therefore, 30 min is a recommended reaction time, but the optimal time needs to be tested by trial experiments.
8. It is important to seal the caps tightly with Parafilm to prevent the volatile products from escaping out of the flasks.

9. Before sterilization, make sure all the caps have been securely put on the desired positions to prevent the steam from ruining the sensitive components. Do not disconnect the dO_2 probe for longer than 10 s after autoclave. Do not autoclave glucose along with the bioreactor media, and add sterilized glucose solution separately.
10. The productivities of the target esters can be characterized as g IBAc/(L · g cells · h) and g IAAc/(L · g cells · h).

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Protocols for the Production and Analysis of Isoprenoids in Bacteria and Yeast

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Abstract

Isoprenoids (a.k.a. terpenes/terpenoids) are the largest group of natural products. They fulfil a wide variety of both essential and non-essential roles in biology; many isoprenoids also have useful industrial applications. In recent years, there has been a significant focus on metabolic engineering of various isoprenoids in microbial cells. Here, we describe methods for lab-scale culturing, sampling and analytics of different isoprenoid classes using specific examples from the following classes: (1) highly volatile isoprenoids that sequester into culture headspaces, e.g. the hemiterpene isoprene; (2) volatile isoprenoids that can be collected in a non-toxic organic phase, e.g. the monoterpene limonene; and (3) non-volatile isoprenoids that accumulate in the cell, e.g. the carotenoid lycopene. Production methods are provided for isoprene, limonene, and lycopene as examples for each class. Specific analytical methods are provided for isoprene, limonene, terpinolene, caryophyllene, amorphadiene, linalool, nerolidol, pinene and lycopene. We focus on yeast and *Escherichia coli* as production organisms. The protocols can be modified for other organisms and products as appropriate.

Keywords: Analytics, Bio-production, *Escherichia coli*, Industrial biotechnology, Isoprene, Isoprenoids, Limonene, Lycopene, *Saccharomyces cerevisiae*, Terpenes, Terpenoids, Yeast

Abbreviations

CDM	Chemically defined medium
ECMet	Extracellular metabolites
GC–MS	Gas chromatography–mass spectrometry
HPLC	High pressure/performance liquid chromatography
ISTD	Internal standard
LLOQ	Lower limit of quantitation
LOD	Limit of detection
OD	Optical density
PTFE	Polytetrafluoroethylene (commonly identified as Teflon®, a DuPont brand name)
rpm	revolutions per minute
SIM	Selected ion monitoring
TB	Terrific broth

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TIC	Total ion current (chromatogram)
ULOQ	Upper limit of quantitation
YPD	Yeast extract peptone dextrose (a.k.a. YEPD)
YPDG	Yeast extract peptone dextrose galactose

1 Introduction

Isoprenoids (a.k.a. terpenes/terpenoids) are the largest group of natural products, with over 70,000 identified to date (www.dnp.chemnetbase.com/intro/). They fulfil a wide variety of both essential and non-essential roles in biology, including but not limited to cell wall biosynthesis, membrane componentry, intracellular and extracellular signalling, biotic and abiotic stress responses, subcellular tRNA and protein targeting, electron transport, pigments (both photosynthetic and non-photosynthetic), semiochemicals, hormones, defence molecules, vitamins, virulence factors and antioxidants. Many isoprenoids also have useful industrial applications, including as agricultural chemicals, fuel components, chemical and polymer feedstocks, food colours, fragrances, pharmaceuticals, nutraceuticals and vitamins [1]. For these reasons, a great deal of experimental work has been done on both naturally produced and engineered isoprenoids.

There are a few examples of microorganisms that naturally produce large amounts of industrially useful isoprenoids (e.g. production of the food colour/pro-vitamin A compound β-carotene by the unicellular green alga *Dunaliella* spp. and the fungus *Blakeslea trispora*). However, natural sources seldom provide sufficient amounts of the desired isoprenoid. Metabolic engineering aims to redirect carbon flux through metabolic networks in order to increase production of desired isoprenoids to achieve rates/yields/titres that are suitable for commercial applications [2]. Gene and organism design for engineering of isoprenoid production in microbes have been reviewed recently [1, 3], as have various aspects of engineering, extraction and/or analysis of various individual classes of isoprenoids [4–7]. Isoprenoid production is commonly engineered in the model microbes *Saccharomyces cerevisiae* (yeast) and *Escherichia coli*, and we will focus on these organisms in this chapter.

There are two distinct metabolic pathways that produce isoprenoids, namely, the mevalonate pathway and the methylerythritol phosphate pathway (Fig. 1; reviewed in Vickers et al. [1]). Yeast harbours a mevalonate pathway, which initiates at acetyl-CoA and proceeds through six steps to produce sequentially the five-carbon (C_5) universal precursor isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP). *E. coli* harbours a methylerythritol phosphate pathway, which initiates at pyruvate

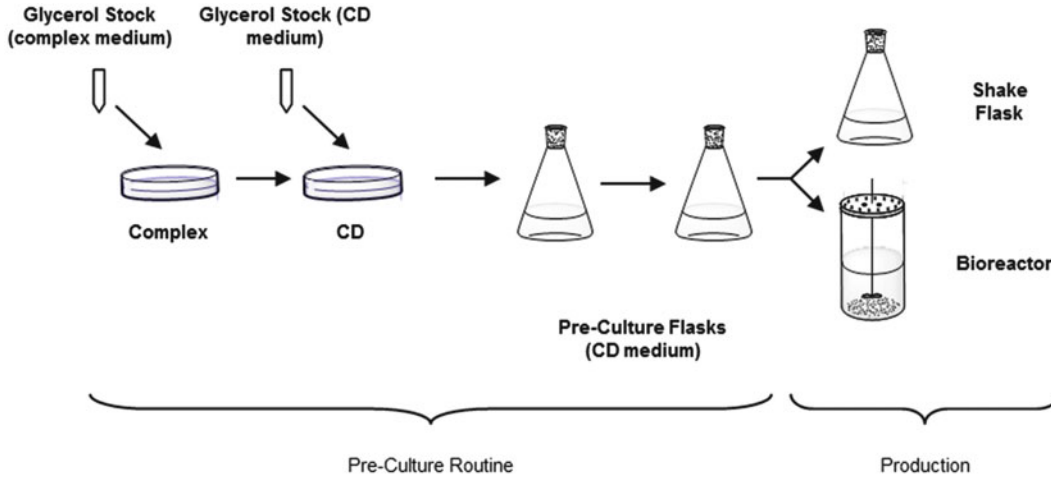

Fig. 1 Simplified metabolic pathways for isoprenoid production. The production of isoprenoids in microorganisms proceeds through either the mevalonate pathway (found in archea and eukaryotes) or the methylerythritol phosphate pathway (found in most bacteria and in plastids). Enzymes: *DTS* diterpene synthase, *FPPS* farnesyl pyrophosphate synthase, *GPPS* geranyl pyrophosphate synthase, *GGPPS* geranylgeranyl pyrophosphate synthase, *HTS* hemiterpene synthase, *MTS* monoterpene synthase, *STS* sesquiterpene synthase. Metabolites: *GA3P* glyceraldehyde 3-phosphate, *A-CoA* acetyl-CoA, *IPP* isopentenyl pyrophosphate (C_5), *DMAPP* dimethylallyl pyrophosphate (C5), *GPP* geranyl pyrophosphate (C_{10}), *NPP* neryl pyrophosphate (C_{10}), *FPP* farnesyl pyrophosphate (C_{15}), *GGPP* geranylgeranyl pyrophosphate (C_{20}). Figure modified from Vickers et al. [1]

and glyceraldehyde 3-phosphate (G3P) and progresses through seven steps to produce both isomers simultaneously. Prenyltransferase enzymes condense DMAPP and IPP to produce the C_{10} precursor geranyl pyrophosphate (GPP), the C_{15} precursor farnesyl pyrophosphate (FPP) and the C_{20} precursor geranylgeranyl pyrophosphate (GGPP). Hemiterpenes (C_5), monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), homoterpenes (C_{16} and C_{11}), triterpenes (C_{30}), tetraterpenes (C_{40}) and longer-chain polyisoprenoids are derived from these universal precursors through the action of synthases and a wide range of modifying and decorating enzymes. Other metabolic pathways frequently converge to produce further isoprenoid derivatives (e.g. a prenyl tail is provided through an isoprenoid pathway, and a quinone head group is provided through the chorismate pathway for vitamin K biosynthesis).

Production and analysis of isoprenoids in microorganisms can be complicated by multiple factors, including (1) many isoprenoids – especially shorter-chain $C_{5–15}$ and some C_{20} compounds – are volatile and may be lost from cultures; (2) the product may be toxic to the production organism; and (3) the production organism may biotransform the product into undesirable derivatives. To circumvent some of these problems, collection and protection of isoprenoids can be improved by adding an organic phase into which the compounds can partition. Highly volatile isoprenoids (such as

the C_5 isoprene) will partition into the headspace and must be recovered from the gas phase of sealed cultures. Volatiles (C_5 and less volatile C_{10}/C_{15}) can also be collected from the off-gas for bioreactor experiments (and in this case, an organic phase is not useful for collection, as sparging the cultures strips the volatiles from both the culture and the organic phase). Organic phases can still be used in anaerobic unsparged bioreactors.

In this chapter, we focus on lab-scale shake-flask experimental protocols for cultivation, extraction and analysis of isoprenoids from the model microbes *S. cerevisiae* and *E. coli*, including isoprenoids belonging to several different classes. The broad techniques described here should, however, be amenable to a wide variety of microbes (minor modifications may be required) and can be transferred in some cases to bioreactor experiments (with the caveats noted above).

Extraction and analysis of each isoprenoid compound depend on the chemical properties of the target compound. The isoprenoid tail moiety is hydrophobic in nature; this often means that target isoprenoids sequester into the headspace, into a non-toxic organic extractant phase or into the cell membrane (for compounds that are not excreted from the cell). The generic protocol for production/extraction can be briefly summarised as follows:

1. The target microorganism is first recovered from stocks by streaking out on solid medium. Single colonies are inoculated into a starter culture, which is grown to mid-log phase.
2. The mid-log starter culture is subcultured into a larger production flask at a low optical density (e.g. $OD_{600/660} = 0.05$). If necessary, an organic extractant phase (such as dodecane) is added. Sealed flasks are used for volatile products to prevent loss.
3. Cultures are incubated under appropriate conditions, and samples are taken at each sampling point.
4. Analysis is performed using appropriate sample preparation and analytical equipment/processes.

The above process will be detailed in the sections below. For ease of presentation, flask-based bioprocess and analytical approaches are broken up into methods appropriate for different classes of isoprenoids, using representative members of each class as examples:

1. Highly volatile isoprenoids that partition easily into the headspace (and/or can be encouraged to do so by increasing the temperature prior to analysis): sealed cultures with gas phase headspace GC–MS analysis. We use the C_5 hemiterpene isoprene as an example.

2. Moderately volatile isoprenoids that will partition into an extractant phase: sealed cultures with an organic extraction phase; GC–MS analysis after sample processing. We use the C_{10} monoterpene limonene as an example. The C_{15} sesquiterpene amorphadiene also falls into this class.
3. Higher-order non-volatile non-secreted isoprenoids that remain in cells: extraction with an organic solvent from liquid culture or cell pellets; analysis by GC–MS, HPLC or spectrophotometry depending on the chemical properties of the compound. We use the C_{40} carotenoid lycopene as an example. The C_{20} diterpene taxadiene also falls into this class.

2 Materials

2.1 Culturing

2.1.1 Pre-culturing

1. Stocks (usually glycerol stocks) of target microorganisms (*see* **Note 1**)
2. Static and shaking incubators with temperature control
3. Plates of solid growth medium (*see* **Note 2**):
   - *S. cerevisiae*: Some isoprenoid products are poorly (if at all) produced by yeast growing on defined medium such as synthetic defined (SD) medium [8] (https://www.bdbiosciences.com/), and cultures in this case may be quite unstable. In this case, use of yeast extract-peptide-dextrose medium is suggested (YPD; 1% yeast extract, 2% peptone, 2% dextrose [8]) or an equivalent rich medium. Auxotrophic selection approaches may preclude this; vectors with antibiotic selection designed for metabolic engineering applications are available as an alternative [9].
   - *E. coli*: For strain testing or selection, use of rich medium such as terrific broth (TB) or SOB [8] is recommended. Characterisation of strains producing isoprenoids should be done in chemically defined medium (CDM) such as M9 [8] or R medium [10] containing an appropriate carbon source. When using the native methylerythritol phosphate (MEP) pathway for isoprenoid production, glycerol has been shown to result in higher yield than glucose [11, 12].
4. Pre-culture flasks (per strain): e.g. 3 × 100 mL baffled shake flasks with 10 mL of appropriate medium (*see* **Notes 3** and **4**)

2.1.2 Production Cultures

1. Sets of three flasks/vials per strain with appropriate liquid medium. Use no more than 20% volume of appropriate selective medium (e.g. 20 mL in a 100 mL flask) to allow for adequate aeration (*see* **Notes 5**–**7**). Products in different classes require different cultivation conditions for production cultures:

- *For highly volatile isoprenoids* (e.g. *isoprene* [C_5]): *see* Sect. **3.2** for culture options. Use either (a) standard baffled shake flasks with sufficient volume for sampling at all time points for non destructive sampling or (b) 20 mL glass headspace GC vials, e.g. Agilent Technologies (http://www.agilent.com) 20 mL vial (part No. 5188-2753) plus PTFE (Teflon®)-lined lid (Part No. 5188-2759) for destructive sampling. If dry cell weight biomass samples are to be taken (*see* Sect. **3.3.1**), preweigh vials (with caps on).
- *For other volatiles* (e.g. *monoterpenes* [C_{10}] *and sesquiterpenes* [C_{15}]): use culture flasks that have a nonrubber seal (e.g. PTFE lid with a PTFE-coated silicone seal to prevent product escape or adsorption) and include one set of flasks for each time point, as sampling is destructive. For example, 100 mL unbaffled shake flasks with PTFE-lined screw caps (DURAN® Erlenmeyer flask with DIN thread www.bacto.com.au; 100 mL, Part No. 21803245; 250 mL, Part No. 21803365. Flasks of 500 mL and 1 L are also available).
- *For non-volatiles* (e.g. *lycopene* [C_{40}]): multiple samples can be taken from each flask, so a single set of unbaffled flasks (*see* **Note 8**) can be used for each strain providing there is sufficient volume for all sampling points and sufficient headspace for adequate aeration.

2. Non-toxic organic solvent (e.g. dodecane (*see* **Note 9**)) if producing a volatile isoprenoid that needs to be sequestered into a suitable liquid organic phase (e.g. monoterpenes [C10] and sesquiterpenes [C15]).

2.2 Sampling

There are two approaches (*see* Sect. **3.2** below for details):

2.2.1 Highly Volatile Isoprenoids

1. If taking time point samples from a single culture rather than destructive samples: 20 mL glass vial (Agilent Technologies www.agilent.com cat. #5188-2753) with 13 mm metal screw cap with septa (Agilent Technologies www.agilent.com cat. #5188-2759)
2. Alternatively, if taking destructive samples: no extra materials are needed as samples are taken directly from the headspace of production cultures (which are already in 20 mL vials)

2.2.2 Volatile Isoprenoids

1. Centrifuge tubes appropriate for sample size (e.g. 15 or 50 mL) (*see* **Note 10**), labelled and pre-weighed.
2. Centrifuge with temperature control and swing-out rotor appropriate for collection tubes.

3. 1.5 mL centrifuge tubes labelled with sample name/number.
4. For biomass measurements: either a spectrophotometer (to measure optical density) or a freeze-drying apparatus and an analytical balance (for cell dry weight measurements). If biomass samples (rather than OD) are to be taken (*see* Sect. **3**), also prepare a set of pre-weighed tubes for freeze-drying and 550 µL H_2O per sample (in bulk).
5. For organic phase samples (where isoprenoids accumulate): glass chromatography vials, e.g. Agilent Technologies (www.agilent.com) 2 mL glass vials (cat. #5190-4030) with PTFE-lined screw cap (cat. #5185-5820). Glass inserts may be required for small sample volumes (Agilent Technologies www.agilent.com cat #5183-2085)

2.2.3 Non-volatile Isoprenoids

1. 1.5 or 2 mL microfuge tubes to harvest cells (*see* **Note 11**)
2. 1.5 mL microfuge tubes for acetone extracts
3. Analytical/HPLC grade acetone

2.3 Analytics

2.3.1 Highly Volatile Isoprenoids

1. For the calibration curve:
   a. Ice box
   b. Acetone (HPLC grade, e.g. Sigma Aldrich www.agilent.com cat. no. 270725)
   c. 10 µL glass syringe, e.g. SGE Analytical Science www.sge.com part no. 002000 for isoprene
   d. Positive displacement pipette or standard pipette equilibrated with acetone for accurate pipetting of this solvent
   e. Calibrant (authentic standard of target isoprenoid), e.g. isoprene (Sigma Aldrich www.sigmaaldrich.com cat no. I19551)
   f. Eight 2 mL microfuge tubes
   g. Eight 20 mL GC vials with lids
   h. ca. 20 mL of culture medium
2. Agilent 7890A gas chromatograph coupled with an Agilent 5975C MSD mass spectrometer (Agilent, www.agilent.com) – or similar GC or GC–MS
3. Varian (www.varian.com) capillary column (Factor FOUR VF-5 ms: 0.25 mm, 0.25 µm, 30 m length with a 10 m fused guard column) – or similar column
4. Ultrapure helium (99.999%) as a carrier gas
5. ChemStation E.01.00.237 software or any newer version (Agilent www.agilent.com) for use with the above GC–MS set-up

6. Autosampler (MPS-2XL) equipped with a heated agitator (Gerstel http://www.gerstel.com); samples can also be injected by hand
7. Syringe for Headspace injections (2.5 mL, GC010055-025-00) (Gerstel http://www.gerstel.com) – or similar, for manual injections
8. Nitrogen (99.999% purity) for headspace syringe rinsing (for use with autosampler)

2.3.2 *Volatile Isoprenoids*

Sample/Calibrant Preparation

1. Ice for samples and sample box/rack with spaces suitable for 2 mL GC vials
2. Fume hood
3. Ethyl acetate (analytical/HPLC grade; www.honeywell-burdickandjackson.com cat. no. AH100-4)
4. Internal standard (e.g. terpinolene; Sigma Aldrich www.sigmaaldrich.com cat no. 86485), caryophyllene (Sigma Aldrich www.sigmaaldrich.com cat no. 22075)
5. Calibrant (authentic standard of target isoprenoid), available from Sigma Aldrich www.sigmaaldrich.com; e.g. limonene (cat. no. 62118)
6. Dodecane ≥99% (available from Sigma Aldrich www.sigmaaldrich.com cat no. D221104)
7. 20 mL glass vial (Agilent www.agilent.com cat #5188-2753) and screw caps (e.g. Agilent www.agilent.com cat #5188-2759)

Analysis

1. Agilent 7890A gas chromatograph coupled with an Agilent 5975C MSD mass spectrometer (Agilent www.agilent.com) – or similar GC or GC–MS
2. Varian www.varian.com capillary column (Factor FOUR VF-5 ms: 0.25 mm, 0.25 μm, 30 m length with a 10 m fused guard column) – or similar column
3. Ultrapure helium (99.999%) as a carrier gas
4. ChemStation E.01.00.237 or any newer version (Agilent www.agilent.com) for use with the above GC–MS set-up
5. Injector (Agilent www.agilent.com part number 7683B) for use with above GC–MS
6. 10 μL syringe (Agilent www.agilent.com part#:9301-0725) for use with above GC–MS
7. Ethyl acetate (HPLC grade) for first step of syringe rinsing
8. Hexane (HPLC grade) for second syringe rinsing (Merck KGaA, 64271 Darmstadt, Germany)

2.3.3 Non-volatile Isoprenoids

1. Ice box.
2. Aluminium foil to cover samples.
3. Acetone (HPLC grade, e.g. Sigma Aldrich www.sigmaaldrich.com cat no. 270725).
4. Positive displacement pipette or standard pipette equilibrated with acetone for accurate pipetting of this solvent.
5. Heat block or water bath able to operate at 55°C.
6. Calibrant (authentic standard of target isoprenoid), e.g. lycopene (Sigma Aldrich www.sigmaaldrich.com cat. no. 75051).
7. Spectrophotometer to detect coloured isoprenoids; for lycopene, set to 475 nm.
8. Glass cuvette with 10 mm path length and ~1,000 μL fill volume.
9. 70% ethanol (*v*/*v*) for rinsing the cuvette.

3 Methods

The methods described here outline an integrated process from cultivation conditions, extraction/collection of the product and analytics for a range of different isoprenoid product classes. Highly volatile isoprenoids are collected from the culture headspace; for isoprenoids that are secreted/excreted, a non-toxic organic solvent (e.g. dodecane) is added at 2.5–10% to sequester the products; and for isoprenoids that accumulate in cells, they are extracted from the biomass. The culture is incubated, and samples are taken at each sampling point. Sample preparation and analysis is then performed.

3.1 Culturing/Production

Production profiles can vary significantly between strains and for different isoprenoids. Initiation of production may be variable, e.g. early-/mid-/late-log phase or even stationary phase (depending on the expression system and the product). An initial growth analysis should be performed to determine growth rates and other elements of culture behaviour on the relevant production medium. It is useful to run an end-stage experiment (e.g., 2–5 days for *E. coli* or 5–7 days for yeast) to prescreen strains (this can be done in parallel with growth analysis). This information can be used to help establish a pre-culturing strategy and design the overall production experiment (including determining how long you will culture and how frequently you will take samples). A time point analysis can then be done to examine production profiles. Sampling may be destructive (see below), so for multiple time point experiments at least three replications for each time point are required (this can mean a large number of flasks per experiment). Negative, nonproducer cultures are included to identify the biological baseline (*see* **Note 12**).

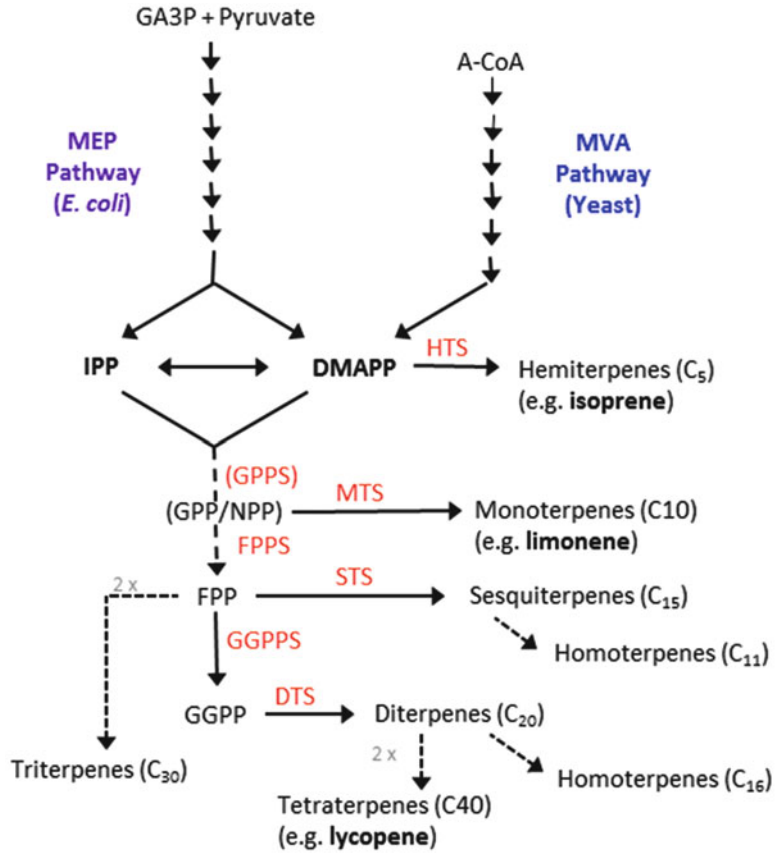

Fig. 2 Pre-culturing and culturing strategies. Glycerol stocks may be prepared in either complex medium or in chemically defined (CD) medium; recovery rates are better from glycerol stocks prepared in complex medium. Stocks should always be streaked on solid media to produce single colonies prior to inoculating shake flaks (as in any case, some strains recover poorly when stocks are inoculated directly into liquid media). Glycerol stocks prepared in complex medium recover poorly when streaked out on CD medium, so an extra step streaking out on complex medium is included prior to re-streaking on CD medium is included. A pre-culture shake flask is then inoculated. If the experimental timing is appropriate, if sufficient biomass is accumulated, and if the culture is reliably in mid-log immediately prior to inoculation, it may be possible to inoculate directly from the first pre-culture shake flask directly into the production flask/bioreactor. Otherwise, a second pre-culture flask is required

We separate the culturing process conceptually into a pre-culturing routine followed by a production culture. Pre-culturing routines can be quite variable and are best established separately for each strain/lab. The protocols described below are generic and can be adapted as required; a specific example is shown in Fig. 2.

3.1.1 Pre-culturing

A pre-culture protocol should achieve several key aims: (1) to ensure that the pre-culture is healthy, synchronised and in mid-log phase prior to inoculation of the production culture; (2) to amplify sufficient biomass from the recovered glycerol stock to inoculate the production culture at a suitable cell density; and (3) to minimise the amount of sub-culturing and time that cells are growing between the glycerol stock and the production culture (this minimises the possibility of random mutations, plasmid loss and other unpredictable effects).

1. Retrieve glycerol stock(s) and streak out on a plate of appropriate selective medium. Incubate at the appropriate temperature until colonies are visible (*see* **Note 13**).
2. Re-streak if necessary (Fig. 2 for comments).
3. Inoculate pre-culture flasks (10 mL in 100 mL flask) (*see* **Note 14**) and incubate at the appropriate speed/temperature (*see* **Note 15**) until mid-log phase is reached (*see* **Notes 16** and **17**).
4. Inoculate second pre-culture flask (if required) and incubate at the appropriate speed/temperature until mid-log phase is reached.

3.1.2 Production Cultures

The production culture is the culture you will monitor for accumulation of your isoprenoid of interest:

1. Check the OD of the pre-culture and confirm that it is in mid-log phase.
2. Inoculate a sufficiently large volume of the production medium to a starting OD of 0.05 and aliquot into the production flasks (*see* **Note 18**).
   - *For highly volatile isoprenoids (e.g. isoprene)*, there are two potential approaches:
     a. If the production rate is expected to be low or is unknown: Incubate 0.5–2 mL (*see* **Note 19**) aliquots in 20 mL glass GC vials. Prepare one production vial per strain per time point (*see* **Note 20**) and sample immediately by GC–MS (*see* **Note 21**). This approach allows for significant build-up of isoprene in production vials and gives an accurate read-out of time point sampling. Biomass can be measured by taking OD readings immediately after sampling or by freeze-drying (if tubes/lids are pre-weighed).
     b. For cultures producing sufficiently high levels of isoprene: incubate the bulk culture in a single unsealed flask stoppered with foam and/or aluminium, and take aliquots at each time point into 20 mL vials for analysis.

Incubate for a sufficient time (*see* Sect. **3.2.1** below) and sample directly onto GC.

- *For other volatiles* (e.g. *monoterpenes* [C_{10}] *and sesquiterpenes* [C_{15}]): use sealed flasks (*see* **Note 22**). Prepare one production flask/vessel for each time point with organic extractant solvent (*see* **step 3** below).
- *For non-volatiles* (e.g. *lycopene* [C_{40}]): we use unsealed flasks stoppered with foam and/or aluminium (*see* **Note 23**). If sufficient volume is used, only one flask per replicate is required as sampling is not destructive.

3. For volatile isoprenoids ($>C_5$): dose in 2.5–10% (v/v culture) of dodecane and seal flask by screwing cap on firmly (*see* **Notes 24–26**).
4. Incubate under appropriate conditions for an appropriate time period. The length of incubation depends on when the target compound is being produced and how long it is produced for. A time-course analysis can be performed to determine the culture production profile.
   - While *E. coli* grows fastest at 37°C, it has been shown for several different isoprenoids that production at 30°C (or lower) results in improved yield [11, 13]. This also reduces product volatilisation and can decrease aggregation of over-expressed proteins.

3.2 Sampling

This section describes sampling/extraction procedures for the isoprenoid of interest. Biomass samples (either OD or cell pellets) should ideally be taken in parallel to track culture growth. Samples for extracellular metabolites (ECMet) can also be taken if suitable analysis facilities are available (*see* Sect. **4** for comments); in this instance, it will be presumed that protocols for sampling and analysis are already available in the operator's lab.

3.2.1 Highly Volatile Isoprenoids

No extraction is required as the product sequesters into the headspace and is sampled directly in the analytics instrumentation (GC or GC–MS). The sampling approach depends on the cultivation approach (*see* Sect. **3.1.2** above):

- For samples growing in replicate 20 mL vials: proceed immediately to GC–MS analysis (*see* Sect. **3.3.1** below).
- For samples growing as a bulk culture: take 2 mL aliquots at each time point into 20 mL GC vials (or 100 μL into 1 mL vials, if production levels are sufficient to allow for a smaller injection volume – see GC–MS analysis Sect. **3.3.1** below). Seal and incubate for 1–4 h (depending on growth and production rates) to generate a production profile (*see* **Note 27**). Measure OD or biomass at the beginning and end of the incubation period.

3.2.2 Volatile Isoprenoids

For volatile monoterpenes and sesquiterpenes, the sampling in this method is destructive (i.e. the entire contents of a flask are harvested at once). This is necessary due to (a) the difficulties in collecting reproducible quantities of dodecane from cultures where the overlay volume is small relative to the culture volume and (b) potential loss of volatile products with repeated opening of flasks. Consequently, for experiments with multiple time points, multiple flasks must be inoculated (*see* **Note 28**).

The method below uses cell dry weight as a measure of biomass because dodecane interferes severely with OD readings. OD readings can be taken with extreme care (after allowing phases to separate), but we have found the risk of dodecane contamination unacceptably high, especially if multiple samples are being processed at one time.

1. Remove flask from the incubator; record the sampling time (*see* **Note 29**).
2. Swirl flask and empty the entire contents into a pre-weighed centrifuge tube of sufficient volume (e.g. 15 mL for 10 mL cultures); record the final weight (tube plus culture) to determine culture volume (*see* **Note 30**).
3. Centrifuge samples in a swing-out rotor at 5,000×*g* for 10 min at 20°C to generate three fractions: cell pellet, medium and dodecane.
4. For dodecane layer (harbouring isoprenoids):
   a. Transfer as much of the upper dodecane layer as possible to a 1.5 mL tube (*see* **Notes 31–34**).
   b. Centrifuge for 3–5 min at maximum speed in a bench centrifuge (can be processed in parallel with biomass samples at **Step 5** below).
   c. Transfer a portion of the dodecane supernatant to a labelled chromatography vial with silicon/PTFE-lined cap for storage (*see* **Note 35**), and store at −80°C until ready for further processing for analysis (see "Calibrant and Sample Preparation").
5. For biomass:
   a. Discard the remaining supernatant (taking care not to disturb the cell pellet).
   b. Add 500 μL H_2O to the cell pellet and resuspend using a bench vortex.
   c. Transfer the resuspended cells to a pre-weighed 1.5 mL centrifuge tube. To maximise the amount of biomass

transferred, both the original tube and the pipette tip should be rinsed:

i. Leave the pipette tip used to transfer the resuspended pellet on the pipette and place on one side.

ii. Using a second pipette with a fresh tip, add a further 500 μL ice-cold H_2O to the 15 mL tube. Swirl the tube to rinse it.

iii. Retrieve the pipette equipped with the original tip, pipette up and down to rinse the tip and then transfer the rinse liquid to the same 1.5 mL centrifuge tube as the first 500 μL resuspended cell pellet.

d. Centrifuge the resuspended cell pellet at 16,000×*g* for 5 min at 4°C (dodecane samples can be processed in parallel; *see* **step 4** above).

e. Immediately pour off the supernatant from the biomass samples (*see* **Note 36**).

f. Freeze samples (*see* **Note 37**), then freeze dry and weigh to determine DCW (*see* **Note 38**).

3.2.3 Non-volatile Isoprenoids

For non-volatile isoprenoids that remain in the cells, sampling is not destructive (with respect to the entire culture), and samples can be taken at several time points from the same flask (*see* **Note 39**). While the protocol described here focuses on lycopene, other carotenoids can be also produced and extracted following the presented method. For non-volatile isoprenoids with significantly different chemical properties, culturing and extraction methods differ and should be modified according to appropriate literature (e.g. diterpenes such as taxadiene are extracted in hexane or ethyl acetate, followed by GC–MS analysis [14]).

General precautions when carrying out quantitative work with lycopene and acetone: (1) Lycopene is sensitive to light, oxygen and heat (*see* **Note 40**). (2) Acetone is highly volatile, and samples containing acetone must be handled on ice in a fume hood; work quickly but carefully to avoid sample loss (*see* **Note 41**). (3) Acetone has a very low surface tension, and additional care and attention must be taken when pipetting (*see* **Note 42**).

Protocol:

1. Transfer 1 mL of each culture into a 1.5 or 2 mL tube. Ideally, take technical duplicates from each flask (*see* **Note 43**).
2. Centrifuge samples at 16,000×*g* for 1 min to pellet the cells. Discard the supernatant (*see* **Note 44**).
3. Wash the cells with 1 mL of water and vortex for complete resuspension.
4. Centrifuge samples at 16,000×*g* for 1 min (*see* **Note 45**).
5. Discard the supernatant carefully using a pipette (*see* **Note 46**).

6. Vortex the pellet to loosen it from the tube wall before proceeding to the next step (*see* **Note 47**).
7. Add 1 mL of acetone per tube and resuspend pellet using a vortex mixer (*see* **Note 48**).
8. Incubate samples at 55°C for 15 min (*N.B. protect samples from light*). Vortex every 5 min to enable complete extraction.
9. Pellet cells for 5 min by centrifugation at 16,000×*g*.
10. Immediately after the centrifuge has stopped, transfer the supernatant (ca. 900 μL) to a fresh 1.5 mL tube; be careful not to disturb the pellet (*see* **Note 49**).
11. Proceed to sample analysis immediately (Sect. **3.3.3**). Keep samples on ice and protected from light (*see* **Note 50**) before measuring lycopene

3.3 Analytics

Successful analysis of samples depends on the chemical properties of each target compound. The protocols listed below are for the example isoprenoids from each class.

3.3.1 Highly Volatile Isoprenoids (e.g. Isoprene): Headspace GC–MS

Calibrant Preparation

Calibrants must be prepared in order to generate a standard curve. The extreme volatility of isoprene makes this preparation fraught with difficulties. The following protocol provides accurate calibration curves given that acetone and isoprene are handled quickly and extreme caution is observed to close all tubes and vials immediately after pipetting and everything is kept ice cold to prevent volatilisation. The 7-point standard curve described here covers a range of 0.3–20 mg/L of isoprene, but can be easily adjusted for cultures producing isoprene titres outside of that range.

1. Prepare a sufficiently large ice box and place in a fume hood.
2. Place the following materials on ice ca. 30 min before beginning calibrant preparation to ensure the temperature is <4°C:
   a. Eight 2 mL microfuge tubes and eight 20 mL GC vials with lids
   b. ca. 20 mL of culture medium and ca. 10 mL of HPLC-grade acetone
   c. Isoprene standard
3. Prepare 100-fold standards of isoprene in acetone:
   a. Prepare a 50 mM (or 3.4055 g/L) stock in a 2 mL tube by adding 10.03 μL of isoprene to 1,990 μL of acetone using a metal syringe. Mix briefly.
   b. Prepare a 2 g/L stock by adding 587 μL of the 50 mM stock to 413 μL of acetone using a positive displacement pipette. This standard (STD #7) will be used to prepare the highest point of the calibration curve, in this case 20 mg/L.
   c. Pipette 500 μL acetone into each of the remaining six tubes.

d. Prepare standards #6 to #1 by performing a serial 1:1 dilution from STD #7 (500 μL of STD #7 + 500 μL of acetone = STD #6 etc.).

e. Keep tubes closed and on ice until needed.

4. Transfer 990 μL (or equivalent amount, depending on the actual culture volume that was used in biological samples) of culture medium into each of the eight GC vials. Ensure screw caps are within easy reach.
5. Prepare a blank by adding 10 μL of acetone directly into the ice-cold medium in the GC vial and immediately screwing the lid on the vial tightly.
6. Prepare isoprene calibrants by quickly adding 10 μL of STD #1 to STD #7 directly into the ice-cold medium in the GC vials and immediately screwing the lid on the vial tightly. Let the vials warm up to room temperature before starting the GC measurement.

GC–MS Analytics

The protocol below is for a specific instrument equipped with an autosampler and heated agitator (*see* Sects. **2** and **3**). It can be modified for different instrumentation/equipment.

1. Incubate sealed cultures/standards in a heated agitator at 45°C for 5 min under constant stirring at 250 rpm.
2. Collect 250 μL of the headspace with a headspace syringe at 60°C while cultures are still in the heated agitator, and inject onto the GC–MS.
3. Inject the samples in a split (20:1) mode with split flow of 50 mL/min and at 250°C using helium as a carrier gas and under a constant total flow of 3 mL/min.
4. Rinse headspace syringe with ultrapure nitrogen for 30 s.
5. After injection, collect cultures and either (a) measure the OD or (b) freeze dry in order to calculate the biomass (*see* **Note 51**).

The compounds are chromatographically separated on a capillary column according to the following oven temperature program and mass spectrometric conditions:

1. Start with oven temperature at 40°C and hold for 2 min.
2. Increase the temperature to 120°C at a rate of 40°C /min.
3. Maintain the ion source, quadrupole and transfer line at 300, 150 and 280°C, respectively.

Isoprene is detected at approximately 1.8 min, in both total ion current (TIC) and selected ion monitoring (SIM) mode using its characteristic ions with mass-to-charge (m/z) ratios of 67, 68 and 69 (the dwell time of the scanning was set at 30 ms for all the ions).

The peak area of the characteristic ions produced by the SIM mode is used for the absolute quantification of isoprene production of the cultures based upon the calibration curve.

Note: Upon fragmentation, isoprene produces other ions as well (such as 53 and 39 m/z); however, 67, 68 and 69 m/z are the only ones that show no ion interference with other volatile compounds contained in the culture.

3.3.2 Volatile Isoprenoids (e.g. Monoterpenes): Standard GC–MS

To prepare for analysis of isoprenoids in dodecane fractions, several steps are performed. First, a diluent with internal standard (to control for variability during dilution and GC–MS analyses) is prepared to dilute the samples. Calibrants, using authentic standards of the target compound at appropriate concentrations, are also prepared. Finally, the samples are diluted to an appropriate concentration to (a) decrease the dodecane concentration (dodecane interferes with analysis) and (b) ensure that the samples are within the calibrant standard curve range. Both the diluent with ISTD and the calibrants are prepared gravimetrically. Achieving the exact desired concentrations is not critical, but it is critical to know the exact final concentration for the ISTD (to determine true dilution) and calibrant (standard curve). Samples are also diluted gravimetrically.

Important considerations for GC–MS analysis (satisfaction of these considerations is embedded in the following protocols) are the following:

- Before the processing of any biological samples, a calibration curve should be built.
- Samples should be processed in a randomised order and preferably in duplicate.
- After biological samples have been analysed, a second, duplicate calibration curve should ideally be built in order to test the reproducibility as well as the stability of the assay.
- Samples for the calibration curve should be processed in an increasing concentration sequence in order to avoid any possibility of interference from analyte carry-over.
- Do not inject dodecane samples directly into the GC–MS; any dodecane must be diluted a minimum of 40-fold prior to injection as dodecane; if injected as a solvent, it will result in an undesirable column carry-over and will also significantly reduce the performance of the ion source in the mass spectrometer over time.
- The calibration curve determines the response of the instrument for the compound(s) under analysis during the analytical acquisition in question. For some instruments, the calibration may hold for future analyses, but it is generally good practice to

calibrate the instrument for each acquisition to ensure robust and reproducible results. The range of the calibration curve is determined by three main measurement points (other than zero) that should be determined during method development: i.e. the limit of detection (LOD), which is the smallest reproducible peak that has a signal/noise value of at least 3; the lower limit of quantitation (LLOQ), which is the smallest peak that has a signal/noise ratio of at least 10 and a reproducibility of <20% CV; and the upper limit of quantitation (ULOQ), which is the largest peak in the linear range, with a reproducibility of <15% CV [15]. The dynamic range of the measurement assay is determined by either the achievable range of the instrument or the necessary biological range if this lies within the achievable range.

Diluent (with Internal Standard)

Prepare the diluent with internal standard (ISTD). The ISTD can be any appropriate compound that is accurately detected using the chromatography approach used for the target isoprenoid and does not produce a peak that interferes with the target isoprenoid. We usually use an alternative isoprenoid, e.g. 10.25 μM terpinolene (*see* **Note 52**) in ethyl acetate. By using a compound similar to the metabolite of interest as an internal standard, we ensure that a) the linear range of the mass spectrometric response is similar to the target compound and b) that chromatographic method will not need modifications due to potential extreme retention time differences. Calibrant stocks are prepared by serial dilution in ethyl acetate, starting with dilution of the stock down to ~10.25 μM. For example, the terpinolene stock is ~5.3 M; dilute in four steps: 0.1 M → 10.25 mM → 1.025 mM → 10.25 μM (*see* **Note 53**). The initial dilutions are prepared in 1 mL volumes, and the final stock (10.25 μM) is prepared in ~16 mL to allow enough diluent for a reasonably large sample set (*see* **Note 54**).

1. Determine required volumes of ethyl acetate and ISTD (*see* **Note 55**) to prepare 1 mL aliquots at the appropriate target concentrations.
2. Place a sample box/rack on ice and allow it to cool.
3. Pre-label 2 mL chromatography vials with the target calibrant concentrations.
4. Place a chromatography vial and vial lid on the centre of the analytical balance, and zero (tare) the balance.
5. Transfer the vial to the fume cupboard, and pipette the appropriate volume of ethyl acetate into the vial.
6. Replace the lid and return the vial to the analytical balance. Record the weight to calculate the actual volume and re-zero the balance.
7. Transfer the vial to the fume cupboard, and pipette the appropriate volume of ISTD into the vial.

8. Replace the lid and return the vial to the analytical balance. Record the weight, vortex the vial to mix and then place it in the chilled sample box/rack on ice.
9. Repeat **steps 1–7** each time using the previous solution to perform a serial dilution down to 1.025 mM (*see* **Note 56**)
10. To prepare the 10.25 μM ISTD/ethyl acetate diluent, place a 20 mL glass chromatography vial and lid on the analytical balance
11. Record the weight of the vial. Do NOT zero the balance
12. Transfer the vial to the fume cupboard and transfer the appropriate quantity of ethyl acetate into the vial to make sufficient diluent for all of your calibrants and samples.
13. Replace the lid and return the vial to the analytical balance. Record the weight; do NOT zero the balance.
14. Transfer the vial to the fume cupboard, and transfer the appropriate quantity of the 1.025 mM ISTD into the vial.
15. Replace the lid and return the vial to the analytical balance. Record the weight, vortex the vial and then place it in the ice box.
16. Calculate the final absolute concentrations based on the weights and the density of ethyl acetate (MW = 88.11 g/mol) and the ISTD (terpinolene MW = 136.23 g/mol).

Calibrant and Sample Preparation

The calibrants (dilutions of authentic standards) should be prepared the same way as samples (*see* **Note 57**). At least five, and ideally seven, calibrant points (concentrations) are required to generate a meaningful calibration curve; each of those seven points should represent a different concentration of calibrant, and they should follow a linear regression (*see* **Notes 58** and **59**). We usually prepare nine calibrants to ensure compliance with these requirements. Table 1 shows an overview of the calibrant working solutions and the final concentrations for analysis.

1. Prepare the calibrant stocks using the same method described in "Diluent (with Internal Standard)" **steps 3–8**, using dodecane as the diluent. Example for limonene (*see* **Note 54** for amorphadiene):
   a. Prepare a 1 M stock solution from the commercial stock.
   b. Use the 1 M stock solution to prepare a 100 mM stock solution.
   c. Use the 100 mM stock solution to prepare 10, 8, 6, 4, 3 and 2 mM working solutions.
   d. Use the 10 mM solution to prepare a 1 mM solution, 400 μM solution, 200 μM solution and 60 μM solution (*see* **Note 60**)

Table 1
Overview of preparation of calibrant working solutions and dilution of them to final concentrations for analysis

Preparation of	C9[a]	C8	C7	C6	C5	C4 (CT[b])	C3	C2	C1
Calibrant working solutions (prepared in dodecane)	8 mM	6 mM	4 mM	3 mM	2 mM	1 mM	400 μM	200 μM	60 μM
Dilute 40-fold with the ethyl acetate/ISTD diluent	↓	↓	↓	↓	↓	↓	↓	↓	↓
Final concentration for analysis	200 μM	150 μM	100 μM	75 μM	50 μM	25 μM	10 μM	5 μM	1.5 μM

[a]C9–C1 are different concentrations of calibrant
[b]CT = control calibrant

2. Retrieve your samples from storage (−80°C freezer) and place them in a sample box/rack on ice. Allow to thaw if frozen.
3. Label three glass chromatography vials for each sample and calibrant: one for the dilution and two for GC–MS technical replicates (*see* **Note 61**) (place glass inserts into the two vials for the technical replicates).
4. *Optional*: Label two additional glass chromatography vials with glass inserts for each calibrant (*see* **Note 62**)
5. Dilute samples and calibrants gravimetrically with diluent (ethyl acetate/ISTD) (*see* **Notes 63** and **64**). Example for 40-fold dilution:
   a. Aliquot 195 μL of the diluent into a 2 mL glass vial and record the weight.
   b. Add 5 μL of sample or calibrant and record the weight. Prepare a blank by mixing 5 μL dodecane with 195 μL diluent (*see* **Note 65**).
   c. *Optional*: Prepare a second vial for the fourth calibrant dilution and label it CT (Calibrant Test) (*see* **Note 66**).
   d. Record the final weight of the diluent vial (*see* **Note 67**)
6. Transfer 50 μL of each diluted sample/calibrant into two labelled technical replicate vials (*see* **step 3** above).
7. Line up your samples in the chilled sample box/rack:
   a. One vial of ~1 mL ethyl acetate
   b. One vial of ~1 mL ethyl acetate plus ISTD (10.25 μM)
   c. Each of the calibrants in duplicate in consecutive order (C0 → C9)
   d. One set of samples, randomised (*see* **Note 68**)
   e. (*optional*) The first vial of CT
   f. The second set of samples in the same order as the first
   g. (*optional*) The second vial of CT
   h. For large numbers of samples: a second set of calibrants in duplicate in consecutive order (C0 → C9)

   Proceed with sample analysis (*see* Sect. **3.3.2** below).

GC–MS Analysis

GC–MS conditions depend on the chemical properties of each compound. Here we present two methods that are capable of measuring two groups of volatile isoprenoids.

Limonene, Terpinolene, Caryophyllene and Amorphadiene

1. Inject 3 μL of the 50 μL sample in a split (50:1) mode with split flow of 50 mL/min at 220°C using helium as a carrier gas and constant total flow of 1 mL/min.
2. Start with oven temperature at 70°C and hold for 13 min.

3. Increase temperature to 300°C at a rate of 50°C and hold for 5 min.
4. Maintain the ion source, quadrupole and transfer line at 300, 150, and 280°C, respectively.
5. The compounds are detected in SIM mode using their characteristic ions (the dwell time of the scanning was set at 30 ms for all the below ions):
   - Limonene: 68, 93 and 136 m/z
   - Terpinolene: 93, 121 and 136 m/z
   - Caryophyllene: 93, 105, 133 and 204 m/z
   - Amorphadiene: 93, 119, 189 and 204 m/z

Linalool, Nerolidol and Pinene

1. Inject 3 μL of the samples in a splitless mode at 220°C using helium as a carrier gas and constant total flow of 1 mL/min.
2. Initial oven temperature is set at 50°C for 2 min.
3. Increase temperature to 200°C at a rate of 10°C/min and hold for 4 min.
4. Increase temperature to 325°C at a rate of 40°C/min and hold for 1 min.
5. The ion source, quadrupole and transfer line should be maintained at 300, 150 and 280°C, respectively.
6. The compounds are detected in SIM mode using their characteristic ions (the dwell time of the scanning was set at 30 ms for all the below ions):
   - Linalool: 71, 74, 93, 121, 136 and 154 m/z
   - Nerolidol: 69, 93, 107, 136 and 161 m/z
   - Pinene: 93, 121 and 136 m/z

3.3.3 Non-volatile, Coloured Isoprenoids (e.g. Lycopene): Spectrophotometry

Lycopene and other carotenoids/isoprenoid pigments can be quantified spectrophotometrically due to their ability to absorb light of a specific wavelength. Lycopene measurements must always be compared to a standard curve of analytical-grade lycopene generated on the same spectrophotometer.

1. Set the spectrophotometer to 475 nm.
2. Using a glass cuvette, prepare blank with acetone. Wait for a few minutes to ensure blank measurements remains constant before measuring samples.
3. Transfer 700 μL of the sample (=acetone extract) into the cuvette and record the absorbance at 475 nm (*see* **Note 69**).
4. Discard acetone waste in adequate container and rinse the cuvette with 70% ethanol.

5. Invert cuvette on paper towel to dry it before measuring the next sample.

3.4 Data Analysis

Spectrophotometric data (non-volatile pigments) can be used directly. For GC–MS data, follow the instructions from your GC–MS instrument manual to retrieve data. As you extract the data for analysis, note the following:

1. Carefully examine controls to determine the level of background contamination and whether this will interfere with data interpretation
2. Overlay all calibrants to examine reproducibility of the GC–MS runs and identify the retention times of the relevant compounds.
3. Overlay your samples with the lowest concentration calibrant to confirm that the peaks share the same retention time and that your samples are above the LLOQ.
4. Use the GC–MS software to integrate under the peaks to obtain peak areas. We recommend that automatic integration is quality controlled by the user; many peak-picking algorithms are prone to inconsistencies depending upon peak quality.
5. Determine the quality of your calibrant data. The quality of your standard curve is critical to the integrity of your data. There are various ways of determining the quality of your standard curve; we use the following three criteria: (1) Technical replicates should not vary by more than 10%. (2) The R^2 value of the calibration linear regression should be >0.98. (3) The formula generated by your calibration curve should accurately back calculate the concentration of the calibrants. The lowest concentration calibrant must be within 20% and subsequent calibrants within 15%. If the lowest or highest concentration calibrant is not accurately back calculated, it may be due to a loss of linearity. Exclude the calibrant from your calibration curve, but be aware this shifts your lower and/or upper limit of quantification (LLOQ/ULOQ) to the next calibrant (and you still need a minimum of 5 points in the linear range of your standard curve). Any calibrants that fail the accuracy check should be removed from the regression; every single calibrant is independent from the rest and so should be assessed independently. Any background contamination, as long as it is consistent (i.e. same amount detected in all blanks), can be handled by subtracting the peak area of the contaminant from the target compound peak.
6. Calculate the concentration of your samples from the standard curve
7. Determine the quality of your sample data using two indicators: (1) Standard deviation and % CV of technical replicate peak integrals should not vary by more than 10%, and peak retention time differences should be negligible for GC–MS

analysis. (2) Biological variation should be reasonable considering the system; biological variation may be expected to be higher than technical variation, but should be sufficiently low to identify differences between treatments.

4 Notes

1. Ideally, individual colonies from the original transformation plates should be isolated as biological replicates, as per Bruschi et al. [16]; this results in three glycerol stocks for each strain. A single colony from each biological replicate is used as a replicate for the production experiments. Where only a single glycerol stock has been prepared, use at least three colonies from that stock as technical replications to provide a source of experimental variation. This will not reflect true biological variation, but rather technical variation for that specific cell line.
2. Use of rich/complex medium generally results in higher titres of isoprenoids.
3. The volume can be upscaled, but keep the medium to a maximum of 20% of the flask volume to ensure adequate aeration. Take care with upscaling yeast cultures, as they can crash if inoculated at very low concentrations.
4. Ensure sufficient volume is available for inoculation of final production culture at target OD (usually 0.05).
5. Prepare sufficient medium in bulk for inoculation and subsequent dispensing.
6. *See* Sect. **3.1** for more information about selection of appropriate production flasks/vials.
7. Keep in mind the oxygen requirements of the culture in closed systems (for some of the cultivation methods listed). If aiming for a fully aerobic fermentation, calculate the maximum culture volume based on the amount of oxygen in the vial and the amount of carbon in the medium that can be converted into biomass. For details, see El-Mansi et al. [17]. As a guideline, we use 0.5 mL for complex medium and 1–2 mL culture volume for CDM (with $\leq$10 g/L carbon source) in 20 mL vials for isoprene production experiments.
8. In our hands, we found that cultures in unbaffled shake flasks produced better than those in baffled shake flasks.
9. Dodecane, although diluted for the final chromatography step, tends to cause a strong reverse solvent effect [18, 19], which can result in chromatographic tailing of volatile isoprenoids. Different solvent mixtures (such as 1:4 toluene to hexane [20]) can be used in order to resolve this problem if it occurs.

10. The size of the tubes depends on sample size. *See* Sect. **3** for details.
11. Some *E. coli* strains form very tight pellets. Resuspension of the pellet is easier in 2 mL tubes in this case.
12. If there are more strains than can be handled in a single production run, include a strain from a previous run to determine reproducibility of the method.
13. Cultivation is usually 37°C/24 h for *E. coli* and 30°C/48 h for yeast; however, this should be modified for strain-specific requirements (e.g. some plasmids may require different incubation temperatures for maintenance; some strains have impaired growth rates).
14. For yeast, use 1–3 colonies to inoculate a 10 mL pre-culture (depending on previously determined growth rates, pre-culture volume and time considerations). For *E. coli*, 1 colony is usually sufficient. Whatever number of colonies you choose, be consistent; aim to keep the amount of biomass used to inoculate reasonably uniform where practicable.
15. For yeast, typically 30°C and 200 rpm; for *E. coli*, typically 37°C and 250 rpm.
16. It is very important to ensure that the pre-culture is in mid-log phase; otherwise, lag phases and/or altered growth profiles can occur in the production culture. Incubation time depends on strain; for yeast, 12 h is normally adequate, but up to 24 h may be required for cultures growing in chemically defined medium (CDM). For *E. coli*, 2–6 h in complex medium and up to 20 h in CDM are usually sufficient. As noted above, some strains suffer growth defects and may take much longer. If the growth rate and profile are known, growth times can be predicted. Depending on the growth rate and experimental timing, it may be necessary to plan for a second pre-culture in order to allow sufficient lab time to properly track the production culture.
17. Aim for an OD_{600} of 0.5–1.0 for *E. coli* or an OD_{660} of <4.0 (typically 2.5–3.0) for yeast.
18. If starting at an OD of 0.05, an initial culture OD reading is not usually meaningful, as the lower linear range limit of many spectrophotometers is around 0.5. The first OD reading is in any case very rarely within the linear range. It is therefore wise to record the OD of the production culture after about 0.5–1 h (*E. coli*) or 1–2 h (yeast).
19. Keep in mind the oxygen requirements of the culture in this closed system. If aiming for a fully aerobic fermentation, calculate the maximum culture volume based on the amount of oxygen in the vial and the amount of carbon in the medium

that can be converted into biomass. For details, see Vickers et al. [9]. As a guideline, we use 0.5 mL for complex medium and 1–2 mL culture volume for CDM (with <10 g/L carbon source).

20. When doing time-course measurements, allow enough time for GC–MS analysis in between sampling of the cultures/replicates. Our isoprene method (see below) takes 12 min; therefore, replicate cultures should be started ca. 15 min apart.
21. It is not recommended to store vials containing isoprene before GC–MS analysis. We have kept vials at 4°C for <2 days without observable losses, but have observed leaking of isoprene upon longer storage/freezing of the vials at −20 or −80°C.
22. As above (*see* Notes 7 and **17**), keep in mind the oxygen requirements of the culture in this closed system. We have used this method primarily for yeast cultures, and we assume that the culture will not remain aerobic for the whole cultivation period. We typically use 10 mL culture in a 100 mL screw-capped flask (*see* Sect. **2.1** above) for lab-scale high-throughput analyses.
23. Baffled or unbaffled flasks may be used. For lycopene production, in our hands we find that unbaffled flasks provide higher titres as long as a maximum 1:5 ratio for culture volume/flask volume is used.
24. Dodecane/culture ratio can be modified according to expected production levels. Where good titres are expected, a 10% (1 mL dodecane per 10 mL medium) overlay is used. For untested products or if a low production titre is expected, this can be decreased to 2.5% (250 μL per 10 mL medium); less than 2.5% hinders recovery efficiency and is not recommended.
25. To ensure accurate pipetting when dosing in dodecane, pre-wet the pipette tip each time (even if you are using the same pipette tip for all samples). Failure to pre-wet the pipette tip can lead to dripping; lost volume will artificially concentrate your sample.
26. NB: Do not autoclave cultures with organic solvents in them; dispose of according to appropriate institutional protocols.
27. Sample simultaneously from the parent culture to measure OD/biomass and for ECMet (biomass can also be measured after taking the isoprene readings).
28. If production levels are high and sufficient organic phase is used (e.g. 10% with a 100 mL culture), it is possible to sample repeatedly from one flask (after allowing flasks to settle for some minutes) for both organic sample and OD. However, this may affect overall culture growth rate, and in general, we do not recommend this approach for users just starting out unless they are using previously engineered strains.

29. If OD samples are to be taken, flasks must be placed on the bench for 5 min to allow the dodecane layer to separate properly. Swirl flasks very gently but thoroughly prior to sampling for OD readings to fully resuspend the biomass without disturbing the stratification of the dodecane. Sample with the flask on an angle well under the dodecane phase. Alternatively, follow the protocol below for biomass by cell dry weight.
30. Try to get as much culture/dodecane into the tube as possible.
31. Minimise transfer of medium.
32. If a dodecane concentration of 2.5% per 10 mL culture (250 μL) is used, approximately 200 μL should be retrievable.
33. Samples are reasonably stable and can be processed in parallel with other centrifugation steps (e.g. at **step 9** below) or while waiting for other samples.
34. ECMet samples can be taken from the supernatant at this stage. Take care to avoid any residual dodecane.
35. Be careful not to disturb the phase interface. For the example above, 100 μL is sufficient. Use a glass insert in the chromatography vial for such small volumes.
36. Be careful not to lose any pellet. Residual liquid is fine; it will sublime away during the freeze-drying step.
37. Samples can be stored frozen prior to freeze-drying.
38. Note that samples taken at low OD will not provide accurate biomass measurements.
39. If sampling multiple time points: in order to maintain a constant level of aeration and minimise evaporative concentration, start with a large enough culture volume.
40. Protect samples from light and high temperature whenever possible. Keep samples on ice and covered with aluminium foil, and only process ca. ten samples at one time to avoid extended handling times.
41. Close all tubes and vials immediately after pipetting.
42. When pipetting acetone, equilibrate the pipette beforehand by pipetting up and down with acetone a few times. Pipette quickly and carefully; otherwise, acetone will drip from the pipette tip
43. Biomass and ECMet samples can also be taken at this stage. For ECMet, take your sample from the lycopene sample after the centrifugation step; this way you need only one sample for both lycopene and ECMet.
44. The pellet is usually quite firm.
45. Snap-freeze here on dry ice or liquid nitrogen, and store at −20°C for later processing if required. Storage of samples at later points in the method is not possible.

46. Be very careful not to disturb the pellet.
47. *Important*: The pellet should become a slurry before adding acetone; this allows easy and reproducible extraction of lycopene.
48. This is our standard acetone extraction volume for *E. coli* strains producing low levels of lycopene. The amount of biomass harvested in **step 1** as well as the amount of acetone in this step can be varied to accommodate for strains producing very low or high amounts of lycopene.
49. Take care not to transfer any cell debris from the pellet into the new tube as this will strongly interfere with the absorbance measurement.
50. For example, with aluminium foil.
51. After sampling, biomass tends to settle to the bottom of the vial. The majority of the supernatant can then be carefully poured off, and the remaining culture freeze-dried and weighed to measure biomass as an alternative to measuring OD (as long as pre-weighed vials were used to incubate samples).
52. We have also use caryophyllene successfully.
53. When performing dilutions, it is important not to perform too large a dilution in one step as this leads to inaccuracy; e.g. a 1/1,000 dilution would be performed in two steps, 1/100 dilution followed by a further 1/10 dilution.
54. As a guide, prepare sufficient volume of diluent to provide 0.5 mL/calibrant and 0.25 mL/sample. The same diluent preparation must be used for ALL calibrants and samples in a single analytical batch as the ratio of ISTD/target compound peak area is used in quantification calculations.
55. Take relative densities and concentrations of solvents/ISTDs into account for these calculations.
56. For example, use the 0.1 M stock to prepare the 10.25 mM stock and the 10.25 mM to prepare the 1.025 mM stock.
57. Example: If samples are to be diluted 40-fold in the diluent prepared in step "Diluent (with Internal Standard)", then calibrants should be prepared in dodecane at 40-fold the target concentration.
58. The recommended *limonene* calibrant concentrations are 1.5, 5, 10, 25, 50, 75, 100 and 150 μM. The recommended *amorphadiene* calibrant concentrations are 0.5, 1, 1.5, 2, 2.5, 4, 6 and 8 ng/μL. These concentrations generate eight point curves, providing more flexibility; however, the concentrations may vary depending on the target concentrations of the compounds in the actual biological samples.

59. Samples in dodecane should be diluted at least 40-fold to minimise interference from the dodecane peak and interference with the ion source.
60. Other stocks can also be used to prepare these dilutions.
61. Technical replicates are separate injections and require separate vials – repeat injections cannot be from the same vial.
62. This provides a second set of technical replicates for the repeat calibration curve at the end of the run; this is required for large sample numbers.
63. Use the same method listed in "Diluent (with Internal Standard)" **steps 3–8**.
64. Remove and replace the lid of the diluent every time you take out an aliquot. Do not allow it to sit open in the fume cupboard as it will volatilise.
65. A negative, no limonene, standard is included to clearly identify the ISTD peak and the true baseline. If contaminants are detected here, they are present in the solvents used for sample prep. This is a critical control, as limonene contamination has been encountered previously.
66. The CT vial is a control injection for when you have excessive numbers of samples. It is a known concentration injection that can be used as a quality control measure to determine whether the calibration curve is still accurately calculating target compound concentrations throughout the run.
67. This value is used to compare the amount missing to the amount removed. While this provides an estimate of how much diluent is volatilising, it does not account for residue discarded with pipette tips.
68. Record the order of your samples!
69. Ensure measurement remains within the previously determined linear range of the assay. If absorption exceeds this range, dilute samples with acetone appropriately.

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Synthetic Biology of Hydrophobic Polymer Production

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Abstract

One-step fermentative synthesis system for the production of polylactic acid (PLA) and PLA copolymers, wherein the latter consist of various 2-hydroxyacids, 3-hydroxyacids, or 4-hydroxyacids, has recently been developed in recombinant microorganisms by an employing engineered polyhydroxyalkanoate (PHA) biosynthesis system. This was accomplished by the construction of basic metabolic pathway for the synthesis of lactyl-CoA and other hydroxyacyl-CoA, which are ultimately used for the synthesis of PLA and PLA copolymers by PHA synthase engineered to accept lactyl-CoA and other hydroxyacyl-CoA as substrates. Here we describe the detailed procedures for the construction of metabolically engineered *Escherichia coli* for the production of PLA and poly(3-hydroxybutyrate-*co*-lactate)[P(3HB-*co*-LA)] in below protocol.

Keywords: *Escherichia coli*, Metabolic engineering, PHA synthase, PLA, PLA copolymer, Polylactic acid, Propionyl-CoA transferase

1 Introduction

Bio-based industry has intensively developed microbial fermentation processes for the production of chemicals, polymers, and fuels, all of which are strong candidates to compensate the defects of petroleum-based materials in respect of environmental problems and to eventually substitute chemically synthesized products. Also, bio-based products are promising solutions for the energy and environmental problems caused by the exhaustion of fossil fuels and CO_2 accumulation in the atmosphere (1). Since cost of target products is one of the most important factors in the commercialization of target products, cost-effective bioprocesses have been developed to reduce product costs by enhancing efficiency of fermentation, purification in chemical grade, and post-processability of bio-based products.

Polyhydroxyalkanoates (PHAs) are biopolyesters synthesized and accumulated by numerous microorganisms as energy and carbon storage materials when bacteria meet nutrient-limiting conditions in the presence of excess carbon substrate (2, 3). Since more

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than 150 kinds of hydroxycarboxylic acids having different functional groups have been identified as possible monomers for PHAs (4), the material properties of PHAs that are much versatile properties of the PHAs are mostly based on the composition of the monomers and can be altered by using different types and combinations of monomers in PHA synthesis (5, 6). Among the 150 different types of monomer constituents of PHAs, the most frequently incorporated monomers into PHAs are (*R*)-3-hydroxycarboxylic acids such as 3-hydroxybuyrate (3HB), 3-hydroxyhexanoate (3HHx), 3-hydroxyoctanoate (3HO), 3-hydroxydecanoate (3HD), and 3-hydroxydodecanoate (3HDD) (2–4). Poly(3-hydroxybutyrate) [P(3HB)] is the best characterized member of PHAs based on its metabolism, production, and properties for its application in the area of biomass-derived plastics.

Recently, the monomer spectrum of PHAs has been broadened to accept 2-hydroxyacids such as lactic acid, 2-hydroxybutyric acid, and glycolic acid as monomer constituents by the development of recombinant *Escherichia coli* and *Ralstonia eutropha* strains capable of producing polylactic acid (PLA), poly(3-hydroxybutyrate-*co*-lactate) [P(3HB-*co*-LA)], and poly(3-hydroxybutyrate-*co*-2-hydroxybutyrate-*co*-lactate) [P(3HB-*co*-2HB-*co*-LA)] (7–14).

For the construction of biosynthesis pathway of lactate-containing PHAs such as P(3HB-*co*-LA), engineered *Clostridium propionicum* propionyl-CoA transferase (Pct_{Cp}) that converts (*D*)-lactate to (*D*)-lactyl-CoA using acetyl-CoA as the CoA donor and engineered *Pseudomonas* sp. 6–19 PHA synthase ($PhaC1_{Ps6–19}$) to accept lactyl-CoA as substrate were employed in recombinant *E. coli* (7). Also, efficiency of lactate-containing PHAs synthesis could be enhanced by genome engineering of *E. coli* XL1-Blue based on systems level metabolic pathway analysis to synthesize more (*D*)-lactyl-CoA available as substrate for the PHA synthase (8).

In this chapter, we describe the basic protocols to explain the procedure for the production of P(3HB-*co*-LA) in recombinant *E. coli*, which include the construction of the expression vector for PHA synthase and propionyl-CoA transferase, genome engineering of an *E. coli* host strain, cultivation of recombinant *E. coli* for the production of P(3HB-*co*-LA), and analysis of P(3HB-*co*-LA) produced in recombinant *E. coli*.

2 Materials (*see* Note 1)

2.1 Construction of Plasmid Expressing PHA Synthase and Propionyl-CoA Transferase

1. Vector: pBluescript II KS (+).
2. Genes of interest.
3. Primers: Some of the primers used in this study are listed in Table 1 (*see* **Note 2**).

Table 1
Primers used in PCR experiments[a]

Primers	Primer sequence	Target gene
Primer 1	**GGATCC** CAAGTACCTTGCCGACATCTATGC	*R. eutropha* PHA biosynthesis genes
Primer 2	**GAATTC** CCTCGCCCCCGCGAGGGCCGC	
Primer 3	**TTCGAA** TAGTGACGGCAGAGAGACAATCAAATC ATGAGTAACAAGAGTAACGATG	*Pseudomonas* sp. 6–19 *phaC*
Primer 4	**CCTGCAGG** TTACCGTTCGTGCACGTACGTGC	
Primer 5	**CCTGCAGG** TTCCCTCCCGTTTCCATTG AAAGGACTACACA ATGAGAAAGGTTCCCATTAT	*C. propionicum pct*
Primer 6	**CATATG** TCAGGACTTCATTTCCTTCAG	
Primer 7	**GAATT**CTACCGTTCGTATAGCATACATTATACGAAGTTATTGCCCTGAACCGACGACCG	Lox66 and Lox 71 Cm gene
Primer 8	**GAATTC**TACCGTTCGTATAATGTATGCTATACGAAGTTATCATCACCCGACGCACTTTGC	
Primer 9	ATGTCGAGTAAGTTAGTACTGGTTCTGAACTGCGGTAGTTCTTCACTGAAATTTGCCATC TACCGTTCGTATAGCATAC	Deletion of the *ackA* gene
Primer 10	TCAGGCAGTCAGGCGGCTCGCGTCTTGCGCGATAACCAGTTCTTCGTTGGTTGGGATAAC TACCGTTCGTATAATGTATG	

[a]Restriction enzyme sites are shown in bold. The sequences of the *ackA* gene are underlined

4. PCR Master Mix, SuPrime HF Premix (2X) (GeNet Bio (http://genetbio.co.kr)) for the amplification of genes of interest. PCR Master Mix from any companies can be used.
5. QIAquick PCR Purification Kit and QIAquick Gel Extraction Kit (Qiagen (http://www.qiagen.com)).
6. Wizard Genomic DNA Purification Kit and Wizard Minipreps DNA Purification Systems (Promega (http://www.promega.com)).
7. Restriction enzymes: *Bst*BI, *Bam*HI, *Eco*RI, *Sbf*I, and *Nde*I (New England Biolabs (http://www.neb.com)) (*see* **Note** 3).
8. T4 DNA ligase (New England Biolabs (http://www.neb.com)).
9. Host strains: *E. coli* XL1-Blue strain (Stratagene (http://www.genomics.agilent.com/en/home.jsp)) is used for cloning.
10. Ampicillin antibiotic.
11. Growth media: LB medium.

2.2 Construction of Host Strains Engineered by Genome Engineering

1. Vector: TOPO plasmid (Invitrogen (http://www.lifetechnologies.com)), pKD46 (15), pJW168 (16).
2. Genes of interest.
3. Primers: Some of the primers used in this study are listed in Table 1.
4. PCR Master Mix, SuPrime HF Premix (2X) (GeNet Bio (http://genetbio.co.kr)) for the amplification of genes of interest. PCR Master Mix from any companies can be used.
5. QIAquick PCR Purification Kit and QIAquick Gel Extraction Kit (Qiagen (http://www.qiagen.com)).
6. Wizard Genomic DNA Purification Kit and Wizard Minipreps DNA Purification Systems (Promega (http://www.promega.com)).
7. Bacteria strains: *E. coli* XL1-Blue strain (Stratagene (http://www.genomics.agilent.com/en/home.jsp)) is used for cloning.
8. Ampicillin, chloramphenicol antibiotics.
9. Growth media: LB medium.

2.3 Cultivation of Recombinant E. coli for the Production of PLA Copolymer

1. Vector: p619C1437-pct540 (*see* **Note** 4).
2. Bacteria strains: Recombinant *E. coli* XL1-Blue that expresses PhaC1437 and Pct540 is used as host strain for biosynthesis of PLA copolymer.
3. Ampicillin antibiotics.

4. Sodium 3-hydroxybutyrate (3HB sodium salt, Acros organics, Geel, Belgium).
5. Growth media: LB medium (Complex) and MR medium (Chemically defined).

2.4 Analysis of PLA Copolymer Produced by Recombinant E. coli

1. Chemicals: Chloroform, methanol, benzoic acid, H_2SO_4, poly (3-hydroxybutyrate), lactate (*see* **Note** 5).
2. Equipment: Agilent 6890 N Gas Chromatography (GC) system (Agilent Technologies, CA, USA) equipped with Agilent 7683 automatic injector, flame ionization detector, and a fused silica capillary column (ATTM-Wax, 30 m, ID 0.53 mm, film thickness 1.20 μm, Alltech, PA, USA).

2.5 Culture Media

1. In our lab, we use products of Difco Korea (http://www.bd.com/) for LB medium containing per liter: 10 g Bacto-Tryptone, 5 g Yeast extract, 10 g NaCl.
2. The MR medium (pH 7.0) contains (per liter) 6.67 g KH_2PO_4, 4 g $(NH_4)_2HPO_4$, 0.8 g $MgSO_4 \cdot 7H_2O$, 0.8 g citric acid, and 5 mL trace metal solution.
3. The trace metal solution for MR medium contains (per liter of 0.5 M HCl) 10 g $FeSO_4 \cdot 7H_2O$, 2 g $CaCl_2$, 2.2 g $ZnSO_4 \cdot 7H_2O$, 0.5 g $MnSO_4 \cdot 4H_2O$, 1 g $CuSO_4 \cdot 5H_2O$, 0.1 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, and 0.02 g $Na_2B_4O_7 \cdot 10H_2O$.
4. Ampicillin (Ap, 50 μg/mL) and chloramphenicol (Cm, 34 μg/mL) were added to the medium depending on the resistance marker of the employed plasmid.

2.6 Preparation of MR Medium

1. In one liter of distilled water, 6.67 g KH_2PO_4, 4 g $(NH_4)_2HPO_4$, 0.8 g citric acid, and 5 mL trace metal solution are dissolved. When these chemicals are dissolved in water, the pH of MR medium is around 5. The pH of medium is adjusted by adding NaOH granules or by adding 10 N NaOH solution.
2. $MgSO_4 \cdot 7H_2O$ is separately autoclaved or filtered. In our lab, 80 g/L of $MgSO_4 \cdot 7H_2O$ is separately made and used for MR medium by adding 1 mL of this stock solution into MR medium lacking $MgSO_4 \cdot 7H_2O$.
3. For the preparation of the trace metal solution, 0.5 M HCl solution is used for dissolution of chemicals. Concentrated HCl solution is used for the preparation of 0.5 M HCl solution. After dissolution of all the chemicals, the color of trace metal solution is yellowish green.

3 Methods

3.1 Construction of p619C1437-pct540

1. The general PCR reaction conditions are: 95°C for 5 min; 30 cycles of: 94°C for 30 s, 55°C for 30 s, 72°C for 1 min 30 s; and a final extension of 72°C for 5 min. The final reaction volume is 20 μL.
2. *Ralstonia eutropha* PHA biosynthesis operon (*phaCAB*), which was amplified from the chromosomal DNA of *R. eutropha* NCIMB11599 using primers 1 and 2, was inserted into pBluescript II KS(+) at *Bam*HI and *Eco*RI sites to make pCnCAB.
3. *R. eutropha phaC* gene in the pCnCAB was replaced by the $phaC1_{Ps6–19}$ gene that was amplified from the genomic DNA of *Pseudomonas* sp. MBEL 6–19 using primers 3 and 4 at the *Bst*BI and *Sbf*I sites to make p619C1-ReAB.
4. *R. eutropha phaAB* gene in the p619C1-ReAB was replaced by the *Clostridium propionicum pct* gene that was amplified by PCR from the chromosomal DNA of *C. propionicum* DSM 1682 using primers 5 and 6 at the *Sbf*I and *Nde*I sites. The resulting plasmid, designated as p619C1-CpPCT, contains the *Pseudomonas* sp. MBEL 6–19 *phaC1* gene and the *C. propionicum pct* gene, in which the expression of the corresponding genes was driven by the *R. eutropha* PHA biosynthesis operon promoter.
5. The details of the construction of plasmid p619C1437-pct540, which expresses the *Pseudomonas* sp. 6–19 PHA synthase gene containing quadruple mutations of E130D, S325T, S477G, and Q481K (PhaC1437) and the *C. propionicum* propionyl-CoA transferase mutant gene containing V193A and four silent nucleotide mutations of T78C, T669C, A1125G, and T1158C (Pct540) under the *Ralstonia eutropha* PHA biosynthesis operon promoter, can be found in the paper published in Biotechnology and Bioengineering (Fig. 1) (7).

3.2 Genome Engineering of E. coli Host Strain

1. The PCR fragment containing the lox71 site, the chloramphenicol resistance gene, and the lox66 site fused together was obtained by PCR using the using primers 7 and 8 listed in Table 1.
2. This PCR fragment was inserted into a TOPO plasmid to make TOPO-Cm-lox66-71.
3. PCR was performed using primers 9 and 10 listed in Table using TOPO-Cm-lox66-71 as a template. In Table 1, the primers for the deletion of the *ackA* gene are listed.
4. The final PCR product was introduced by electroporation into *E. coli* harboring pKD46 expressing the λ-Red recombinase.

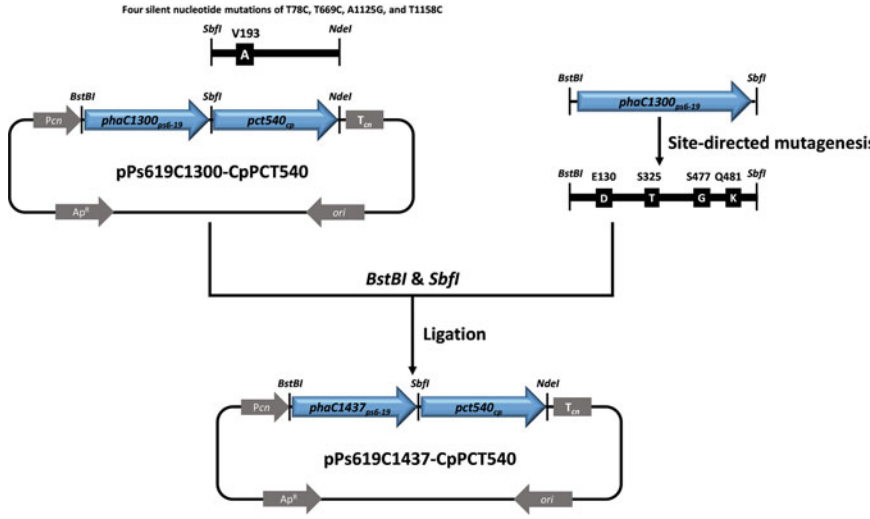

Fig. 1 Schematic diagram of the construction of p619C1437-pct540

5. The mutants in which gene inactivation occurred by double homologous recombination were selected on the LB agar plate containing 34 μg/mL chloramphenicol and subsequently screened by direct colony PCR. About ten colonies were streaked for maintenance of mutants at LB agar plate containing 34 μg/mL chloramphenicol, in which each colony was numbered. Colony PCR was usually carried out following the general PCR reaction conditions except that colony picked by sterilized a toothpick was used a template by inoculating (putting bacteria picked by toothpick) PCR tube.
6. To construct marker-free mutant strains, the antibiotic selection marker was eliminated by using a helper plasmid, pJW168, expressing the Cre recombinase and harboring ampicillin resistance gene and a temperature-sensitive replication origin.
7. The chloramphenicol resistant mutants were transformed with the pJW168, and ampicillin-resistant transformants were selected on LB agar plates containing 100 μg/mL ampicillin and 1 mM IPTG (for the expression of Cre recombinase) at 30°C. Those colonies that lost the chloramphenicol resistance were selected. Among them, positive colonies were cultivated in LB medium without antibiotic markers at 42°C and then were examined for the loss of all antibiotic resistance markers by colony PCR (Fig. 2).

3.3 Culture Conditions for Flask Cultures

1. Transform *E. coli* XL1-Blue competent cells with p619C1437-pct540 vector and plate the transformed cells on a LB agar plate containing 50 μg/mL ampicillin. Leave for 16 h at 37°C until colonies of transformed bacteria are clearly visible.

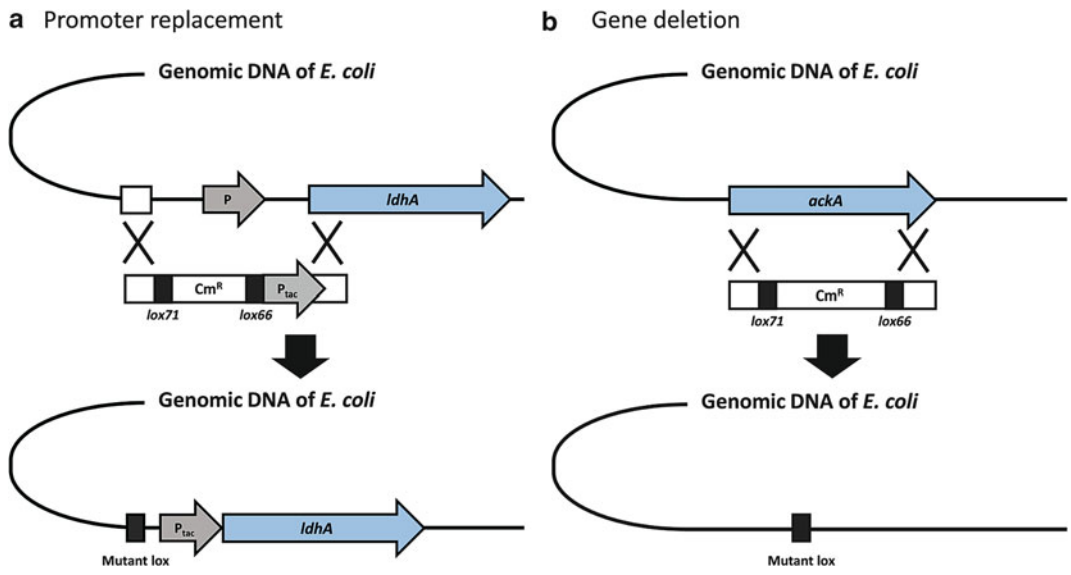

Fig. 2 Schematics of genome engineering of *E. coli* host strain by PCR-mediated homologous recombination using λ-Red recombinase

2. Inoculate 2 mL of LB containing 100 μg/mL ampicillin in a 15 mL tube by picking single colony from the plate.
3. Grow bacteria shaking (250 rpm) at 37°C to an OD 600 nm (optical density) of 0.6–0.8 (about 12 h).
4. Transfer 1 mL of LB culture medium into 100 mL of MR medium supplemented with 20 g/L of glucose, 2 g/L of sodium 3-hydroxybutyrate, and 50 μg/mL ampicillin in a 250 mL flask. Glucose, $MgSO_4 \cdot 7H_2O$, and 3HB sodium salt were sterilized separately (*see* **Note** 6).
5. Continue culture growth for further 96 h at 30°C shaking (250 rpm).

3.4 Collection of Recombinant E. coli for the Analysis of Polymers

1. After cultivation, collect all *E. coli* XL1-Blue strain harboring p619C1437-pct540 by centrifugation at 6,000×*g* for 10 min. 100 mL culture medium is divided into two Falcon™ 50 mL conical centrifuge tubes.
2. Discard the supernatant and wash cells two or three times with 50 mL of distilled water to remove organic acids such as lactic acid produced during cultivation (*see* **Note** 7).
3. Dry the cells in the 95°C oven for more than 24 h to remove water.

3.5 Analysis of Polymers by GC

About 30 mg of dried cell pellet was subjected to methanolysis with benzoic acid as an internal standard in the presence of 15% sulfuric acid. The resulting methyl esters of constituent lactate and carboxylic acids were assayed by GC according to the method of a previous report (17).

1. Prepare methanolysis solution containing per liter: MeOH 970 mL, H_2SO_4 30 mL, Benzoic acid 8 g.
2. Put about 30 mg of dried cell pellet in Pyrex cap tube.
3. Add 1 mL of methanolysis solution and 2 mL of chloroform into Pyrex cap tube containing 30 mg of dried cell pellet and vortex.
4. Do methanolysis reaction to produce 3-hydroxybutyric acid methyl ester and lactic acid methyl ester for more than 4 h (in our lab, overnight reaction is preferred) in 95°C oven.
5. After methanolysis, cool down Pyrex cap tube in room temperature and add 1 mL of distilled water into Pyrex cap tube.
6. Vortex Pyrex cap tube for 1 min and wait until two layers separate. 2 mL of upper layer consists of water and methanol and 2 mL of lower layer consists of chloroform that contains 3-hydroxybutyric acid methyl ester and lactic acid methyl ester.
7. Use 1 mL of chloroform for GC analysis.

4 Notes

1. In the list of Materials (Subheading 2), we identify the names of vendors from which we usually purchase reagents and chemicals, but we do not have any intention to endorse these specific vendors. We provide the URLs of the vendor sites which are now commonly used.
2. After PCR reaction, we usually insert these PCR products into TOPO plasmid for sequencing. Thus, no additional sequences are added at 5′ ends of primers besides restriction enzyme recognition sites to ensure functional restriction of PCR products.
3. Instead of *Bst*BI that needs high temperature for the restriction reaction, *Sfu*I (http://lifescience.roche.com/) can be used.
4. Any expression system can be used for the expression of PHA synthase and propionyl-CoA transferase. However, it has been reported that expression of PHA biosynthesis genes under weak constitutive promoter in high copy number plasmid is favorable for high-level production of PHA.
5. For GC analysis, lactate and P(3HB) are used for the preparation of a standard curve for the analysis of P(3HB-*co*-LA) produced in recombinant *E. coli*.
6. Sodium 3-hydroxybutyrate is added to the culture medium as the direct precursor of 3-hydroxybutyryl-CoA, which is generated by propionyl-CoA transferase. Adding sodium 3-hydroxybutyrate more than 2 g/L might inhibit cell growth. Instead of adding sodium 3-hydroxybutyrate, expression of *R. eutropha phaAB* genes that are involved in the synthesis of 3-hydroxybutyryl-CoA from acetyl-CoA can be used as an alternative.

7. Since lactate and sodium 3-hydroxybutyrate exist in the culture medium, these might be detected as monomer constituents after methanolysis reaction if they are not thoroughly removed by washing with distilled water. In our lab, collected cells are usually washed three times with distilled water.

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Methods for Recombinant Rhamnolipid Production

Till Tiso, Andrea Germer, Benjamin Küpper, Rolf Wichmann, and Lars M. Blank

Abstract

Rhamnolipids are glycolipidic microbial biosurfactants with potential, e.g., as emulsifier or foaming agents in industrial applications. Currently rhamnolipids are produced with pathogenic wild-type strains such as *Pseudomonas aeruginosa* during growth on hydrophobic substrates (e.g., plant oils). The combination of complex regulation of secondary metabolites in wild-type strains like the quorum sensing system in *P. aeruginosa*, costly substrates, and low production rates is hindering the market success of rhamnolipids. Challenges include sophisticated fermentation, time-consuming production, and laborious and expensive downstream processing. Recombinant rhamnolipid production is capable of overcoming all these downsides.

Here we present a protocol for the heterologous production of rhamnolipids. This method offers advantages such as (1) easy expression regulation by IPTG induction, (2) glucose as carbon source, and (3) a nonpathogenic host. The most prominent genes responsible for rhamnolipid synthesis, emanating from *Pseudomonas* or *Burkholderia* species, are already identified. We describe a method for cloning and expressing the genes *rhlA* and *rhlB* from *P. aeruginosa* in vector pVLT33 allowing for the production of mono-rhamnolipids. Furthermore, we present cultivation methods for the constructed strain, fermentation procedures for upscaled production of the product of interest, qualitative as well as quantitative analytical methods, and finally purification protocols.

Keywords: Analytics, Biosurfactant, Foam fractionation, Purification, Recombinant *Pseudomonas putida*, Recombinant rhamnolipid production

1 Introduction

As rhamnolipids are surface-active agents (surfactants), they show high potential for diverse applications [1, 2]. Only to mention a few examples, they can be used as detergents or foaming agents in washing and household cleaning agents, as emulsifying agents in the food and cosmetic industry, and in bioremediation [3]. Rhamnolipids can even be utilized in cutaneous wound healing [4] and other pharmaceutical applications [5]. Moreover, rhamnolipids present an eco-friendly alternative to chemically synthesized detergents as they are biodegradable, can be produced from renewable

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resources, and thus are regarded as "green" chemicals [6]. Furthermore, they show antimicrobial effects [7, 8] and play a role in plant defense against microbes [3].

Rhamnolipids are glycolipids. That means that they consist of a glycosidic and a lipidic part. Thus, they are amphiphilic molecules composed of one or two rhamnose units that are linked by a β-glycosidic bond to one or two β-hydroxy fatty acids (Fig. 1). Rhamnolipids are classified as mono- or di-rhamnolipids depending on the number of rhamnose molecules.

Naturally occurring rhamnolipid structures vary in fatty acid chain lengths (8–24 carbon atoms) and saturation grade (up to two unsaturations) leading to about 60 discovered structures [6].

The main rhamnolipid producers are *Pseudomonas* and *Burkholderia* species, which are representatives of the γ- and β-proteobacteria, respectively. Wild types like *P. aeruginosa* produce rhamnolipids with hydroxy fatty acid chain lengths of mainly ten carbon atoms [9–11], whereas for *Burkholderia* species, the major congener contains C_{14} chains [12, 13]. Rezanka et al. [14] reported thermophilic strains producing rhamnolipids containing hydroxy fatty acids with chain lengths of up to 24 carbon atoms.

Most reports about rhamnolipid production from bacterial wild-type strains focus on *P. aeruginosa*, which is an opportunistic pathogen featuring a complex regulation of the expression of the genes responsible for rhamnolipid production involving a quorum sensing system that is activated under phosphate or nitrogen limiting conditions. Müller et al. [15] achieved a titer of 39 g/L rhamnolipids after 90 h of cultivation with sunflower oil as carbon source. The maximum rhamnolipid concentration reported was 112 g/L using soybean oil after a fermentation period of 11 days [16]. However, these titers have not been published a second time.

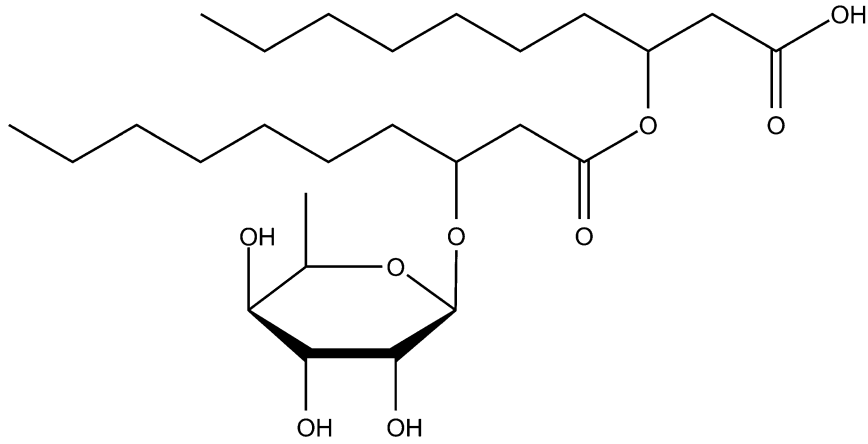

Fig. 1 Molecular structure of a rhamnolipid

The motivation to construct a recombinant rhamnolipid-producing strain is to overcome some of the described challenges of the wild type: First, the strains are cultivated using plant oils as sole carbon and energy source resulting in low production rates. Second, downstream processing requires costly [10, 17] and labor-intensive methods in order to separate the rhamnolipids from the resulting emulsion of oil and culture broth. Third, for industrially safe production, the pathogenicity of the strain is an obstacle. There are many reports about rhamnolipid-producing isolates other than *P. aeruginosa*, but their definite identification and pathogenicity require further investigation [6].

Therefore, in our approach for recombinant production of rhamnolipids, *P. putida* KT2440 was chosen as production host. Being the host of the first host-vector biosafety (HV1) system for gene cloning in gram-negative soil bacteria, it was certified as a safety strain [18], and thus, authorization procedures should be significantly simpler and less time-consuming. It furthermore shows only little change in growth rate in the presence of rhamnolipid concentrations of up to 90 g/L [19]. *P. putida* KT2440 contains both necessary pathways for the precursors for rhamnolipid biosynthesis, which for fatty acid availability was thought to be fatty acid de novo synthesis solely, but newer investigations showed that it is β-oxidation or a combination of both [20] and formation of activated rhamnose. We introduced the *P. aeruginosa* genes *rhlAB* (PA3479 and PA3978, respectively) annotated as rhamnosyltransferase chain A (although it was shown that it is not a glycosyltransferase [21]) and rhamnosyltransferase chain B, respectively, into *P. putida* KT2440 using vector pVLT33. With this recombinant construct, mono-rhamnolipid titers of up to 0.22 g/L could be obtained. As techniques for high-cell-density fermentations with *P. putida* KT2440 exist [22, 23], increasing the titers and thus the space-time yield of rhamnolipid-producing fermentations is promising.

A further benefit of *P. putida* KT2440 as host strain for the recombinant production of rhamnolipids is its close relationship to the natural producer *P. aeruginosa*. Ochsner et al. [24] expressed *rhlAB* under the control of the *tac* promoter in different host strains and showed that *P. putida* KT2442 featured the highest rhamnolipid titer. In general, *Pseudomonas* species are very tolerant toward difficult substrates or products that might show toxic effects to other host strains. Thus, the production of rhamnolipids with *P. putida* bears great potential for industrial applications and might develop for the production of "green" chemicals.

2 Materials

2.1 Strain Development

2.1.1 Strain

- Microtiter plate-based cultivation system with online growth monitoring like the BioLector by m2p-labs GmbH (Baesweiler, Germany)
- *Escherichia coli* DH5α
- *P. putida* KT2440
- *P. aeruginosa* PA01 (handle with care, as *P. aeruginosa* is an opportunistic pathogen)

2.1.2 Expression Cassette

- DNA isolation Kit (e.g., DNeasy Blood and Tissue Kit, QIAGEN, Hilden, Germany)
- DNA polymerase (e.g., PfuTurbo by Stratagene, Waldbronn, Germany)
- A suitable expression plasmid (we use the broad-host-range plasmid pVLT33 [25] with the ori from plasmid RSF1010 [26] and the promoter $lacI^q$/Ptac [27])
- *P. aeruginosa* PA01 (handle with care, as *P. aeruginosa* is an opportunistic pathogen)
- Primers
- Restriction enzymes
- Ligase (e.g., T4 DNA Ligase, Fermentas, Thermo Fisher Scientific, Waltham, MA, USA)
- Miniprep kit (e.g., QIAprep Spin Miniprep Kit, QIAGEN GmbH, Hilden, Germany)
- Competent *E. coli* DH5α for transformation
- Antibiotic (e.g., kanamycin or tetracycline)

2.1.3 Electroporation

- Electrocompetent host organism
- Sterile 300 mM sucrose solution
- 15 mL falcon tube
- 1–10 ng of plasmid DNA
- Electroporation cuvette (0.2 cm gap)
- Electroporator (e.g., Gene Pulser Xcell by Bio-Rad Laboratories Inc., Hercules, CA, USA)
- 1 mL prewarmed (30°C) LB medium
- Selective LB agar plates (e.g., with kanamycin or tetracycline)

2.1.4 Screening

Blood Agar Plates

- Blood agar base (e.g., from Carl Roth, Karlsruhe, Germany)
- Deionized water
- Defibrinated sheep blood (e.g., from Fiebig-Nährstofftechnik, Idstein, Germany)

- Petri dishes
- Possibly antibiotics

CTAB Plates

- Cetyltrimethylammonium bromide (CTAB, IUPAC name: hexadecyl-trimethyl-ammonium bromide)
- Mineral salts agar medium (KH_2PO_4 1.4 g/L, Na_2HPO_4 1.8 g/L, $NaNO_3$ 4 g/L, $MgSO_4 \times 7\ H_2O$ 0.8 g/L, $CaCl_2 \times 2\ H_2O$ 0.2 g/L, agar 30 g/L; adjust pH to 6.7 and autoclave)
- Trace elements solution ($FeSO_4 \times 7\ H_2O$ 2 g/L, $MnSO_4 \times H_2O$ 1.5 g/L, $(NH_4)_6Mo_7O_{24} \times 4\ H_2O$ 0.6 g/L; sterilize by filtration)
- Dye solution (CTAB 4 g/L, methylene blue 0.1 g/L; sterilize by filtration)
- 50% (m/vol) glucose solution (*see* Sect. 3.2.1)
- Sterile deionized water
- Petri dishes
- Antibiotics as required

2.2 Production of Rhamnolipids

- 10 mL volumetric flask (not a graduated cylinder)
- 500 mL graduated cylinder

2.2.1 Stock Solutions

- Kanamycin sulfate (or kanamycin) (store at 4°C)
- Tetracycline hydrochloride (or tetracycline) (light sensitive, store at −20°C and in a dark place)
- Isopropyl β-D-1-thiogalactopyranoside (IPTG)
- Glucose monohydrate (or glucose)
- Deionized water
- Pure ethanol
- 0.2 μm syringe filter

2.2.2 Media

Complex Medium

- Lysogeny broth (LB) medium [28]: 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl; autoclave

Minimal Media

- M9 medium [29]; prepare three solutions:
  - M9 salts: $Na_2HPO_4 \times 2\ H_2O$ 64 g/L, KH_2PO_4 15 g/L, NaCl 2.5 g/L, NH_4Cl 5.0 g/L, autoclave
  - 1 M $MgSO_4$, sterilize by filtration
  - US trace elements solution: 37% fuming HCl 82.81 mL/L, $FeSO_4 \times 7\ H_2O$ 4.87 g/L, $CaCl_2 \times 2\ H_2O$ 4.12 g/L, $MnCl_2 \times 4\ H_2O$ 1.50 g/L, $ZnSO_4 \times 7\ H_2O$ 1.87 g/L, H_3BO_3 0.30 g/L, $Na_2MoO_4 \times 2\ H_2O$ 0.25 g/L, $CuCl_2$

× 2 H_2O 0.15 g/L, Na_2EDTA × 2 H_2O 0.84 g/L, sterilize by filtration

- Wilms-MOPS (WM) medium [30]; prepare four solutions:
  - MOPS buffer: MOPS 104.65 g/L, adjust pH to 7.4, autoclave
  - WM salts (KH_2PO_4 150 g/L, $(NH_4)_2SO_4$ 250 g/L, NH_4Cl 75 g/L, Na_2SO_4 100 g/L, $MgSO_4$ × 7 H_2O 50 g/L, sterilize by filtration)
  - Vitamin solution: thiamin × HCl 10 g/L, sterilize by filtration and store at 4°C
  - Trace elements solution: $ZnSO_4$ × 7 H_2O 0.54 g/L, $CuSO_4$ 0.31 g/L, $MnSO_4$ × H_2O 0.3 g/L, $FeCl_3$ × 6 H_2O 41.76 g/L, Titriplex III 33.39 g/L, $CoCl_2$ × 6 H_2O 0.54 g/L, $CaCl_2$ × H_2O 1.98 g/L, sterilize by filtration

2.2.3 Cultivation

Cultivation in Shake Flasks

- Bacterial strain
- Medium
- Test tubes or small shake flasks without baffles (*see* **Note 1**) and aluminum caps (cotton plugs are also suited for sufficient aeration)

High-Throughput Cultivation

- Bacterial strain
- Medium
- Microtiter plate (MTP)-based shaking system like System Duetz [31] (http://www.enzyscreen.com/1427673.htm)
- Microtiter plates:
  - "Polypropylene square 24-deepwell microplates" (CR1424a) with the appropriate lids "sandwich cover for our own-manufactured polystyrene square 24-deepwell MTPs" (CR1224b)
  - "Polypropylene square 96-deepwell microplates" (CR1496a) with the appropriate lids "sandwich cover for 96-deepwell MTPs CR1496a" (CR1296b)

Fermentation in a Lab Scale Fermenter

- Bacterial strain
- Medium
- Glucose stock solution
- Fermenter with a volume of 1–5 L (e.g., KLF2000, Bioengineering AG, Wald, Switzerland, with a maximum volume of 3.2 L)
- pH probes and control
- pO_2 probe and control

- Medium-feeding system, which replaces the medium and the nutrients as well as glucose
- The required equipment to guide the foam out of the fermenter over the headspace or a mechanical foam destroyer (foaming out is to be preferred)
- Shake flasks and test tubes for pre-cultures
- 2N NaOH
- 2N HCl or H_3PO_4

2.3 Scaling-Up of Rhamnolipid Production

- Equipment that is adequate to handle the new liquid volume scale like pumps for the pH correction solutions and the feeding procedure
- Adequate container for the foam to be collected and collapsed (*see* **Note 2**)
- Elongated tubes for bases and acids (*see* **Note 3**)

2.4 Analytics

2.4.1 Orcinol Assay

- Vacuum centrifuge
- Benchtop thermo shaker (e.g., by DITABIS AG, Pforzheim, Germany)
- Spectrophotometer (e.g., Genesys 10 UV by Thermo Fisher Scientific, Waltham, MA, USA)
- Ice cold ethyl acetate
- Deionized water
- Orcinol solution (1.6% orcinol in deionized water)
- Sulfuric acid (60%)

2.4.2 Thin-Layer Chromatography

- TLC silica gel 60 aluminum sheets (e.g., Merck KGaA, Darmstadt, Germany)
- Blow drier
- TLC chamber with lid
- Rhamnolipid standard containing mono- and di-rhamnolipids (e.g., JBR425, Jeneil Biosurfactant Co., LCC, Saukville, USA)
- Running buffer: mixture of chloroform, methanol, and acetic acid in a ratio of 65:15:2
- Dye solution: anisaldehyde, sulfuric acid, and acetic acid in a ratio of 1:2:100
- TLC sprayer (e.g., TLC sprayer unit from Carl Roth GmbH & Co., Karlsruhe, Germany)

2.4.3 High-Performance Liquid Chromatography

Sample Preparation

- Acetonitrile
- 4 mm syringe filter with 0.2 µm pore size and a filter membrane of regenerated cellulose (*see* **Note 4**)

Chromatography

- HPLC system
  - Binary gradient HPLC pump
  - Autosampler
  - Column oven
  - Corona charged aerosol detector (Corona CAD) (Thermo Fisher Scientific Inc., Waltham, MA, USA)
  - Nitrogen generator
  - Additional for identification of unknown compounds: triple quadrupole mass spectrometer with electrospray ionization
- Reversed-phase (RP) C18 column. Choose one of the following (*see* **Note 5**):
  - NUCLEODUR C18 Gravity with a precolumn cartridge of 4 mm length with a particle size of 3 µm and dimensions of 150 × 4.6 mm from Macherey-Nagel GmbH & Co. KG (Düren, Germany)
  - Kinetex RP C18 column with a SecurityGuard ULTRA Cartridges UHPLC C18 precolumn (Phenomenex Inc., Torrance, CA, USA)
- Also applicable but with poorer resolution are RP C8 columns
  - ProntoSIL 120-3-C8 SH with the dimensions of 150 × 2 mm and a particle size of 3 µm (Bischoff Chromatography, Leonberg, Germany)
  - Luna C8(2) (dimensions: 4.6 × 150 mm, particle size: 5 µm) by Phenomenex Inc. (Torrance, CA, USA)
- Acetonitrile LC-MS grade
- Purified water (at least ultrapure water of "Type 1," as defined by various authorities (e.g., ISO 3696), e.g., using a Millipore device) containing 0.2% formic acid

2.5 Purification

- Vacuum dead-end sterile filters (0.2 µm)

2.5.1 Cell Separation

- Cross-flow filtration or continuous centrifuges like a disk-stack separator (*see* **Note 6**)

2.5.2 Liquid Extraction

- 37% HCl

Precipitation by Acidification

- Acidified water acidified to pH 3 with 37% HCl
- Ethyl acetate
- Separating funnel

- Rotary evaporator
- 0.05 M $NaHCO_3$, acidified to pH 2 with 37% HCl

Extraction

- Chloroform/ethanol (2:1 v/v)
- Rotary evaporator

2.5.3 Adsorption/ Desorption

- Isocratic pump. May be a binary gradient pump (*see* **Note 7**)
- Fractionator
- Conductivity meter
- Hydrophobic adsorbent Europrep 60–60 C8 (Knauer GmbH, Germany)
- Pressure-resistant column
- Ethanol
- H_2O
- Rotary dryer
- Freeze-drier

3 Methods

3.1 Strain Development

For recombinant rhamnolipid production, two elements are required. On one hand, the genes coding for the rhamnolipid-producing enzymes have to be acquired. On the other hand, an enzyme expression system composed of an expression vector and a suitable host organism has to be chosen.

3.1.1 Strain

An adequate strain has to feature at least two characteristics. Firstly, it must not be sensitive to the desired product, and secondly, it has to be able to synthesize the required precursors.

1. Check for precursor providing pathways by using databases like KEGG [32].
2. Examine resistance to the product of choice by using a microtiter plate-based cultivation system with online growth monitoring like the BioLector by m2p-labs GmbH (Baesweiler, Germany). The advantage of such a system is that only small amounts of the sample are required and different concentrations or strains can be investigated in parallel. Record the growth curve and check if deviations from the uninfluenced growth like lag phase elongation, decreased growth rate, or lower final OD occur.

A strain meeting all the mentioned prerequisites for rhamnolipid synthesis is *P. putida* KT2440 [33, 34]. It synthesizes dTDP-L-rhamnose and β-hydroxydecanoyl-acyl carrier protein and furthermore is able to grow in the presence of at least 90 g/L rhamnolipids.

3.1.2 Expression Cassette

For an expression system enabling the host organism to produce rhamnolipids, first of all a suitable backbone has to be chosen. Elements like ori (origin of replication) and antibiotic resistance genes should be adjusted to the respective microorganism. We decided to use the broad-host-range plasmid pVLT33 [25] with the ori from plasmid RSF1010 [26] and the promoter $lacI^q$/Ptac [27]. The genes for rhamnolipid production were taken from *P. aeruginosa* PA01.

1. Isolate genomic DNA from the donor organism using a DNA isolation kit.
2. Isolate the vector of choice using a Miniprep kit.
3. Amplify the whole *rhlAB* operon with a DNA polymerase. Use primers with adequate restriction sites.
4. Digest *rhlAB* PCR product and the vector in separated reaction tubes with the selected restriction enzymes.
5. Ligate both DNA molecules.
6. Transform the ligation into competent *E. coli* DH5α using, for example, electroporation described in Sect. 3.1.3 (*see* **Note 8**).
7. Cultivate cells in a liquid culture and isolate plasmid using a Miniprep kit.
8. Transform *P. putida* KT2440 using electroporation as described in Sect. 3.1.3.

3.1.3 Electroporation

For transformation of plasmids into *P. putida*, we use a modified protocol for electroporation of *P. aeruginosa* originally invented by Choi et al. [35] (*see* **Note 9**):

1. Grow a 5 mL overnight culture in LB medium at 30°C with orbital shaking.
2. Harvest cells by centrifugation at 6,000 rpm for 2 min in a 15 mL falcon tube.
3. Resuspend pellet in 2 mL of sterile 300 mM sucrose solution after thoroughly removing all supernatant by pipetting (*see* **Note 10**).
4. Centrifuge again and resuspend in 1 mL of 300 mM sucrose solution; again only after thoroughly removing all supernatant by pipetting.

5. Repeat centrifugation and resuspend cells in 100 µL of 300 mM sucrose solution. The cells are now prepared for electroporation. Store on ice until use.
6. Add 1–10 ng of plasmid DNA to electrocompetent cells and mix gently by stirring with pipette tip (*see* **Note 11**).
7. Transfer DNA/cell suspension into an electroporation cuvette (0.2 cm gap). Ensure that the liquid has contact to both electrodes.
8. Wipe dry the cuvette, position it in the electroporator, and apply the following pulse: 2.5 kV, 200 or 400 ohm, 25 µF.
9. Immediately after the electroporation, add 1 mL prewarmed (30°C) (*see* **Note 9**) LB medium and transfer the suspension to a reaction tube.
10. Incubate for 2 h at 30°C.
11. Spread 50 µL of the transformed cells on a selective plate containing your antibiotic of choice (*see* **Note 12**).
12. Centrifuge the remaining culture (16,000 g, 10 s) and discard supernatant by pouring.
13. Resuspend cells in remaining supernatant and spread 50 µL on a second selective plate. Incubate plates at 30°C. After 12 h small colonies should be visible. In some cases longer incubation times of up to 48 h are necessary.

3.1.4 Screening

Screening for rhamnolipid-producing strains will be needed in early stages of research with recombinant rhamnolipid-producing bacteria. While in principle all the methods listed in Sect. 3.3 can be used, it is preferable to use a more simple procedure, as these analytical procedures rely on supernatants of cultures of rhamnolipid-synthesizing bacteria. The methods described here utilize the cells themselves, accelerating the identification of rhamnolipid-producing strains.

Blood Agar Plates

This assay depends on the ability of the surfactant to lyse red blood cells. This hemolysis causes a clear halo around rhamnolipid-producing bacteria [36].

1. Autoclave 40 g/L blood agar base in deionized water.
2. After cooling down to 50°C, add 7% defibrinated sheep blood (*see* **Note 13**). Also add antibiotic if needed in the respective concentration.
3. Pour approx. 20 mL of medium into petri dishes.
4. Store plates at 4°C.
5. Divide the plate in squares of approx. 5 × 5 mm by drawing lines on the backside of the plate.

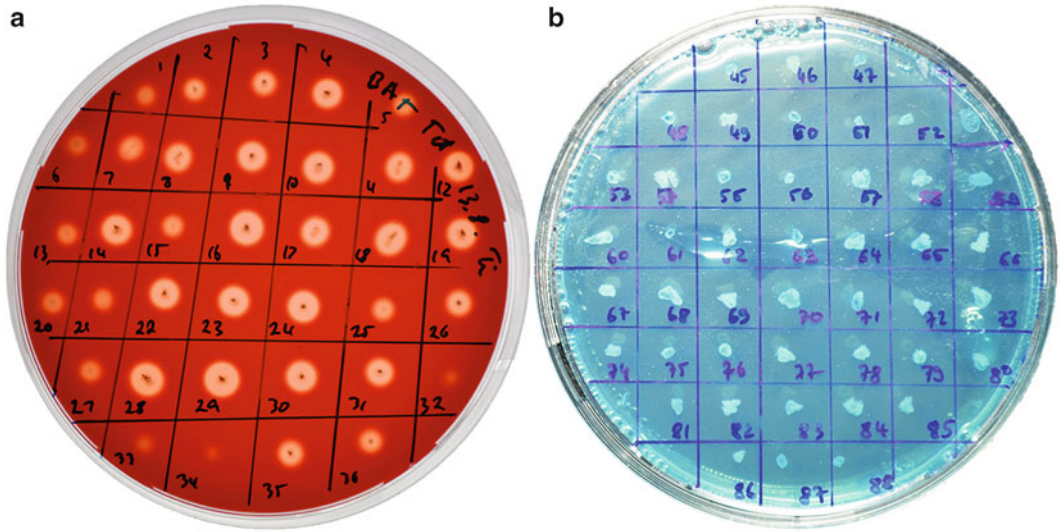

Fig. 2 Plate assay to detect rhamnolipid production. (**a**) Blood agar plate with hemolytic halos around bacterial cultures. (**b**) CTAB plate with light blue colonies. Rhamnolipid formation can be detected by *dark blue spots* inside the colonies

6. Use a toothpick to transfer the bacteria to the plate. To guarantee reproducible colonies, only slightly prick the plate with the toothpick.
7. Incubate the plate overnight at 30°C or until colonies are formed.
8. Afterward store plate in refrigerator at 4°C and incubate until halos start to arise. Usually this happens after one day at 4°C. The halo diameter is directly proportional to the surfactant production. An example can be seen in Fig. 2.

CTAB Plates

This assay is based on the anionic properties of the rhamnolipids, which form an insoluble ion pair with cationic substances [36]. A combination of cetyltrimethylammonium bromide (CTAB, IUPAC name: hexadecyl-trimethyl-ammonium bromide) and methylene blue yields dark blue colonies [36].

1. For 1 L CTAB plate medium, mix 500 mL liquid mineral salts agar medium, 2 mL trace elements solution, 50 mL of dye solution, and 40 mL of 50% (m/vol) glucose solution. Add sterile water up to 1 L. For plasmid-based expression systems, also add the respective antibiotic. Make sure to add the antibiotic only after cooling down to hand warm temperature. The plates should have a light blue color.
2. Pour approx. 20 mL of medium into petri dishes. For quantification of rhamnolipid production, use the exact same amount of liquid agar, to ensure same thickness of plates.

3. Store plates in darkness, as dyes are light sensitive.
4. Apply cultures as described before. Colonies of rhamnolipid-producing strains will be dark blue.

3.2 Production of Rhamnolipids

Efficient rhamnolipid production is highly dependent on the culture conditions. The culture conditions are basically defined by the vessel, the shaking conditions, and the used medium, which depend on the experimental requirements.

3.2.1 Stock Solutions

Antibiotics

- Kanamycin (50 g/L)
  - Weigh 60.12 mg kanamycin sulfate (or 50 mg kanamycin) in the volumetric flask.
  - Fill up with deionized water to 10 mL.
  - Sterilize by filtration.
  - Aliquot and freeze (*see* **Note 14**).
  - Store at 20°C.
- Tetracycline (10 g/L)
  - Weigh 108 mg tetracycline hydrochloride (or 100 mg tetracycline) in the volumetric flask.
  - Add 5 mL ethanol.
  - Fill up with deionized water to 10 mL.
  - Sterilize by filtration.
  - Aliquot and freeze (*see* **Note 14**).
  - Store at 20°C in a dark place as tetracycline is light sensitive.

Inducer (400 mM) (e.g., IPTG (Isopropyl β-D-1-Thiogalactopyranoside))

1. Weigh 953 mg IPTG in the volumetric flask.
2. Fill up with deionized water to 10 mL.
3. Sterilize by filtration.
4. Aliquot and freeze (*see* **Note 14**).

Glucose (50% (m/vol))

1. Weigh 275 g glucose monohydrate (or 250 g glucose) in the graduated cylinder.
2. Fill up with hot deionized water to 500 mL.
3. Sterilize (*see* **Note 15**).

3.2.2 Media

Depending on the planned experiments, complex media or minimal media (*see* **Note 16**) can be used. For a maximum yield of rhamnolipids, use complex media supplemented with an additional substrate like glucose. The bacteria will then grow with the components of the complex medium and utilize the sugar for rhamnolipid synthesis [19]. To determine applicability of different substrates or even for experiments with labeled substrates, minimal media are to be used.

If you use a plasmid-based expression system for recombinant rhamnolipid production, add antibiotic marker in adequate concentrations. Derivatives of, for example, vectors pVLT33 and pVLT31 [25] require the addition of 50 μg/mL kanamycin and 20 μg/mL tetracycline, respectively. If you applied inducible promoters, add an inducer. If you use the pVLT vectors, add IPTG (isopropyl β-D-1-thiogalactopyranoside) to a final concentration of 0.4 mM (guaranteeing full induction) from the beginning of the fermentation to induce the taq-promoter.

LB Medium

The preferred complex medium for recombinant rhamnolipid production with *P. putida* KT2440 is lysogeny broth (LB) medium. To further enhance rhamnolipid production, add 10 g/L glucose.

M9 Minimal Medium

For 1 L of M9 medium, mix 200 mL of M9 salts solution, 2 mL 1 M $MgSO_4$, and 2 mL US trace elements solution. Add your carbon source, which in our case is 10 g/L glucose from a 50% (w/v) solution, and add sterilized water to 1 L.

Wilms-MOPS (WM) Minimal Medium

For 1 L of WM medium, mix 400 mL of MOPS buffer, 20 mL of WM salts, 1 mL of vitamin solution, and 1 mL of trace elements solution. Add your carbon source, which in our case is 10 g/L glucose from a 50% (w/v) solution, and add sterilized water to 1 L.

3.2.3 Cultivation

There are different options for cultivation of microorganisms available. Depending on your application, shake flasks, microtiter plates, or even fermenters can be chosen. For rhamnolipid production and comparison of a small number of different strains or media, we use shake flasks. If you aim for a screening of, for example, different hosts, knockout mutants, or promoter libraries, use a microtiter plate system, for example, System Duetz. For rhamnolipid production on a bigger scale, use fermenters. Traditionally 30°C works best for *P. putida*.

Cultivation in Shake Flasks

Flasks (*see* **Note 1**) should be filled with max. 10% of the nominal volume to ensure sufficient oxygen supply. It also is important to use adequate shaking speed and throw. Use at least a throw of 25 mm and a speed of 200 rpm. Even better are 50 mm and 300 rpm.

1. Prepare a pre-culture using small flasks (100 mL) or test tubes (fill test tubes with 5 mL of medium) (*see* **Note 17**).
2. Inoculate pre-culture with a sterile toothpick directly from the cryo-culture or from a plate.
3. Shake as stated above (tubes in an inclined rack).
4. Incubate pre-culture overnight.

5. Inoculate main culture with ~1 mL from the pre-culture to an OD_{600} of 0.1.
6. Incubate for 24 h or until glucose is depleted.

High-Throughput Cultivation

For high-throughput screening of a big number of different microorganisms, use a microtiter plate (MTP)-based shaking system like System Duetz [31]. Use a throw of 50 mm and a shaking speed of 300 rpm in your shaker. Only these conditions will guarantee required oxygen supply. Two formats are available for recombinant rhamnolipid production (*see* **Note 18**).

1. Prepare a pre-culture in the MTPs by filling with the appropriate amount of your medium of choice.
2. Inoculate pre-culture with a sterile toothpick directly from the cryo-culture or from a plate.
3. Shake as stated above.
4. Incubate pre-culture overnight.
5. Inoculate main culture from the pre-culture to an OD_{600} of 0.1.
6. Incubate for 24 h or until glucose is depleted.

Fermentation in a Lab Scale Fermenter (*see* **Note 19**)

The advantage of an aerobic cultivation of microorganisms in a fermenter is the option to adjust the pH and to control the pO_2 via controlling the agitation, aeration, and pressure automatically. Fill the fermenter with a maximum of about 65% of the maximal volume (*see* **Note 20**).

1. Prepare a pre-culture using shake flasks as mentioned before and use in total 10% of the desired culture volume in the fermenter (*see* **Note 21**).
2. Inoculate by pouring the complete pre-culture in the fermenter.
3. To keep the lag phase short, start with 10 g/L of glucose. The feeding should only start when needed.
4. When using the foaming-out method, keep the glucose concentration low and use a recycle of the foam. The foam is wet and hence the nutrients are removed with the rhamnolipids if no recirculation is used.
5. Replace liquid loss in the fermenter by a medium-feeding system, which replaces the medium and the nutrients as well as glucose. This is especially convenient when you plan to ferment in a "fed-batch" or in a continuous mode wherein the foam represents the outlet flow and the medium feed represents the inlet flow to keep the working volume constant.

6. Control the pH value and hold it on a value of about 7 ± 0.2 by the addition of 2N NaOH and 2N HCl or H_3PO_4.
7. Keep the pO_2 value above 10% anytime by adjustment of the air flow, the pressure in the fermenter, and the stirring speed, to guarantee aerobic conditions. Start with a fixed aeration rate and leave the process at outlet pressure. Adjust the pO_2 only by an increase of the stirring speed until the maximum is reached. After that the only adjustment is done via the flow of sterile filtered air (*see* **Note** 22).
8. Take samples every once in a while to check if the cells are still vital and if any nutrients are missing or are decreasing in their concentration so that counteractions can be initiated on time. Also check for possible contaminations even when using antibiotics in the medium.

3.3 Scaling-Up of Rhamnolipid Production

The scaling-up of a fermentation process means the increase of the liquid volume by holding other parameters like aeration rate or the specific performance input of the stirrer constant for a comparable successful fermentation. Of course there are new requirements for the fermentation and especially for the downstream processing, which will be discussed in Sect. 3.5. The main aspects of the fermentation are explained in Sect. 3.2.3. For the setup at a larger scale, you need an equipment that is adequate to handle the new liquid volume scale like pumps for the pH correction solutions and the feeding procedure. When using the foaming-out procedure as mentioned before, prepare an adequate container for the foam to be collected and collapsed (*see* **Note** 2).

In Fig. 3 (left), you can see that the foam is led out of the fermenter into a container. The foam contains cells, glucose, and

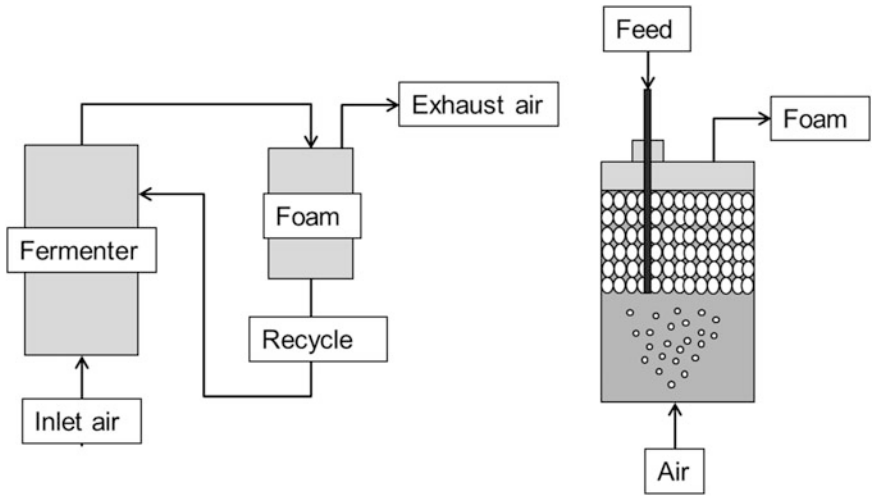

Fig. 3 Possible fermentation setup (*left*) and injection tube in fermenter top (*right*)

other nutrients that should not be wasted for an economically feasible process. Therefore, use a recycle stream to hold the concentration of cells on a high level to maintain the maximum number of biocatalysts for maximum productivity and to avoid a loss of nutrients. The instrumental setup of controlling the pH value as well as the support with additional nutrient solution and carbon source needs special attention regarding the equipment (*see* **Note 3**).

Scaling-up of the fermentation requires additional equipment for downstream processing especially for the next step after the cultivation: the cell separation. This will be discussed in Sect. 3.5.1.

3.4 Analytics

A very important part of recombinant rhamnolipid production is the analysis of the produced surfactant. There are basically two different aspects interesting for the experimenter: The spectrum of the different rhamnolipid congeners and the total concentration of rhamnolipids in the sample. According to the information you need, the analytical method should be chosen. You should also consider the number of samples you want to analyze (*see* **Note 23**).

3.4.1 Orcinol Assay (see Note 24)

The orcinol assay can be used to determine the total concentration of rhamnolipids [37]. Heating with sulfuric acid will lead to dehydration of the sugar which then reacts with orcinol to yield a colored ingredient. As no preceding separation is applied, this method cannot differentiate between different rhamnolipid species. The dying by orcinol is furthermore very unspecific and works with any neutral sugar molecule. For that reason extraction with ethyl acetate as the first step is required. This will separate lipidic compounds from the fermentation broth. In combination these two steps will exploit the amphiphilic character of the molecule and yield a good estimation of the total rhamnolipid concentration.

1. Remove cells from the culture by centrifugation at 13,000 g for 2 min.
2. For extraction add 500 µL of ice cold ethyl acetate to 100 µL of supernatant and vortex well. As distribution of rhamnolipids in the two phases depends on an equilibrium vortex for at least 10 s.
3. Centrifuge at 17,000 g and 4°C for 30 s for phase separation.
4. Transfer the organic (upper) phase to a new tube.
5. Repeat the procedure with the remaining aqueous phase for two more times.
6. Pool the organic phases.
7. Evaporate solvent in a vacuum centrifuge. The residue should be a dark brown very viscous liquid.
8. Resuspend rhamnolipids in 100 µL of deionized water and vortex well.

9. Add 100 µL orcinol solution and 800 µL sulfuric acid (60%).
10. Incubate at 80°C and 1,000 rpm orbital shaking in a benchtop thermo shaker for 30 min.
11. After cooling to room temperature, measure the samples at 421 nm in comparison to different concentrations of a rhamnolipid standard using a spectrophotometer.

3.4.2 Thin-Layer Chromatography

Thin-layer chromatography (TLC) can serve as a quick and easy method for qualitative analysis of rhamnolipids [38]. The advantage of this method is that various samples can be analyzed at the same time (depending on the size of the used plate up to 20) and no sample preparation is required. A downside is that separation of rhamnolipid species by TLC only yields four fractions, depending on number of sugar and lipid moieties.

1. Centrifuge to remove cells at 13,000 g for 2 min.
2. Draw a line 1 cm apart from the long edge of sheet and mark the sites where to spot on the samples. The distance should be at least 0.95 cm, depending on sample volume.
3. Spot 10 µL of supernatant on the markings.
4. Blow-dry to evaporate the liquid.
5. Depending on the expected concentration spot 3–10 times 10 µL onto the plate (*see* **Note 25**).
6. As reference use 10 µL of any commercial rhamnolipid extract containing mono- and di-rhamnolipids.
7. Fill chamber with a 0.5 cm high layer of running buffer and wait approximately 30 min until the air in the chamber is saturated. Make sure that the spotted samples are not submerged in the running buffer.
8. Before positioning the TLC plate into the chamber, ensure that its surface is completely dry.
9. Close chamber with lid and wait until the running buffer front is just below the top edge of the plate.
10. Take out the plate and blow it dry.
11. To visualize the rhamnolipids on the TLC plates, apply the dye. For homogenous staining use a sprayer from a distance of 10 cm or completely dip plate into staining solution.
12. Blow-dry the sheet and heat it for 5 min at 120°C. Document results promptly, as spots fade quickly. The result should look like the example in Fig. 4.

3.4.3 High-Performance Liquid Chromatography

High-performance liquid chromatography analysis is the most comprehensive method for rhamnolipid analysis. Quantification not only can be carried out absolutely, but also separation is far

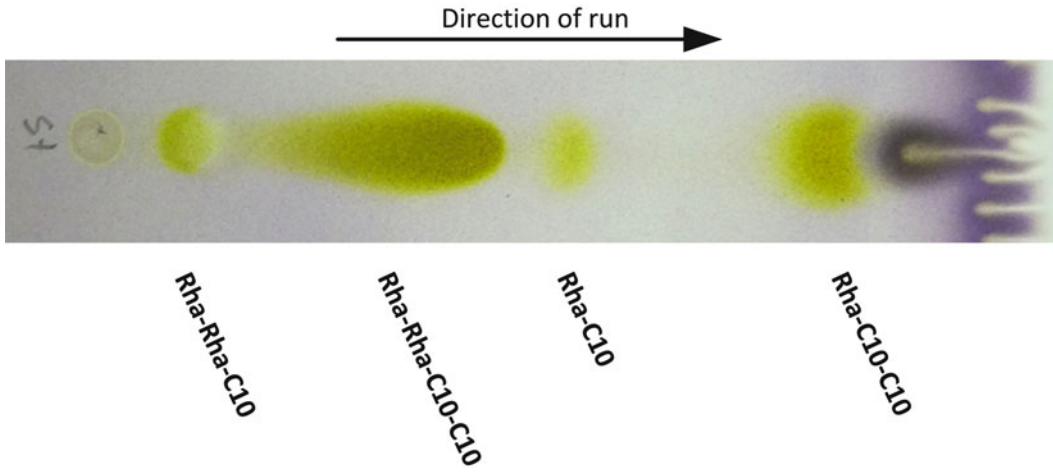

Fig. 4 Thin-layer chromatography plate of a rhamnolipid reference sample

more sophisticated, facilitating separation of rhamnolipid congeners differing only in the length of the attached hydroxy fatty acids. It nevertheless is highly dependent on the available standards. Qualitative analysis of rhamnolipid species can also be carried out by subsequent mass spectrometry.

Sample Preparation

Prior to analysis samples should be prepared for HPLC measurements. This procedure has various advantages. The most important is to prevent clogging of the column. Clogging may happen for different reasons like due to particles in the sample which can be removed by centrifugation and filtration. Harder to remove are compounds, which are solved in the aqueous sample but precipitate due to the changing conditions (e.g., solvent, temperature, pH value) during the HPLC analysis, for example, proteins. The second important benefit of purified samples is that essential peaks are not superimposed by interfering peaks. For very sensitive detectors, purification might even be inevitable. Especially mass spectrometers (MS) rely on absolute clean and salt-free samples.

1. Centrifuge culture samples at 13,000 g for 2 min to separate cells and cell residues.
2. Mix 500 μL of the supernatant with 500 μL of acetonitrile and vortex well.
3. Incubate overnight at 4°C to facilitate precipitation of any residual material.
4. Centrifuge again at 13,000 g for 5 min.
5. Filter with a 4 mm syringe filter with 0.2 μm pore size and a filter membrane of regenerated cellulose. The samples are now ready to be analyzed.

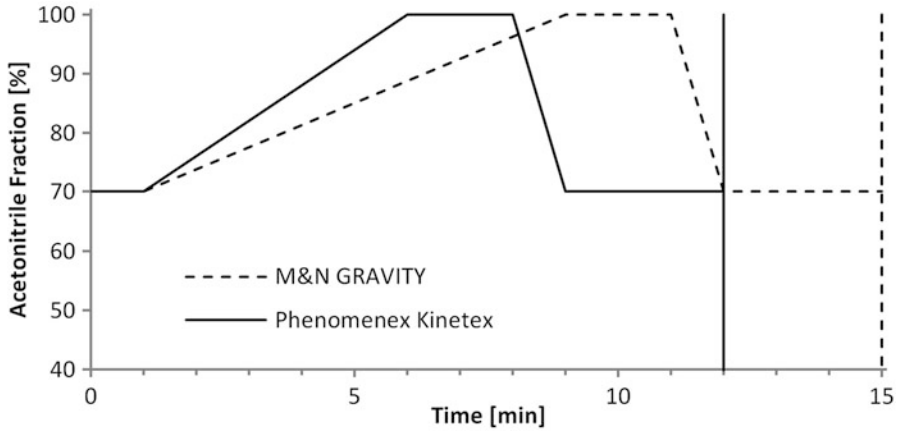

Fig. 5 Gradient programs chosen for the two different RP C18 columns. While the *dashed line* shows the program for the M&N column, the continuous line presents the program for the Phenomenex column. The *vertical lines* mark the end of the corresponding program

Chromatography

Reversed-phase high-performance liquid chromatography corona charged aerosol detection (RP-HPLC-CAD) was used for rhamnolipid quantification.

1. Use the following flow gradient: Start with 70% of acetonitrile and 30% 0.2% formic acid. After 1 min increase acetonitrile concentration to 100% within 8 min. Hold at 100% for another 3 min. After 11 min total running time, start decreasing acetonitrile concentration to 70% during 1 min. Keep concentration for another 3 min. The described courses of acetonitrile concentration can be seen in Fig. 5.
2. Set the column oven temperature to 40°C.
3. Set the flow rate to 1 mL/min.
4. Use 5 μL as injection volume.

Quantitative Evaluation

Figure 6 shows a chromatogram resulting from RP-HPLC-CAD analysis of a rhamnolipid reference sample. The most prominent compound is the Rha-C10-C10, which is a mono-rhamnolipid with two C10 hydroxy fatty acid chains. Smaller fractions include Rha-C10-C8, Rha-C12-C10, and also a rhamnolipid with one unsaturation in the lipid carbon chain: Rha-C12:1-C10. The position of the lipid chains, as well as the position of the double bond, is unknown.

Qualitative Analysis

For characterization of rhamnolipids and to identify the peaks you see in your RP-HPLC-CAD analysis, perform high-performance liquid chromatography electrospray ionization mass spectrometry (HPLC-ESI-MS) analysis.

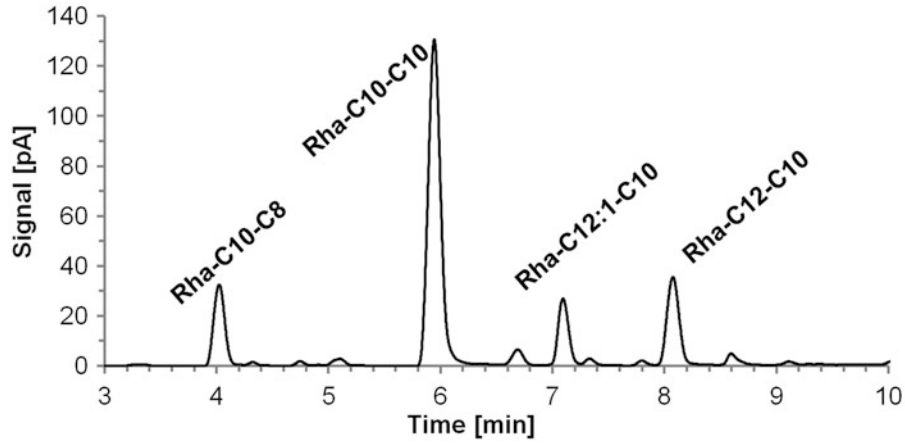

Fig. 6 Chromatogram of RP-HPLC-CAD of a rhamnolipid reference sample. The most prominent rhamnolipid species are indicated

1. Use negative enhanced mass spectrum mode scanning from 200 to 1,000 Da for the MS.
2. Apply a flow injection analysis with a reference sample to optimize the following parameters: IS −4,500 V, declustering potential −100 V, curtain gas (N_2) 10 arbitrary units (au), source temperature 500°C, nebulizer gas (N_2) 50 au, and heater gas (N_2) 20 au.
3. Set collision energy and third quadrupole-entry barrier to −5 and 8 V, respectively.
4. Apply negative enhanced product ion scan mode for structural elucidation MS/MS experiments, in which product ions are generated in the second quadrupole by collision-activated dissociation of selected precursor ions of the first quadrupole and mass analyzed in a linear ion trap. The collision energy should range from 30 to 70 V.

3.5 Purification

Purification of rhamnolipids can be required for different reasons, for example, to prepare samples for analysis. But also production of the surfactant on a bigger scale might play a role if you, for example, want to produce reference samples for analytical purposes or do more research on the substance itself.

3.5.1 Cell Separation

- In a small scale up to 1 L of liquid volume, use vacuum dead-end sterile filters (0.2 μm) or batch centrifugation at 9,000 g and 10°C for 10 min [39] or even better at 15,000 g and 10°C for 5 min.
- Other techniques especially for larger-scale fermentations are cross-flow filtration or continuous centrifuges like a disk-stack separator (*see* **Note 6**).

3.5.2 Liquid Extraction (see **Note 26**)

As described by Déziel et al. [40], rhamnolipids can be precipitated by acidification. This is valid for di- as well as for mono-rhamnolipids.

Precipitation by Acidification

1. Remove cells by centrifugation for 5 min at 15,000 rpm and 10°C. Opposite to the aforementioned procedure, this is sufficient for liquid extraction.
2. Acidify supernatant with 37% HCl to a pH of 3.
3. Incubate overnight at 4°C.
4. Recover the precipitated rhamnolipids by centrifugation at 15,000 rpm and 4°C for 45 min.
5. Resuspend in 15 mL acidified water.
6. Extract this suspension three times with 15 mL ethyl acetate by thoroughly mixing the two phases for at least 15 min and subsequent phase separation in a separating funnel.
7. Combine the three organic phases and evaporate the ethyl acetate in a rotary evaporator.
8. Solve the residue in 15 mL of the 0.05 M $NaHCO_3$.
9. Incubate overnight at 4°C.
10. Final recovery of the precipitate can be done by centrifugation for 60 min at 15,000 rpm and 4°C.

Extraction

This protocol originates from Wang et al. [39].

1. Remove cells by centrifugation for 5 min at 15,000 rpm and 10°C.
2. Mix the chloroform/ethanol mixture and the cell-free culture broth 1:1.
3. Stir for at least 15 min to ensure equilibrium.
4. Separate organic (upper) phase.
5. Repeat extraction with the aqueous phase two more times and combine the organic phases.
6. Evaporate the solvent in a rotary evaporator.

3.5.3 Adsorption/Desorption

From all possible unit operations regarding a capture of the extracellular rhamnolipids from a fermentation broth, the process of adsorption/desorption is a very easy to handle and very efficient procedure, which is also very cost effective and can be scaled-up in volume easily. In comparison to a liquid-liquid extraction, much lower amounts of solvents are necessary for a sufficient capture step. The hydrophobic adsorbent Europrep 60–60 C8 (Knauer GmbH, Germany) is a silica-based adsorbent which was successfully tested with a recovery of about 97% regarding rhamnolipids [41].

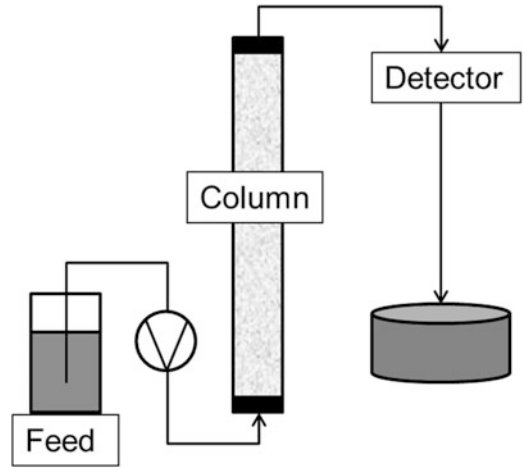

Fig. 7 Setup of the adsorption/desorption device

1. Fill the adsorbent into a pressure-resistant column (Fig. 7).
2. Equilibrate the adsorbent using ethanol for about 15 min with 2–6 bed volumes per hour.
3. Wash the bed with H_2O in the same way until no organic solvent is present anymore. After that the bed is ready to use.
4. Pump the cell-free fermenter broth over the adsorbent until a breakthrough of rhamnolipids is detected (take samples or use an online detection system).
5. Wash again with H_2O to clean the bed from any hydrophilic substances like inorganic salts, for example. For best results, use a conducting meter to see if some ionic substances are still released from the bed. The rhamnolipids will stick to the adsorbent via their hydrophobic fatty acid chains and will not be washed out.
6. Proceed with the desorption process by application of ethanol or aqueous ethanol solutions using a flow of 2–6 bed volumes per hour (*see* **Note** 7).
7. Fractionate the outlet flow adequately.
8. Pool the fractions or handle them individually for the further procedure. Due to a product purity of 75–80% regarding rhamnolipids, it is also possible to evaporate the organic solvent directly. For recycling of the solvent, use a rotary dryer.
9. Use a freeze-drier for final drying to a slightly gray-yellowish solid.

4 Notes

1. If you use shake flasks, avoid baffles. As rhamnolipids are surface active, foaming is a big problem in baffled flasks.

   A good choice for the production of rhamnolipids is a main culture of 50 mL in a 500 mL Erlenmeyer flask.

2. When using the foaming-out procedure as mentioned before, prepare an adequate container for the foam to be collected and collapsed because the amount of foaming is excessively higher than at a scale 10 times smaller. The content of air in the foam depends on the mode of operation of the fermentation and can vary between 70 and 95%. Foam with a low amount of liquid is much more stable and will need much longer time to collapse and to be harvested or pumped back into the fermenter. That is why it is absolutely necessary to offer a high volume of space for the foam.

3. The instrumental setup for the controlling of the pH value as well as the support with additional nutrient solution and carbon source needs special attention regarding the equipment. Usually these streams are led into the fermenter through inlets in the fermenter top cover. During the fermentation and dependent on the residence time of the air, the foam may become very stable. When stable foam is observed, it is necessary to use long tubes to guarantee that all liquids like acid, base, or glucose are not pumped onto the rising foam, but rather into the medium below its surface. If nutrients, but even worse acid or base, accumulate on the foam, stable fermentation operation is impossible, because cells adhered to the foam will lyse. This will lead to an increase in the viscosity of the collapsed foam as well as to a loss of cells as functional biocatalysts.

   When using a recycle stream, elongated tubes are not necessary for the addition of glucose and other nutrients because over time they will reach the fermenter again, whereas for the addition of bases and acids, the elongated tubes are indispensable.

4. If you have a lot of samples, consider using a filter in 96-well plate format (e.g., 96-Square Well, 0.45 μm Glass Fiber Filter Plate from Phenomenex Inc., Torrance, CA, USA) with a vacuum manifold. Also centrifugation can be carried out in 96-well plates.

5. The NUCLEODUR C18 Gravity column by Macherey-Nagel is to be preferred if you need robust and reliable chromatography. The Kinetex RP C18 column by Phenomenex however features better separation performance. Due to different particles the Kinetex column with the same dimensions can perform

the separation in a shorter time. As a trade-off and owing to the smaller particles (2.6 μm), the column is more susceptible to clogging. Do not use it, if you do not have absolutely clean samples.

6. Both devices are not sufficient for the separation of *Pseudomonas* cells from fermentation broth. Disk-stack separators need very high rotational velocities and produce again new foam on the retentate side as well as on the cleared liquid side. Cross-flow sterile filtration units are easy to handle and produce a completely cell-free filtrate phase, but due to the micellar character of the surfactants within this range of pH [42], these micelles are also retained by the sterile filter membrane so that the concentration of rhamnolipids in the filtrate is very low. It is advantageous that on the other hand an enriched phase is received on the retentate side. This means that it is possible to concentrate the product by cross-flow filtration and that it is only necessary to keep the retentate phase for further purification techniques like batch centrifugation.
7. When using pure ethanol, all hydrophobic substances will be released from the adsorbent as soon as it comes in contact with this organic solvent. One of which is the colorant pyoverdine, produced by this *Pseudomonas* species. The desorption process needs thus to be adapted to the required purity. The yellowish brown color will not affect the performance of the rhamnolipids, but will affect the acceptance of the customer depending on the desired purpose. By using pure ethanol for desorption, you will obtain a yellowish brown solution that contains all substances including mono- and di-rhamnolipids and fatty acids as well as the colorant. Use a gradient desorption to separate the colorant from the desired product beginning with an EtOH:H_2O ratio of 0:100 and ending with 100:0 and fractionate the outlet flow adequately. With this scheme, the main part of the colorant remains in the first fractions, while the rhamnolipids show a majority in concentration in the following fractions and the fatty acids appear primarily in the last fractions. In this case you will need a binary gradient pump.
8. Do not transform directly to *P. putida* as genetic stability and copy number both are significantly increased in *E. coli* DH5α (depending on the used plasmid).
9. *E. coli* can be transformed using any standard protocol used in your lab. If you use the protocol described in Sect. 3.1.3, make sure to adjust the temperature to 37°C instead of 30°C.
10. Thorough removal of the supernatant is paramount. Electroporation can only work with a salt-free solution. Rather remove some of the cells than risk the chance of a failed transformation. You can also use salt-free LB medium for the overnight culture,

avoiding salts right from the beginning. Also take care that the DNA solution is free of salts. If you want to apply an increased amount of plasmid DNA for more transformants, use alcohol precipitation to get rid of the salts from the Miniprep kit.

11. You can also mix the solution by pipetting. In this case use a 1 mL pipette tip and cut off the lower 5 mm to reduce shear forces which will destroy plasmid DNA. Also avoid vortexing.
12. We use kanamycin (50 μg/mL) or tetracycline (20 μg/mL). Both yield good results.
13. Make sure to mix the blood well by shaking before withdrawal from the vessel. As the blood is oxygen sensitive, also avoid opening the lid. A lid with a septum and the use of syringe and needle are recommended. Do not add the blood before cooling down the medium. The plate should be homogeneously red, and neither should it be transparent nor should any particles be visible. If you find any of these irregularities, you should discard the plates and start new.
14. Choose aliquot based on the amount of substance you use daily. It is best to avoid repeated freeze/thaw cycles.
15. If you want to perform absolute quantitative experiments, use a filter (0.2 μm) for sterilization. If you do not aim for 100% accuracy, autoclave the solution. As a small fraction of the glucose will caramelize, the composition will most likely not be reproducible.
16. We tested different minimal media. The two media that worked best are described below. The main difference in these media is the applied buffer. The phosphate buffer applied in M9 medium is somewhat troublesome in HPLC measurements, as it elutes near to the carbon sources *Pseudomonas* uses. Wilms-MOPS medium on the other hand involves cumbersome preparation.
17. Use the same medium as you aim to use in the main culture to reduce lag phase. You do not have to add IPTG in a pre-culture. Also, if you use complex media, any additional carbon source (e.g., glucose) is not required.

    If your strains are growing badly in your minimal medium of choice, you can also prepare an additional pre-pre-culture in complex medium to increase cell titers when inoculating. Incubate the pre-pre-culture as stated above. Afterward wash the cells with your minimal medium and inoculate the pre-culture with minimal medium with the washed cells.
18. While the 24-well MTPs can be filled with up to 2 mL of the culture, the 96-well MTPs only hold 1 mL of the culture. If you carry out an endpoint assay with a lot of different cultures, use the 96-well format. If you want to take samples, use the 24-well plates, as the higher culture volume facilitates sampling. If you

really require sampling, you should consider using as many plates as you need samples. By harvesting one whole plate, you will have sufficient sample material even if you use 96-well plates.

19. A considerable difference to cultivation in shake flasks is the aeration. In fermenters this usually happens via aeration tubes. This method ensures enough oxygen supply to the cells which would not be possible via diffusion. As a consequence of this bubbling, a lot of foaming occurs, especially with rhamnolipids in the medium. This can be exploited by foam fractionation which can be the first step in purification of the rhamnolipids [41, 43].
20. This maximal culture volume of 65% of the total volume is important. As mentioned above, the aerobic fermentation process for the production of rhamnolipids leads to an excessive foaming, which cannot be held under control with the usage of antifoam reagents. At low concentrations, they are useless, and at high concentrations, they have a negative effect on the growth of the microorganisms, with significantly reduced rhamnolipid production. With a small headspace, your fermenter will overflow very quickly.

    Even with a large headspace, it is necessary to actively guide the foam out of the fermenter to keep the fermentation running without the risk of blocking and contaminating the air outlet and filters, etc. Another option would be the use of a mechanical foam destroyer.
21. For 1 L of fermentation medium, use 100 mL of pre-culture with a high concentration of productive cells. Make sure you use the microorganisms while they are in the exponential growth phase and have not fully consumed the glucose yet so that the time for adaptation to the new growth condition will be minimal. Keep in mind, to only fill flasks up to 10% of their maximum volume. If you do not have big enough flasks, use more than one. If you inoculate your flasks from a well-grown overnight culture in a tube, the pre-culture should grow for about 6 h if you use complex medium. In minimal medium this might need some 10–15 h.

    To keep the lag phase short, use the same medium in the pre-culture as in the fermenter.
22. Keep the aeration rate as low as possible. Especially when you use a salt medium, you have to be careful with aeration during the lag phase, because the organisms need CO_2 for the anaplerotic reactions. A high aeration rate leads to stripping of CO_2, resulting in an extended lag phase. Low aeration is furthermore important to prevent the medium from unnecessary foaming. Modern fermentation controller systems are able to increase the stirring speed depending on the actual pO_2 value.

23. To check if there are rhamnolipids in your sample at all, use thin-layer chromatography (TLC). For approximate concentration determination and a large number of samples, use the orcinol assay in 96-well plate format. TLC can also handle high number of samples, but does not allow for quantification. Nevertheless, it provides rough information about the spectrum of different rhamnolipid congeners. For a comprehensive but time-consuming analysis of your samples, choose high-performance liquid chromatography (HPLC) measurement. This method provides quantitative as well as qualitative data.
24. Unfortunately this assay is not suitable to determine absolute concentrations, as the results of this procedure are difficult to reproduce. Nevertheless, it delivers reasonable results when comparing samples from one measurement. It is thus suited, for example, for screening approaches, where ranking of strains, mutants, or clones is aspired.
25. Depending on the expected concentration, spot 3 to 10 times 8 μL onto the plate. With recombinant strains you probably will only need to use 1–3 times the 8 μL as titers should range from a couple of 100 mg/L to a few g/L. With wild types use ten times, as concentrations of surfactants are usually rather low.
26. Liquid extraction requires only standard laboratory equipment. It is thus easier to establish. The downside is that it is more laborious and furthermore yields in a high viscous liquid with very poor water solubility. Using adsorption/desorption is less laborious but requires more and above all more expensive equipment. The decisive advantage is nevertheless that the product will be a powder, easy to handle and furthermore easily water soluble. The favorable method for purification is thus adsorption/desorption.

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Purification of Peptide Antimicrobials and Thioether-Stabilized Molecules Produced In Vivo by Lantibiotic Modification Machineries

Manuel Montalban-Lopez, Andrius Buivydas, and Oscar P. Kuipers

Abstract

The presence of posttranslational modifications is a key feature for the biological activity and stability of many natural products, including lanthipeptides. Lanthipeptides are a group of posttranslationally modified peptides containing lanthionine residues in their structures. Among lanthipeptides, the subgroup of lantibiotics is receiving a renewed interest in the last years for their high antimicrobial activity. The development of production platforms for novel lantibiotics and peptides containing posttranslational modifications is taking place in parallel to the understanding of the mechanistic aspects of the enzymes responsible for these modifications. It is now possible to combine pieces (i.e., enzymes and regulatory elements) of different biosynthesis routes of natural products in order to modify peptides of interest in a predictable manner. In this chapter we detail the production of lanthipeptides and thioether-stabilized peptides using two well-established production platforms for *Escherichia coli* and *Lactococcus lactis*. These are based on the dehydration and cyclization reactions carried out by the enzymes NisB and NisC, respectively, on peptides fused to the nisin leader peptide. Furthermore, we provide appropriate protocols for the purification and characterization of the peptides produced and modified with these biosynthetic tools.

Keywords: Lanthipeptide, Lantibiotic, Nisin, Posttranslational modification, Synthetic biology

1 Introduction

Lanthipeptides constitute a group of ribosomally produced and posttranslationally modified peptides (RiPPs) that display diverse activities [1, 2]. They show as a main feature the presence of lanthionine, which consists of the linkage of two amino acids (i.e., a dehydrated serine or threonine residue and a cysteine) via a thioether bond. The best characterized and studied lanthipeptides are those with antimicrobial activity, which are referred to as lantibiotics (*lan*thionine-containing an*tibiotics*). Lantibiotics display activity against a wide range of Gram-positive food spoilage and pathogenic bacteria (*Listeria*, *Streptococcus*, *Clostridium*,

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Staphyloccus, and many others) [3–6]. The challenge that multidrug-resistant bacteria such as methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecalis* pose in clinics nowadays and the activity of lantibiotics against such organisms has brought them to a front position in antibiotic research [7]. Their potent antimicrobial activity, which is in the concentration range of antibiotics conventionally used in therapeutics, and the conserved activity against multidrug-resistant microorganisms clearly indicate a potential medical use [3, 8]. In fact, the scarce pharmacokinetic and pharmacodynamic studies carried out on lantibiotics point at their applicability. Furthermore, the interest of lantibiotics in therapeutics is due not only to their antimicrobial activity but also to the possibilities that their biosynthesis machinery offers for the synthesis of thioether-stabilized peptide drugs (vide infra).

The biosynthesis of lanthipeptides requires different steps. First, a serine or threonine residue is dehydrated rendering dehydroalanine (Dha) or dehydrobutyrine (Dhb), respectively. In a second step, the thiol group of a cysteine is coupled to the dehydroamino acid in a regio- and stereospecific manner to produce the lanthionine (cysteine coupled to Dha) or methyllanthionine (cysteine coupled to Dhb) ring [2]. Depending on the enzymes that catalyze these reactions, lanthipeptides are divided into four different classes. In class I two separate enzymes are responsible for the dehydration (LanB) and cyclization (LanC). In class II, III, and IV lanthipeptides, a single multidomain enzyme can do both conversions (LanM, LanKC, and LanL, respectively). The posttranslational modification (PTM) enzymes recognize specific regions within the leader peptide of the lanthipeptide and this favors the transition from an inactive state to an activated one [9–12]. Additional posttranslational modifications can take place in lanthipeptides, which increase their chemical diversity and have a great impact on their activity [13, 14].

The substrate specificity of the lanthipeptide dehydratases and cyclases has been extensively studied. The modification machinery of lacticin 481 (Fig. 1) has been the most thoroughly studied LanM lantibiotic synthetase. It has been proved that the enzyme LctM can modify in vitro and in vivo diverse peptide substrates fused to the lacticin 481 leader peptide including peptides containing noncanonical amino acids [15–19]. Nevertheless, this promiscuity of the LanM synthetases does not hold true for all the studied LanMs. It has been shown that the lichenicidin and haloduracin machineries are more restricted regarding their substrate tolerance [20].

In our lab we focus on the nisin modification machinery, which belongs to the class I lantibiotics (Fig. 1). Nisin is the prototype of class I lantibiotics, and its mechanism of action, biosynthesis, regulation, and applications have been extensively studied [21–23]. The nisin gene cluster consists of 11 genes encoding the immunity

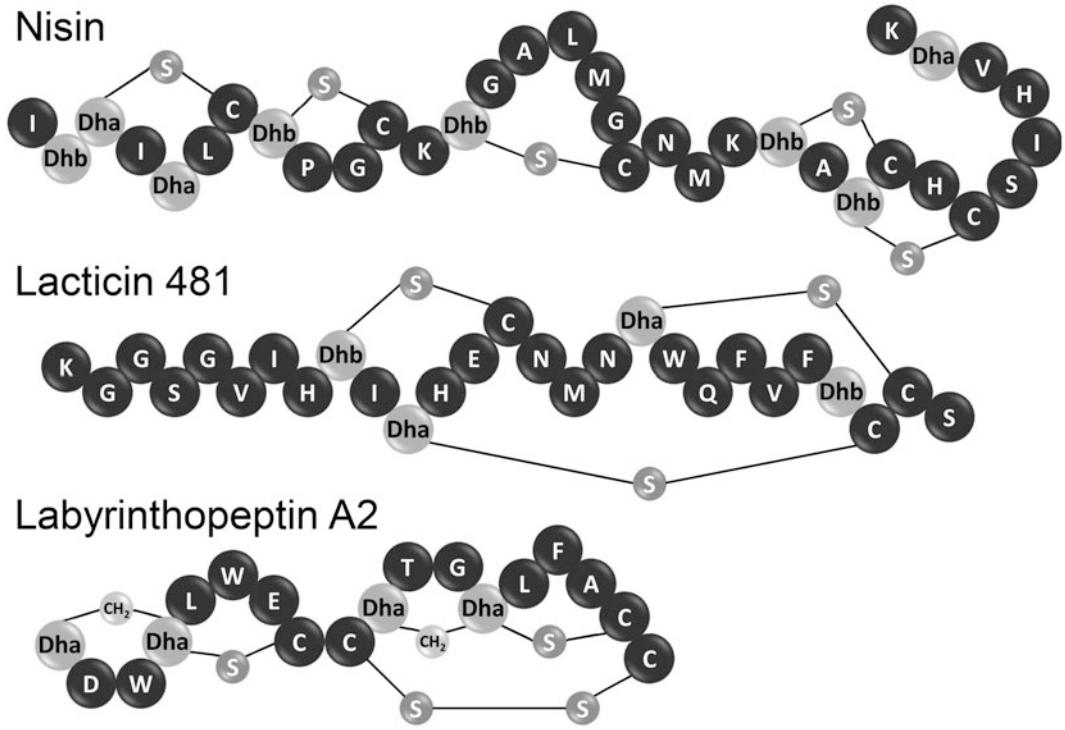

Fig. 1 Structure of nisin, lacticin 481, and labyrinthopeptin as representatives of class I, II, and III lanthipeptides, respectively

proteins NisI and NisFEG; a two-component system autoinduced by nisin, NisRK; the structural peptide NisA; and the biosynthesis machinery composed of NisB (dehydratase), NisC (cyclase), NisT (transporter), and NisP (leader peptidase) [24–28]. The two-component system NisRK is widely used for the overexpression of proteins in diverse Gram-positive organisms [29, 30]. The nisin dehydratase, transporter, and cyclase have been characterized for their substrate specificity proving their broad substrate tolerance [31–33] and applications in biotechnology for extracellular protein display [34], biosynthesis of novel lantibiotics [35, 36], and the biosynthesis of lanthionine-containing peptide hormones with increased stability and potency [37, 38]. NisBTC can modify and transport different peptide sequences fused to the nisin leader peptide which functions as a recognition motif for the enzymes and at the same time keeps the modified peptide inactive until it is cleaved by proteolysis [10, 12, 27, 39, 40].

The dissection of the nisin machinery into regulatory (NisRK) and enzymatic modules (NisBTC) provides very useful tools for synthetic biology studies focused on the production of novel bioactive molecules [14]. In this way, lantibiotics present in genomes of bacteria can be mined in silico with appropriate tools such as Bagel3 [41] and expressed using the nisin biosynthesis machinery

as a plug-and-play system. This basic system has been extended with other PTM enzymes from other lantibiotic gene clusters such as the reductase LtnJ from the lacticin 3147 gene cluster or the oxidative decarboxylase GdmD from the gallidermin cluster [35]. These preliminary works provide a look into the chemical diversity that can be achieved in ribosomally produced peptides using adequate enzymatic modules.

In this chapter we collect a set of techniques that allow the production and purification of novel compounds modified with the nisin modification machinery in both *Escherichia coli* and *Lactococcus lactis*. We describe a basic system for *L. lactis* composed of two plasmids where one is carrying the genes *nisBTC* under the control of the nisin-inducible promoter and a second vector contains the structural gene to be expressed (Fig. 2). This basic system has been recently extended with the insertion of a zinc-regulated promoter controlling the expression of the structural gene and the insertion of additional PTMs [35, 42]. This demonstrates the versatility of the *L. lactis* platform for the design and production of posttranslationally modified peptides. Similarly, we provide insight into one of the production platforms designed for *E. coli*, which has also dissected the PTM enzymes of the nisin cluster into two vectors with the enzymes and the structural peptide under the control of the T7 promoter (Fig. 3). We include the description of protocols for the design and construction of genes of interest encoding for the structural peptide, the induction of the expression strains, the purification of the target peptides by immobilized metal affinity chromatography or cation exchange chromatography, and the characterization of the peptide modification extent by mass spectrometry.

2 Materials

2.1 Construction of Expression Vectors for L. lactis

- pNZE-empty [35], pNZnisA-E3 [32], pCZ-Cm [42], pIL3BTC [43], pIL3EryBTC [35] (Fig. 2).
- *L. lactis* NZ9000 [44] (*see* **Note 1**).
- Designed primers or synthetic genes encoding for the peptide of interest.
- Phusion polymerase (Thermo Fisher Scientific, http://www.thermofisher.com) and thermal cycler.
- Media:

  M17 (Difco): 5.0 g pancreatic digest of casein, 5.0 g soya peptone, 5.0 g beef extract, 2.5 g yeast extract, 0.5 g ascorbic acid, 0.25 g magnesium sulfate, 19.0 g di-sodium-glycerophosphate.

  SM17: M17 containing 0.5 M sucrose.

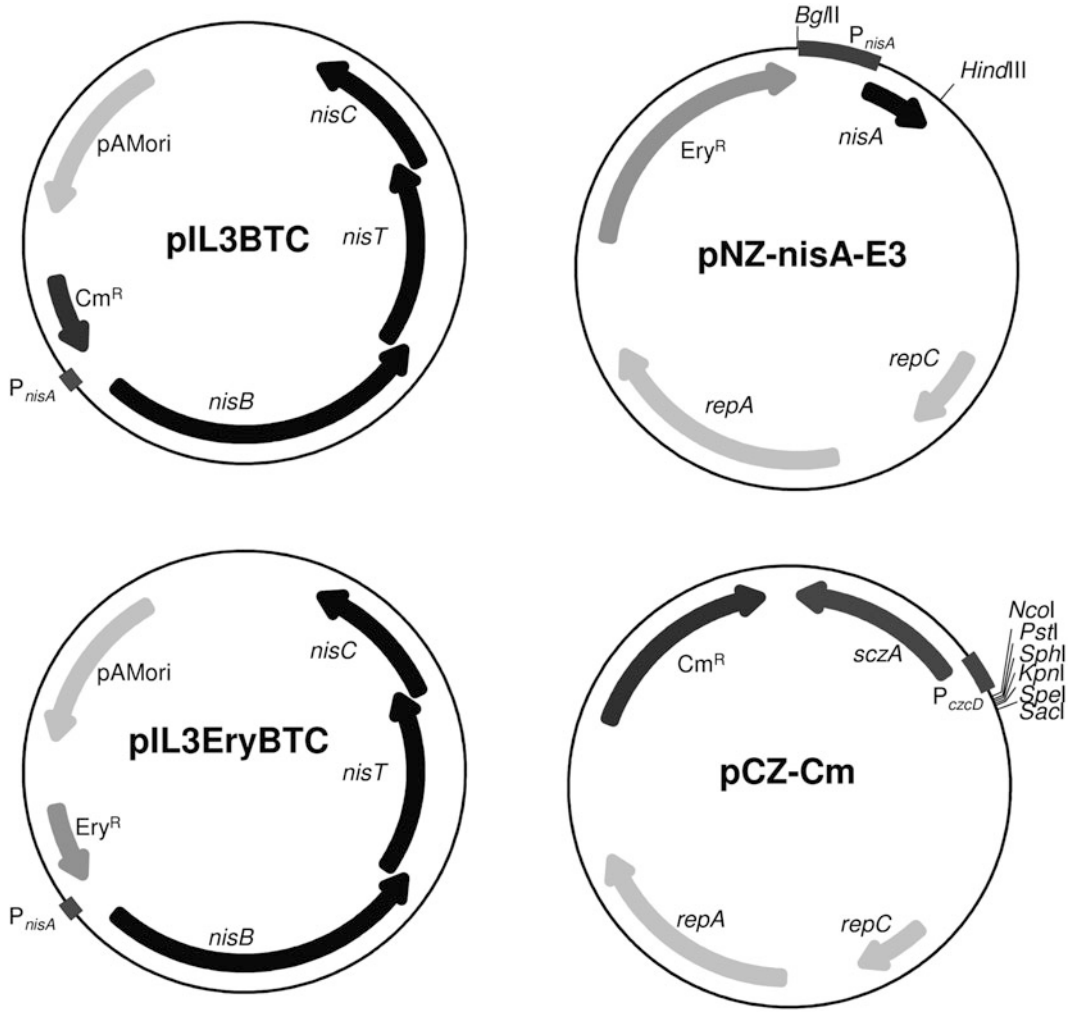

Fig. 2 Plasmid system for the expression of lanthipeptides in *L. lactis*

SGGM17: M17 containing 0.5 M sucrose, 0.5% glucose, 1% glycine.

SGM17MC: M17 containing 0.5 M sucrose, 0.5% glucose, 20 mM $MgCl_2$, 2 mM $CaCl_2$.

GSM17agar: M17 containing 0.5 M sucrose, 0.5% glucose, and 1.5% agar.

M17 can be purchased as a powder mixture of the different components. In our lab we use M17 from Difco. For the preparation of SM17 with or without agar, weigh the amount of powder required and add sucrose to a final concentration of 0.5 M. SM17 can be autoclaved at 121°C for 15 min and stored up to 3 months. Glucose and glycine are added from a 20% stock solution

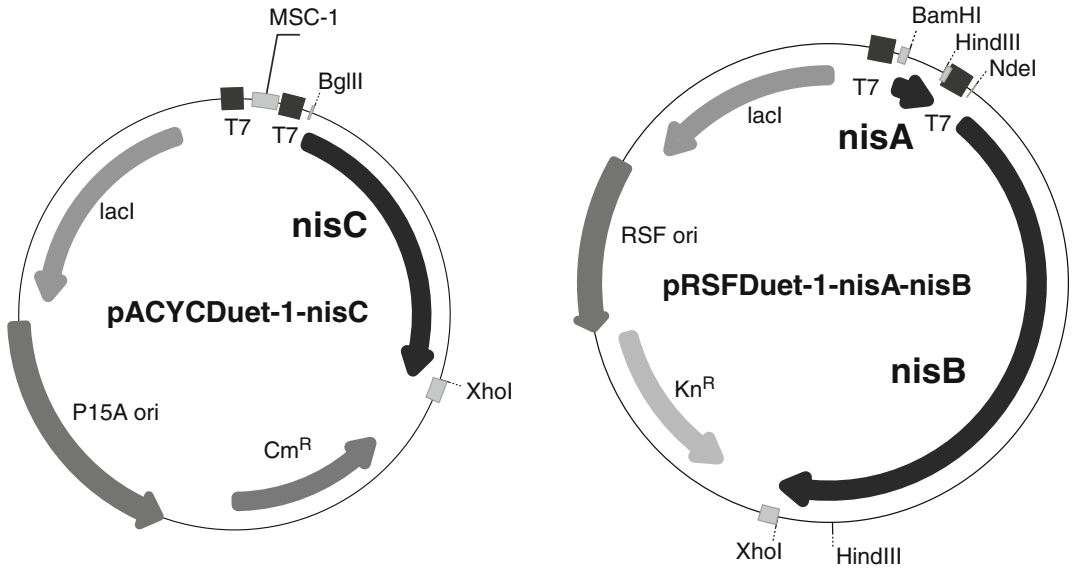

Fig. 3 Plasmid system for the expression of lanthipeptides in *E. coli*

(*see* **Note** 2). $CaCl_2$ and $MgCl_2$ are added from 0.1 and 1 M solutions, respectively.

- Buffers and general reagents:

  0.5 M sucrose (*see* **Note** 3).

  Sterile 1 M $MgCl_2$.

  Sterile 0.1 M $CaCl_2$.
- Antibiotics: erythromycin 5 mg/mL (1,000×) and chloramphenicol 5 mg/mL (1,000×). Dissolve the antibiotic in 70% ethanol and store at −20°C.
- Sterile electroporation cuvettes (2 mm separation between the electrodes) and electroporator. In our lab we purchase both from Bio-Rad.

2.2 Protein Expression in *L. lactis*

- Spectrophotometer.
- Nisin stock solution – 5 μg/mL (1,000×). Mix commercial nisin powder (Sigma-Aldrich, 2.5% m/m purity) with 0.05% acetic acid (*see* **Note** 4) and shake for 1 h in a rotor. Centrifuge to remove insoluble particles and filter the supernatant. Aliquot and freeze at −20°C. Avoid freezing and thawing repeatedly, making small volume aliquots.
- Sterile 0.5 M $ZnSO_4$ (only required if the zinc-inducible system is used).

- Antibiotics: erythromycin 5 mg/mL (1,000×) and chloramphenicol 5 mg/mL (1,000×). Dissolve the antibiotic in 70% ethanol and store at −20°C.
- Media:

  GM17: M17 containing 0.5% glucose (*see* **Note** 2).

  Chemically defined medium (CDM). This medium is prepared according to Poolman and Konings (1988) [45] with minor modifications. It is composed of five different solutions that are mixed before use.

  – Vitamin mix (100×, composition per liter): 0.2 g nicotinic acid, 0.1 g thiamine hydrochloride, 0.1 g riboflavin, 0.1 g calcium pantothenate, 1.0 g 4-aminobenzoic acid, 1.0 g biotin, 0.1 g folic acid, 0.1 g cyanocobalamine, 0.5 g orotic acid, 0.5 g 2-deoxythymidine, 0.5 g inosine, 0.25 g DL-6,8-thioctic acid, 0.5 g pyridoxamine dihydrochloride, 0.2 g pyridoxal HCl. Mix the components, adjust the pH to 7.0, and filter-sterilize (*see* **Note** 5). Store in the dark at −20°C.

  – Amino acid mix (20×, composition per liter): 3.5 g glycine, 4.75 g L-alanine, 2.5 g L-arginine, 7.0 g L-asparagine, 7.8 g L-glutamine, 3.0 g L-histidine, 4.25 g L-isoleucine, 9.5 g L-leucine, 8.75 g L-lysine, 2.5 g L-methionine, 5.5 g L-phenylalanine, 13.5 g L-proline, 6.75 g L-serine, 4.5 g L-threonine, 1.0 g L-tryptophan, 6.5 g L-valine. Mix the amino acids, adjust the pH to 7.0, and filter-sterilize.

  – Metal mix (400×, composition per liter). Solution 1: 160.0 g $MgCl_2$, 20.0 g $CaCl_2$, 2.0 g $ZnSO_4 \cdot 7H_2O$ (*see* **Note** 6). Solution 2: 1.2 g $CuSO_4 \cdot 5H_2O$, 0.08 g $CoCl_2$. Solution 3: 2.0 g $FeCl_2 \cdot 4H_2O$. Solution 4: 14.0 g $MnSO_4$. Filter-sterilize each solution and keep in the dark at −20°C.

  – Base mix (100×, composition per liter): 1.0 g adenine, 1.0 g uracil, 1.0 g xanthine, 1.0 g guanine. Dissolve in milliQ water and add a few drops of 0.2 M NaOH to facilitate the solubilization. Filter-sterilize and store in the dark at −20°C.

  – Basic medium (composition per 920 mL): 0.29 g tyrosine, 13.6 g KH_2PO_4, 25.1 g K_2HPO_4, 0.6 g ammonium citrate, 1.0 g sodium acetate. Adjust the pH to 6.5, autoclave, and store in the dark at −20°C.

  Add 10 mL of vitamin mix, 50 mL of amino acid mix, 2.5 mL of each one of the four metal solutions, and 10 mL of the base mix to 920 mL of basic medium. When this is properly mixed, add glucose at a final concentration of 0.5% and 0.25 g cysteine.

  Minimal expression medium (MEM) [43]: similarly to CDM, MEM is prepared as separate solutions in order to facilitate the solubilization of all the components, facilitate the

sterilization, and allow long-term storage. The vitamin mix is sterilized by filtration and can be frozen in aliquots at −20°C (*see* **Note** 5). The buffer and nutrient solutions are prepared separately in 80% and 20% of the final volume, respectively, and mixed before autoclaving at 121°C for 15 min. Glucose is added at a final concentration of 0.5% from a filter-sterilized 20% solution (*see* **Note** 2). The vitamins are added just before use.

- Vitamin mix (1,000×, composition for 100 mL): 0.01 g of biotin, 0.1 g of folic acid, 0.1 g of riboflavin, 0.1 g of nicotinic acid, 0.1 g of pantothenic acid, 0.2 g of pyridoxal.
- Buffer solution (composition per liter): 2.0 g $(NH_4)_2SO_4$, 7.48 g $Na_2HPO_4 \cdot 2H_2O$, 3.0 g KH_2PO_4, 1.0 g NaCl.
- Nutrient solution (composition per liter): 10.0 g casamino acids, 2.0 g sodium acetate, 0.08 g asparagine, 0.3345 g $MgCl_2 \cdot 4H_2O$, 0.0165 g $CaCl_2 \cdot 4H_2O$, 0.00054 g $FeCl_3 \cdot 6H_2O$.

2.3 Construction of Lantibiotic Expression Vectors for E. coli (See Note 7)

- Vectors for the expression of peptides containing lantibiotic modifications (*see* **Note** 8): pACYCDuet-1 and pRSFDuet-1 (EMD Millipore, http://www.emdmillipore.com).
- PCR cleanup kit (Macherey-Nagel, http://www.mn-net.com).
- FastDigest restriction enzymes (Thermo Fisher Scientific, http://www.thermofisher.com).
- T4 ligase (Thermo Fisher Scientific, http://www.thermofisher.com).
- Phusion high-fidelity DNA polymerase (Thermo Fisher Scientific, http://www.thermofisher.com).
- Bacterial strain: *E. coli* DH5α (Invitrogen, http://www.lifetechnologies.com) is used for cloning. Electrocompetent cells were prepared according to standard protocols [46].
- Antibiotics: 50 mg/mL kanamycin (dissolved in MilliQ water and filtered) and 25 mg/mL chloramphenicol (dissolved in 70% ethanol). The antibiotic stocks are aliquoted and stored at −20°C. The final concentration in the media is 50 and 25 μg/ml, respectively.
- LB-Lennox broth (Formedium, http://www.formedium.com) (composition per liter): 10.0 g tryptone, 5.0 g yeast extract, 5.0 g NaCl. When solid media were required, 1.5% agar was added. Autoclave at 121°C for 15 min.
- Plasmid miniprep kit (Macherey-Nagel, http://www.mn-net.com).

2.4 Expression of Peptides Containing Lantibiotic Modifications in E. coli

- Expression vectors: pRSFDuet-1 containing *nisA* and *nisB* and pACYCDuet-1 containing *nisC* (Fig. 3).
- Terrific broth (Formedium, http://www.formedium.com) (composition per liter): 12.0 g tryptone, 24.0 g yeast extract, 4.0 ml glycerol (*see* **Note** 9). Autoclave at 121°C for 15 min.
- Antibiotics: 50 mg/mL kanamycin and 25 mg/mL chloramphenicol. Dissolve kanamycin in water (filtering is required) and chloramphenicol in 70% ethanol. Aliquot and store at −20°C. The final concentration in the medium is 50 μg/mL for kanamycin and 25 μg/mL for chloramphenicol.
- Expression strain: *E. coli* BL21(DE3) (Invitrogen, http://www.lifetechnologies.com) (*see* **Note** 10).
- Isopropyl β-D-1-thiogalactopyranoside (IPTG): 1 M in milliQ water. Filter-sterilize and store at −20°C.
- Sonication lysis buffer (http://www.embl.de): 50 mM TrisHCl, pH 7.5, 200 mM NaCl, 5 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF).
- Ultrasonic homogenizer (Sonics Vibra-Cell VCX130, www.sonics.com). Optionally a French press could be used to disrupt cells.

2.5 Purification of Lanthionine-Containing Peptides Using Cationic Exchange Chromatography

- Dilution buffer: 100 mM lactate.
- Wash buffer: 50 mM lactate pH 4.0 (*see* **Note** 11).
- Elution buffer: 50 mM lactate 1 M NaCl pH 4.0.
- Storage buffer: 0.2 M sodium acetate + 20% ethanol.
- 75% acetic acid.
- 2 M NaCl.
- 1 M NaOH.
- HiTrap SP HP 5 mL prepacked column (GE Healthcare, http://www.gelifesciences.com).

Prepare all the solutions according to the recipe using MilliQ water and filter them with a 0.45 μm filter to remove any particles that can damage the column.

2.6 Purification of Lantibiotics on Nickel-NTA Column

- Ni-NTA resin (Qiagen, http://www.qiagen.com).
- Lysis buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 7.0.
- Wash buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 7.0.
- Elution buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 7.0.
- 20% ethanol.

2.7 Salt Removal of Semipurified Lanthionine-Containing Peptide

- MilliQ water.
- PD-10 G-25 Sephadex prepacked gel filtration columns (GE Healthcare, http://www.gelifesciences.com).

2.8 Cleavage of the Leader Peptide

- Sequencing-grade trypsin (Sigma-Aldrich, www.sigma-aldrich.com) dissolved in onefold cleavage buffer.
- Tenfold cleavage buffer: 50 mM $CaCl_2$ 500 mM Tris pH 6.5.

2.9 Reversed-Phase HPLC Separation of the Lanthionine-Containing Peptide

- Chromatographer: we use a semipreparative Agilent 1200 series HPLC (www.agilent.com).
- Solvent A: 0.1% trifluoroacetic acid in MilliQ water.
- Solvent B: acetonitrile 0.1% trifluoroacetic acid.
- Analytical C-12 column Jupiter Proteo 90Å, 4 μm, 250 × 4.6 mm, with a precolumn of the same material (Phenomenex, www.phenomenex.com).
- Semipreparative C-12 column Jupiter Proteo 90Å, 4 μm, 250 × 10 mm, with a precolumn of the same material (Phenomenex, www.phenomenex.com).

2.10 Determination of the Peptide Mass Using MALDI MS

- Mass spectrometer: Applied Biosystems Voyager DE Pro mass spectrometer.
- 50% acetonitrile, 0.1% trifluoroacetic acid.
- Matrix: α-cyano-4-hydroxy-cinnamic acid. Dissolve the matrix in 50% acetonitrile 0.1% trifluoroacetic acid at a final concentration of 5 mg/mL (*see* **Note** 12).
- Peptide standard (Sigma-Aldrich): ACTH fragment (2,465.19 Da), insulin oxidized β-chain (3,494.65 Da), bovine insulin (5,735.00 Da). Dissolve each peptide standard in 100 μL of 50% acetonitrile 0.1% trifluoroacetic acid to obtain a 100 pmol/μL solution and mix the three solutions (final concentration 33.3 pmol/μl of each peptide). Aliquot in small volumes (we use 10 μL per tube) to prevent repeated thawing and freezing and store at −20°C.

3 Methods

3.1 Protocol for the Construction of Lantibiotic Expression Vectors in L. lactis

3.1.1 Preparation of Competent Cells [47]

1. Inoculate *L. lactis* NZ9000 in SGGM17 (*see* **Note** 13) and incubate overnight at 30°C.
2. Use the overnight culture to inoculate fresh SGGM17 at a final concentration of 4% (*see* **Note** 14).
3. Grow the cells until they reach an OD_{600nm} of 0.4–0.6.
4. Centrifuge the culture at 8,000 × *g* for 10 min in a precooled centrifuge at 4°C (*see* **Note** 15).

5. Discard the supernatant and carefully resuspend the cells in 0.5 volumes of ice-cold 0.5 M sucrose containing 10% glycerol. Avoid vortexing. Centrifuge in the same conditions as **step 4**.
6. Repeat **step 5** two more times.
7. Resuspend the cell pellet in 0.01 volumes of ice-cold 0.5 M sucrose containing 10% glycerol.
8. Prepare 40 μL aliquots in sterile cold tubes and freeze them at −80°C if they are not going to be used immediately (*see* **Note** 16).

3.1.2 Transformation

1. Mix the plasmid DNA with an aliquot of competent cells pipetting smoothly (*see* **Note** 17). Leave on ice for 1 min.
2. Transfer the cells to an ice-cold electroporation cuvette (*see* **Note** 18).
3. Place the cuvette in the electroporator and apply an electrical pulse at 200 Ω, 2.5 kV, 25 μF.
4. Immediately after the pulse add 1 mL of SGM17MC.
5. Keep on ice for 5 min.
6. Incubate 2 h at 30°C.
7. Plate the cells on SGM17agar with the appropriate antibiotic.

3.1.3 Construction of a Peptide Fused to Nisin Leader Peptide

The expression of lantibiotics, dehydrated and thioether-stabilized peptides in *L. lactis*, is based on a two plasmids system (Fig. 2). The first one is a pIL253-based vector that can harbor large inserts and carries the PTM enzymes NisB, NisC, and NisT. The second plasmid is a pSH71-based vector that can replicate in diverse bacteria including *E. coli* and *L. lactis*. Although the pSH71-derived vector can serve as a shuttle vector, the easy manipulation and transformation of *L. lactis* using electroporation in an osmotically controlled environment make the intermediate cloning in *E. coli* not necessary and saves time (*see* previous protocol).

In either case, the control of the expression is achieved with the use of the nisin-controlled expression system (NICE). This system requires the insertion of a nisin-inducible promoter (either P_{nisA} or P_{nisF}) in front of the gene(s) of interest and the presence of NisRK in the producer strain to sense the addition of nisin. NisRK can be encoded in an additional plasmid or in the chromosome. Due to the presence of two plasmids in the system, we recommend the use of strains with *nisRK* integrated in the chromosome such as *L. lactis* NZ9000, which is an MG1363 derivative with *nisRK* integrated in the *pepN* locus [48]. A recently developed system includes in addition to the nisin-inducible system a zinc-inducible promoter so that the enzymes and the structural peptide can be induced separately [42]. This is an advantage when additional posttranslational modification enzymes are included in the system and induction timing needs to be controlled.

Cloning a Synthetic Construct

The existing vectors containing the gene *nisA* or its derivatives do not contain restriction sites in their sequence that allow the cloning of small fragments fused to the leader peptide. Considering the small size of the genes encoding the core peptide to be fused to the nisin leader peptide, we suggest ordering synthetic constructs encompassing the promoter, the leader peptide, and the core peptide. This facilitates the cloning process and selection.

1. Design the synthetic DNA containing a *Bgl*II site, the promoter P_{nisA}, and the ORF encoding the nisin leader peptide and the core peptide, followed by a *Hind*III site.
2. Digest the synthetic DNA and the vector pNZE-empty with *Bgl*II and *Hind*III.
3. Ligate the vector and the DNA.
4. Transform the ligation mixture into *L. lactis* NZ9000.
5. Select positive clones by colony PCR with the primers pNZEmf (CAATTCCTTAAAACATGCAGG) and pNZrev (CAATCAAAGCAACACGTGC).
6. Transform a right clone into *L. lactis* NZ9000 (pIL3BTC) and select transformants on SGM17-agar containing 5 μg/ml erythromycin and 5 μg/ml chloramphenicol.

Cloning a Small Peptide Sequence

1. Design a reverse primer that hybridizes with the nisin leader peptide coding sequence and carries a tail coding for the peptide of interest and a phosphorylated forward primer that hybridizes with the region downstream the *nisA* gene in pNZnisA-E3 (Fig. 2).
2. Perform a round PCR that amplifies the whole vector pNZnisA-E3 using a high-fidelity polymerase such as Phusion (Thermo Fisher Scientific. http://www.thermofisher.com).
3. Clean the PCR product with an appropriate kit. We use the Macherey-Nagel kit (Macherey-Nagel, http://www.mn-net.com).
4. Ligate the PCR product to circularize the amplified vector.
5. Transform the ligation mixture into *L. lactis* NZ9000 and select transformants on SGM17-agar containing 5 μg/ml erythromycin.
6. Select positive clones by colony PCR with the primers pNZEmf (CAATTCCTTAAAACATGCAGG) and pNZrev (CAATCAAAGCAACACGTGC).
7. Transform a right clone into *L. lactis* NZ9000 (pIL3BTC) and select transformants on SGM17-agar containing 5 μg/ml erythromycin and 5 μg/ml chloramphenicol.

3.2 Protocol for Protein Induction in L. lactis

1. Inoculate *L. lactis* NZ9000 containing the pIL3BTC and the pNZE-derivative where the gene of interest is cloned in GM17 containing 5 μg/ml erythromycin and 5 μg/ml chloramphenicol. Grow overnight at 30°C (*see* **Note** 19).
2. Mix the components of the expression medium (MEM for nisin-induced expression or CDM if zinc induction is used) and prewarm them at 30°C.
3. Inoculate the expression medium with the overnight culture at a final concentration of 4%.
4. Grow the cells until an OD_{600nm} between 0.4 and 0.6.
5. Induce the culture with either 5 ng/mL nisin or 5 ng/mL nisin and 1 mM $ZnSO_4$.
6. Incubate 3 h at 30°C.
7. Centrifuge the culture at 8,000 × *g* for 10 min at 4°C.
8. Retain the supernatant.

3.3 Construction of Lantibiotic Expression Vectors in E. coli (See Note 7)

The expression vector pRSFDuet-1 contains a 6xHis tag in the beginning of the first polylinker between *Nco*I and *Bam*HI restriction sites. This enables the introduction of 6xHis tag before the nisin leader peptide and the peptide to be expressed. Here we present the expression system with the nisin biosynthesis genes; however it is not the only possible version (*see* **Note** 8).

All PCR and DNA restriction products have to be cleaned with the PCR cleanup kit to ensure efficient further manipulations (e.g., ligation or restriction).

1. Amplify the genes of choice (*nisA*, *nisB*, and *nisC*). When employing PCR, use high-fidelity polymerase, e.g., Phusion (*see* Subheading 2.3), and follow the manufacturer's instructions.
2. Digest *nisA* DNA fragment with *Bam*HI and *Hind*III and ligate it into previously *Bam*HI/*Hind*III digested pRSFDuet-1. For the ligation use T4 ligase and follow manufacturer's instructions.
3. Transform the ligation mix into *E. coli* DH5α and plate on LB-kanamycin agar plates (*see* Subheading 2.4). Incubate plates at 37°C for 16 h or until visible colonies appear.
4. Check approximately 10 colonies for positive clones by colony PCR. Choose a positive clone and inoculate it into LB-kanamycin medium (*see* Subheading 2.4). Incubate overnight at 37°C with vigorous shaking.
5. Pellet the overnight culture and use the plasmid miniprep kit to isolate the plasmid. Verify if cloning was successful by sequencing.

6. Clone *nisB* DNA fragment into the obtained pRSFDuet-1 *nisA* containing vector using *Nde*I and *Xho*I restriction enzymes. Follow the instructions from **steps 2–5**.
7. Clone *nisC* DNA fragment into pACYCDuet-1 vector using *Bgl*II and *Xho*I restriction enzymes and following the instructions from the previous **steps 2–5**. LB-chloramphenicol agar plates (*see* Subheading 2.4) must be used instead LB-kanamycin ones for selection.

3.4 Expression of Lanthionine-Containing Peptides in E. coli

The methods presented here are based on previous methodology but with minor modifications [49].

1. Transform *E. coli* BL21(DE3) with both pRSFDuet-1 containing *nisA* and *nisB* and pACYCDuet-1 containing *nisC* and plate on LB-agar plates containing kanamycin and chloramphenicol (*see* **Note** 20). Incubate plates at 37°C for 16 h or until visible colonies appear.
2. Grow an overnight culture from a single colony.
3. Inoculate terrific broth (*see* **Note** 9) containing kanamycin and chloramphenicol (see Subheading 2.4). Grow cells at 37°C with vigorous shaking until OD_{600nm} reaches 0.4–0.6.
4. Induce the culture with 1 mM IPTG and continue growing cells at 18°C for an additional 16 h (*see* **Note** 21).
5. Pellet the cells by centrifugation at 4°C, 8,000 × *g* for 15 min. Resuspend the cell pellet in 0.2 culture volumes of sonication buffer (*see* Subheading 2.4). It is possible to store the cell suspension at −20°C for several weeks; otherwise proceed with cell lysis as described in the next steps.
6. Chill the suspension on ice. Lyse the cells using an ultrasonic homogenizer. Use 10 times 10 s pulses with a pulse intensity of 65% leaving 30 s intervals in between for cooling.
7. Centrifuge the lysate at 18,000 × *g* for 30 min at 4°C.
8. Collect the supernatant and discard the cell pellet.

3.5 Protocol for Cation Exchange Chromatography (See Note 22)

1. Dilute the supernatant with 1 volume of dilution buffer and filter through a 0.45 μm membrane.
2. Wash the HiTrap SP HP column with 5 column volumes of wash buffer.
3. Wash the column with 5 column volumes of elution buffer.
4. Wash the column with 5 column volumes of wash buffer.
5. Run the supernatant through the column (*see* **Note** 23).
6. Wash the column with 5 column volumes of wash buffer.

7. Elute the peptide with elution buffer. The first 4 mL fraction corresponding to the column dead volume can be discarded. Collect the next 8 mL.
8. Regenerate the column:
   (a) Wash with 3 volumes of MilliQ water.
   (b) Wash with 3 volumes of 1 M NaOH.
   (c) Wash with 3 volumes of MilliQ water.
   (d) Wash with 3 volumes of 2 M NaCl.
   (e) Wash with 3 volumes of MilliQ water.
   (f) Wash with 3 volumes of 75% acetic acid.
   (g) Wash with 3 volumes of MilliQ water.
   (h) Wash with 5 volumes of storage buffer.

Keep a sample of **steps 1**, **5**, and 7 to monitor the production and purification process either by Tricine-SDS-PAGE [50] or antimicrobial tests [35] if the lanthionine-containing peptide has inhibitory activity.

3.6 Purification of Lantibiotics by Immobilized Metal Affinity Chromatography on a Nickel-NTA Column

The method presented here is based on [39].

1. Equilibrate 1 mL of 50% superflow Ni-NTA resin (*see* **Note** 24) with 10 volumes of lysis buffer (*see* Subheading 2.6) in a 50 mL tube by mixing on a rotor for 30 min.
2. Let the resin settle down, remove the buffer, and repeat **step 1**.
3. Resuspend the column material in 4–8 mL cytoplasmic fraction obtained previously (protocol 3.4) and transfer it into a 15 mL tube. Add lysis buffer to a final volume of 12 mL.
4. Allow the His-tagged peptide to bind to the column material on a rotor at 4°C for 2 h. After binding transfer the column material to a gravity column.
5. Wash the column twice with 10 volumes of wash buffer (*see* Subheading 2.6).
6. Elute the peptide with 10 column volumes of elution buffer taking fractions of approximately 0.5 mL (*see* Subheading 2.6).
7. Wash the column with 10 volumes 20% ethanol for storage.

Take a sample of the lysate before applying the Ni-NTA resin and from **steps 4** and **5** to monitor the production and purification process by Tricine-SDS-PAGE [50] or antimicrobial tests [35] if the lanthionine-containing peptide has inhibitory activity.

3.7 Desalting Protocol

1. Wash the PD-10 column with 25 mL MilliQ water.
2. Apply up to 2.5 mL sample and discard the flow-through.
3. Elute the peptide with 3.5 mL MilliQ water.

4. Repeat **steps 1–3** to desalt all the peptide fractions obtained from either cation exchange chromatography or immobilized metal affinity chromatography.

3.8 Cleavage of the Leader Peptide

1. Mix 9 volumes of peptide solution with 1 volume of tenfold cleavage buffer.
2. Add 1 μg of sequencing-grade trypsin (*see* **Note** 25).
3. Incubate at 37°C for 2 h (*see* **Note** 25).

3.9 HPLC Separation of the Lanthionine-Containing Peptide

1. Equilibrate the column with 10 column volumes of 20% solvent B (*see* Subheading 2.9).
2. Inject the digestion mix.
3. Run the separation gradient:
   (a) Analytical column: 8 min 20% solvent B, 13 min 25% solvent B, 38 min 60% solvent B, 39 min 95% solvent B, 44 min 95% solvent B, 45 min 20% solvent B. Use a constant flow at 1 mL/min.
   (b) Semipreparative column: 8 min 20% solvent B, 13 min 25% solvent B, 38 min 60% solvent B, 39 min 95% solvent B, 44 min 95 % solvent B, 45 min 20 % solvent B. Use a constant flow at 2.5 mL/min.
4. Collect the peaks for further analysis or storage.
5. Remove the solvent from the sample (e.g., freeze-dryer) and store at −20°C (*see* **Note** 26).

3.10 Protocol for Mass-Spectrometry Determination Using MALDI-TOF

1. Dissolve the matrix.
2. Wash the MALDI target with 50% acetonitrile 0.1% trifluoroacetic acid and dry.
3. Spot 1 μl of sample or protein standard mix on the target and let it dry.
4. Optional: if the samples contain salts (this is not necessary for HPLC purified samples or protein standard), it is possible to use zip-tip C_{18} (Millipore) before spotting them on the target or wash with MilliQ water after spotting. To wash with MilliQ water, spot 5 μL MilliQ water on the sample once that it is dried and immediately remove it with a tissue by capillarity. The results are usually better with zip-tip treatment.
5. Spot 1 μL of the dissolved matrix on the sample and let it dry.
6. Determine the mass of the peptides in the mass spectrometer. We use 20,000 V acceleration, 94% grid, 0.05% guide wire, 100 ns delay time in linear mode acquisition.
7. Calibrate the mass data with appropriate software using the peptide standard as a reference. We use external calibration

and Data Explorer 4.0.0.0 (Applied Biosystems) for the analysis.

8. Compare the mass values determined experimentally with the theoretical values (*see* **Note** 27).

4 Notes

1. The induction in *L. lactis* using nisin requires the two-component system NisRK, which is integrated in the genome of strain NZ9000.
2. Glucose cannot be autoclaved together with M17 or SM17; therefore filter sterilization of a 20% concentrated stock is recommended.
3. It is advisable to use a fresh solution and not store it for longer than 1 month. Use MilliQ water for its preparation to minimize the presence of ions that can prevent the transformation.
4. Nisin is more soluble and stable at acidic pH. It is less stable at neutral pH and unstable at alkaline pH. The commercial powder contains a high proportion of insoluble particles that have to be removed.
5. The vitamin mix is sensitive to light. Store protected from light.
6. Prepare the metal mix without zinc if the zinc-inducible system for *L. lactis* is going to be used.
7. The lantibiotic expression system in *E. coli* briefly described here is based on Shi and coworkers methodology with some modifications [49].
8. Other optional vectors could be used for heterologous lantibiotic production in *E. coli*: (a) single plasmid pET28a, pET28b, or pRSFDuet-1 [51]; (b) single fosmid (e.g., pCC2FOS) or in combination with other expression vectors (e.g., pET24a(+) [52]); (c) pRSFDuet-1 in combination with pCDFDuet-1 [53].
9. LB could be considered to be used instead of terrific broth; however it might affect the protein production levels.
10. Different expression strains might give different lantibiotic production levels (e.g., lower nisin amounts are produced in *E. coli* Rosetta-gami 2(DE3)) (EMD Millipore, http://www.emdmillipore.com) compared to *E. coli* BL21(DE3) when using the same experimental setup (unpublished results Buivydas A, Moll GN, Kuipers OP).
11. In order to increase the storage time of this solution, we recommend the preparation of 0.5 M lactic acid pH 4.0 and dilute it tenfold just before use.

12. α-Cyano-4-hydroxy-cinnamic acid is light- and oxygen-sensitive. Therefore, prepare it fresh, and after mixing the power with the solvent, cover with aluminum foil.
13. Antibiotics can be added when competent cells containing already one plasmid are being used.
14. Due to the presence of glycine in the medium, the growth speed is reduced. Thus, we recommend using prewarmed medium at 30°C to avoid additional delay.
15. It is important that the cells are in mid-exponential phase to obtain optimal competence. Therefore, it is necessary chilling them and work all the time on ice and with ice-cold media and material (e.g., plastic tubes, cuvettes, etc.) from this point.
16. The transformation efficiency can be improved if the cells are frozen in liquid nitrogen. However, it is not essential. Normal efficiency values of 10^4–10^5 CFU/μg DNA can be achieved using this procedure.
17. If the cells were frozen, they must be thawed on ice. Do not add the DNA on frozen cells and keep the DNA volume below 4 μL. The DNA sample must contain a low salt concentration. The usual elution buffers in commercial DNA extraction kits have low salt concentration and can be used directly. Otherwise desalt your sample by precipitation or dialysis against MilliQ water on a 0.22 μm filter membrane (Millipore) for 20–60 min.
18. The separation gap between the electrodes for transformation of *L. lactis* is 2 mm. Make sure there are no air bubbles in the cells after placing them in the cuvette and dry the outside electrode with a tissue.
19. In the system using nisin and zinc as inducers, the selected vectors are pIL3EryBTC and a pCZ-Cm derivative containing the structural genes [35, 42].
20. We recommend transforming *E. coli* BL21(DE3) simultaneously with the two plasmids by electroporation, instead of preparing competent cells containing already one of the plasmids.
21. Induced cultures could be incubated, alternatively, at 37°C. However the protein production level might be lower, though sufficient for antimicrobial activity tests, MS, and/or Western blot analyses.
22. This protocol is designed for lantibiotics, which have a pI above 6.0. If the pI of the produced protein is lower, anionic exchange chromatography is advised instead of reducing the pH of the solutions in this protocol.
23. We use a peristaltic pump to load the column at a flow rate of 5 mL/min.

24. The volume of 50% superflow Ni-NTA column resin is used in ratio 1:4 with cleared cell lysate (*see* Subheading 3.4). 4 ml of a lysate could be obtained from 20 to 200 mL of initial cell culture volume depending on the expression level of 6xHis-tagged protein.
25. Trypsin cleaves preferentially in the arginine residue located at the end of the nisin leader peptide. Lanthionine rings confer protection of the peptide against proteolysis, reducing the possibilities of degradation. However, it might be necessary to reduce the incubation time and enzyme to peptide ratio if positively charged residues are exposed to cleavage in a particular peptide of interest.
26. It is preferable storing a dried sample rather than keeping it in organic solvent. In the latter case, store at −80°C and not −20°C.
27. During data analysis it is crucial to calculate the mass differences introduced by the posttranslational modification(s) taking place. For instance, each dehydration in the peptide causes a reduction of 18 Da in the mass, whereas reduction of dehydroalanine by a LanJ enzyme increases the mass 2 Da. There are no devoted tools developed for this purpose; thus the data analysis needs to be precise.

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Engineering Transcription Factor-Based Biosensors for the Detection of Intracellular Products

Solvej Siedler

Abstract

The screening of novel enzymes and efficient production strains for the production of small molecules becomes more and more important for fast and reliable strain construction. Increased concentrations of small molecules often do not lead to a measurable phenotype, which impedes rapid detection of improved production strains. Metabolite biosensors based on transcription factors are currently an upcoming method for detection of intracellular metabolites in vivo. This technique couples the response of a transcription factor to the readout of a fluorescent reporter protein. The described example is based on the transcriptional repressor QdoR from *Bacillus subtilis*, which is inactivated by some flavonoids (e.g., kaempferol). The constructed biosensor showed over sevenfold increase of the fluorescent signal after addition of the effector and was successfully applied for detection of kaempferol production in vivo in *Escherichia coli* cells containing a flavonol synthase from *Arabidopsis thaliana* (fls1).

Keywords: Flavonoids, Metabolite biosensors, QdoR, Transcriptional regulator

1 Introduction

One of the major bottlenecks in metabolic engineering processes is the screening for the best production strain, because metabolite concentrations are seldom associated with a measurable phenotype. Chromatographic methods (e.g., HPLC and GC) offer despite the accuracy only a low throughput of ~500 samples per day, whereas colorimetric assays increase the throughput about 1,000-fold. Nevertheless, taking the diversity of constructed libraries (e.g., metagenomics, random mutagenesis) in mind, only a small fraction of the sample space can be analyzed. In nature, thousands of transcription factors have been evolved to sense different substrates precisely, thereby analyzing the metabolite concentrations in vivo in bacterial cells.

Taking advantage of those specialized elements can be achieved through coupling the signal to the readout of a fluorescent reporter protein. One major advantage of using fluorescent markers is the

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ability to use fluorescence-activated cell sorting (FACS) for high-throughput screening and thus drastically shorten the time for analyzing millions of mutant cells [1, 2]. Specific biosensors are foreseen to revolutionize the field of metabolic engineering by enabling the screening of libraries in the sizes of 10^9–10^{10}. In recent years, several examples for functional biosensors and metabolic switches have been developed, e.g., for detection of amino acid producing cells [1, 3], for screening of NADPH-dependent enzymes [4], or for redirection of the central carbon metabolism [5].

In *Escherichia coli*, the number of homologous transcriptional regulators is limited, but modifying the substrate specificity of such regulators by laboratory evolution [6, 7] or using heterologous regulatory mechanisms can be a promising alternative [8].

Plant secondary metabolites are an important source of new drugs and nutraceuticals. Phenylpropanoids, especially flavonoids and stilbenes, were shown to have antioxidant, antiviral, antibacterial, anticancer, and immunosuppressive activities [9–11]. For the efficient identification of functional genes, novel tools such as biosensors need to be applied.

In this chapter, we describe the general parameters which have to been taken under consideration for construction of metabolite sensors and the evaluation and application thereof using a flavonoid responsible biosensor as an example (Fig. 1).

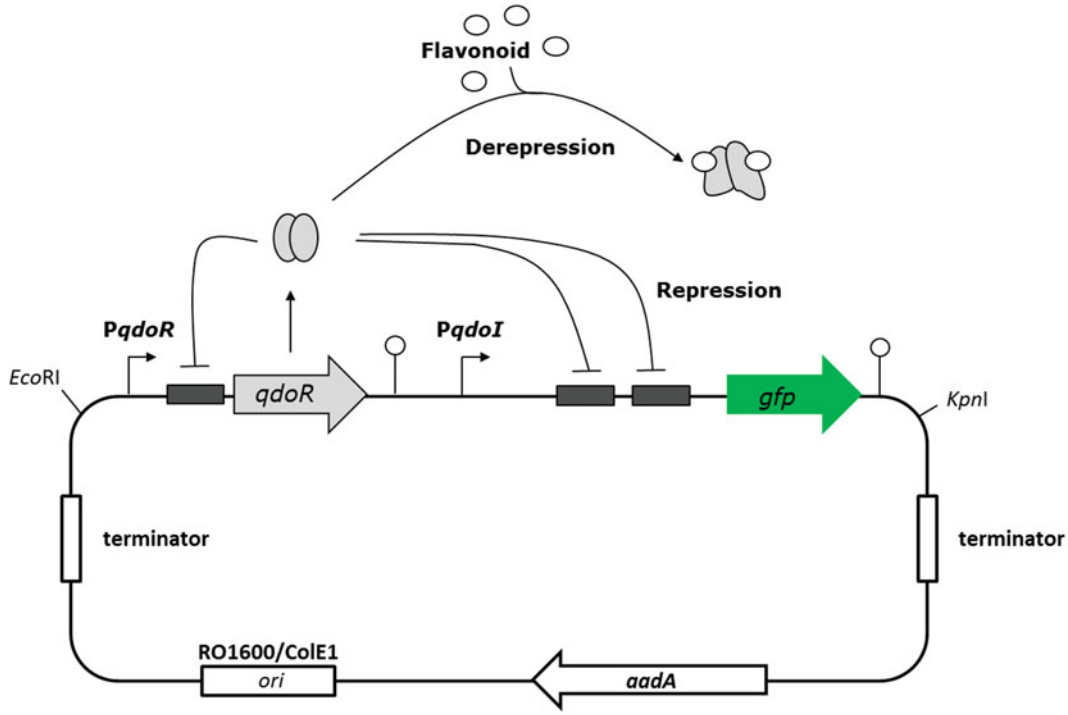

Fig. 1 Map of p441-QdoR. Spectinomycin-resistance and colE1 replicon-based medium copy number plasmid

2 Materials

2.1 Construction of p441-QdoR

1. Vectors: pSEVA441 (http://seva.cnb.csic.es/SEVA/Contact_us.html) previously digested with *Kpn*I and *Eco*RI.
2. Synthetic DNA construct QdoR-GFP (*see* **Note 1**).
3. Primers: pSEVA-seq-for (5′-AGCGGATAACAATTTCACACAGGA-3′), pSEVA-seq-rev (5′-TGGGACAACTCCAGTGAAAAGTTC-3′).
4. Restriction enzymes *Kpn*I and *Eco*RI (Thermo Scientific: www.thermoscientificbio.com).
5. Gelelectrophoresis 1% agarose.
6. Gel Extraction Kit (Macherey & Nagel: http://www.mn-net.com/).
7. T4 DNA ligase (New England Biolabs: http://www.neb.com).
8. Bacterial strains: *E. coli* DH5α strain (GibcoBRL, Life Technologies (http://www.lifetechnologies.com)) is used for cloning.
9. Spectinomycin antibiotic (*see* Subheading 2.5).
10. Growth media: yeast extract-tryptone x2 (2xYT) (*see* Subheading 2.6).

2.2 Validation of Functionality In Vivo

1. Vectors: p441-QdoR (*see* **Note 2**)
2. Bacteria strains: *E. coli* BL21(DE3)
3. Antibiotics: spectinomycin
4. 2x YT growth media
5. Kaempferol, dihydrokaempferol, and naringenin (*see* Subheading 2.5)

2.3 Whole-Cell Biotransformation and Flavonoid Extraction

1. Vectors: p441-QdoR and pRSF-FLS1 (*see* **Note 3**)
2. Bacteria strains: BL21(DE3)
3. Antibiotics: spectinomycin, kanamycin (*see* Subheading 2.5)
4. 2xYT growth media (*see* Subheading 2.6)
5. M9 media (*see* Subheading 2.6)
6. Isopropyl β- D-1-thiogalactopyranoside (IPTG): use 0.5 mM for overexpression (*see* Subheading 2.5).
7. Methanol

2.4 HPLC Analysis

1. Discoverys HS F5-5 column (4.6 mm by 150 mm; 5.0-mm particle size; Sigma–Aldrich)
2. Buffer A: 10 mM ammonium formate pH 3.0
3. Acetonitrile
4. Kaempferol and dihydrokaempferol as standard

2.5 General Buffers and Reagents

1. Spectinomycin: 50 μg/ml in water. Store at −20°C (Sigma (http://www.sigmaaldrich.com)).
2. Kanamycin: 50 μg/ml in water. Store at −20°C (Sigma (http://www.sigmaaldrich.com)).
3. Kaempferol: 10 mM in DMSO. Store at −20°C (Sigma (http://www.sigmaaldrich.com)).
4. Naringenin: 10 mM in DMSO. Store at −20°C (Sigma (http://www.sigmaaldrich.com)).
5. Dihydrokaempferol: 10 mM in DMSO. Store at −20°C (Sigma (http://www.sigmaaldrich.com)).
6. Isopropyl β-D-1-thiogalactopzranoside (IPTG) (Sigma (http://www.sigmaaldrich.com)): 1M in sterile double distilled (MilliQ) water stored in 1 ml aliquots at −20°C.

2.6 Bacteria Growth Media

These may be purchased from any supplier of common bacterial growth medium components or preprepared media. Here we use products of Becton-Dickinson (http://www.bd.com/).

1. 2xYT: 16 g Bacto-Tryptone, 10 g yeast extract, 5 g NaCl/L water.
2. LB: 10 g Bacto-Tryptone, 5 g yeast extract, 10 g NaCl/L water.
3. M9 minimal medium: [12] containing 10 g l^{-1} glucose.

To prepare solid media, Bacto-agar at the final concentration of 2% was added to the solutions. Following autoclaving, media were supplemented with antibiotics. The final concentrations of the antibiotics used in this study were as follows: spectinomycin: 50 μg/ml, kanamycin: 50 μg/ml.

3 Methods

The protocol describes the construction, validation, and application of a metabolite sensor in *E. coli*.

3.1 Construction of pQdoR-Gfp

For construction of the biosensor, the DNA region containing the native promoter and open reading frame of the repressor (QdoR) were used together with the native promoter regions of their target genes controlling the expression of green fluorescent protein (GFP) (*see* **Note 1**). The codon usage and promoter structure of *E. coli* were taken under consideration (*see* **Note 4**). In principle every vector backbone can be used (*see* **Note 5**).

1. The construct should be amplified by PCR or subcloned by using the restriction sites *Kpn*I and *Eco*RI for 1 h at 37°C.
2. Purify the resulted PCR product or restriction digest by gel extraction or PCR cleanup.

3. Clone the digested QdoR-GFP insert to previously *Kpn*I/*Eco*RI digested pSEVA441 vector by standard ligation procedure and transform it into *E. coli* DH5α competent cells.
4. The success of the cloning should be verified by sequencing using the pSEVA-seq-for/rev primers.

3.2 Validation of Functionality In Vivo

Analysis of the metabolite sensor response to a set of different flavonoids in *E. coli*:

1. Transform BL21(DE3) competent cells with p441-QdoR vector and plate the transformed cells on a LB-agar plate containing 50 μg/ml spectinomycin. Leave for 16 h at 37°C until colonies of transformed bacteria are clearly visible.
2. Inoculate 5 ml of 2xYT + 50 μg/ml spectinomycin in a cultivation tube with a single colony.
3. Grow shaking (250 rpm) at 37°C to an OD600 = 0.7.
4. Transfer 1 ml aliquots to new cultivation tubes and add different flavonoids or DMSO as negative control to the culture to 0.1 mM final concentration (*see* **Note 6**).

   For examination of the dynamic range of the biosensor, add different concentrations in the range from 0.005 to 0.1 mM kaempferol to your culture.
5. Continue culture growth for 20 h at 37°C shaking (250 rpm).
6. Transfer 200 μl of the sample to a 96-well plate and measure the absorbance at 600 nm and the fluorescence (excitation 485 nm and emission 515 nm) of the sample.
7. For calculation of the specific fluorescence, subtract a blank vial with medium from the individual values and calculate the quotient of the fluorescence and the OD_{600} (Fig. 2).

3.3 Whole-Cell Biotransformation and Flavonoid Extraction

1. Transform BL21(DE3) competent cells with p441-QdoR and pRSF-FLS1 or pRSF-duet (as a negative control) vectors and plate the transformed cells on a LB-agar plate containing 50 μg/ml spectinomycin and 50 μg/ml kanamycin. Leave for 16 h at 37°C until colonies of transformed bacteria are clearly visible.
2. Inoculate 3 ml of 2xYT + 50 μg/ml spectinomycin and 50 μg/ml kanamycin in a cultivation tube with a single colony.
3. Grow shaking (250 rpm) at 37°C for 16 h.
4. Take a fresh tube and inoculate 4 ml 2xYT to an OD600 of 0.05.
5. Grow shaking (250 rpm) at 30°C to an OD600 = 0.6
6. For induction add IPTG to 0.5 mM final concentration.
7. Continue culture growth for 5 h at 30°C shaking (250 rpm).

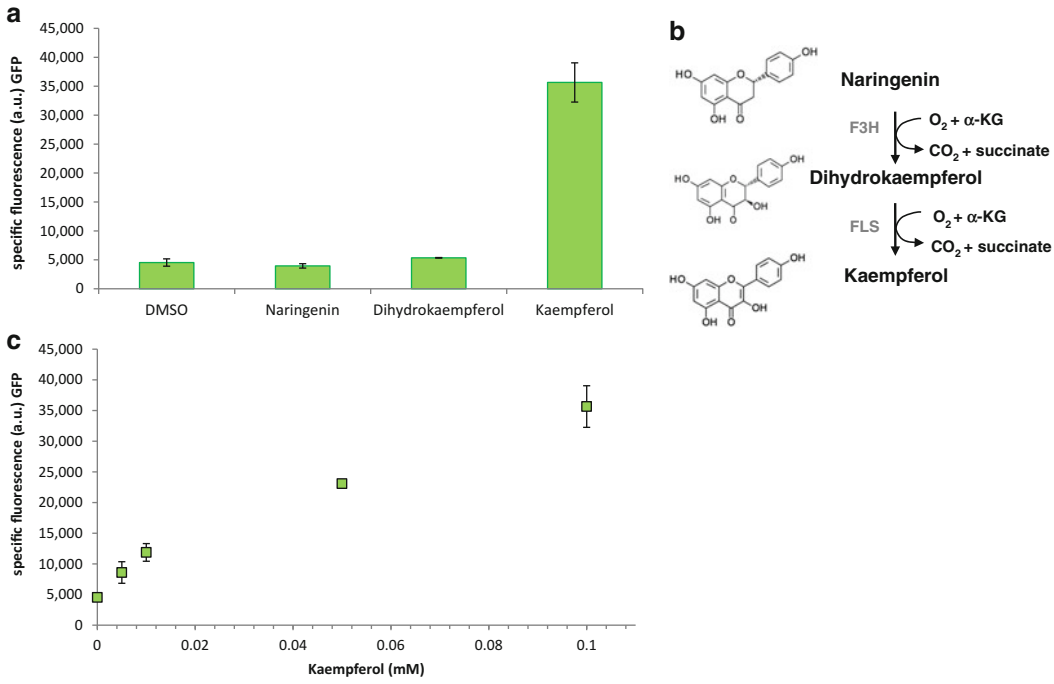

Fig. 2 (**a**) Effector spectrum of the constructed biosensor. The specific fluorescence of *E. coli* cells harboring pQdoR-GFP after cultivation in the presence of 0.1 mM of various flavonoids. DMSO was used as a control since flavonoids were dissolved in it. (**b**) Schematic representation of the flavonoid synthetic pathway from naringenin to kaempferol. (**c**) Fluorescence intensity as a function of kaempferol concentration. Mean values and standard deviations from three independent experiments are shown. Abbreviations: *DMSO* dimethyl sulfoxide, *α-KG* α-ketoglutarate, *F3H* flavanone 3-hydroxylase, *FLS* flavonol synthase

8. Harvest cells in a bench centrifuge at 10,000 × *g* for 2 min.
9. Resuspend the pellet in M9-minimal medium + 50 μg/ml spectinomycin and 50 μg/ml kanamycin supplemented with 0.5 mM IPTG and 0.25 mM dihydrokaempferol. After incubation of 24 h at 30°C, measure the fluorescence in a plate reader as stated in 3.2.6 (**Note 8**). The suspension can be stored at −20°C or metabolite extraction can be directly performed.
10. Add 1 equiv. volume of methanol to the cell suspension.
11. Incubate 30 min at room temperature.
12. Centrifuge at 16,000 × *g* for 5 min to sediment cells and cell debris. Retain the supernatant for HPLC analysis.

3.4 HPLC Analysis

1. Use a Discovery HS F5-5 column (4.6 mm by 150 mm; 5 μm particle size; Sigma-Aldrich).
2. Flow rate of 1 ml/min was used with a linear gradient of 10 mM ammonium formate pH 3.0 buffer (phase A) and acetonitrile (phase B) by the following method: 0–2 min (5% B),

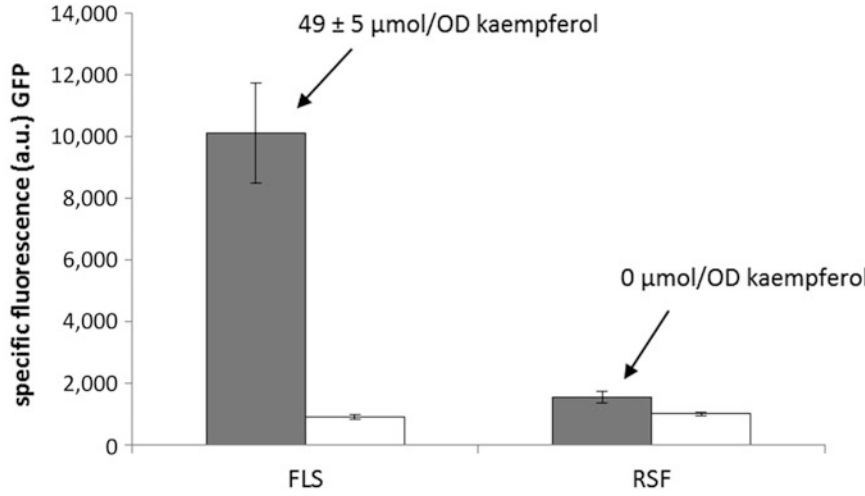

Fig. 3 Correlation between concentration of kaempferol produced and specific fluorescence measured 16 h after the addition of 200 µM dihydrokaempferol in *E. coli* cells containing plasmids p441-QdoR and pRSF-FLS or empty plasmid pRSF. 200 µM dihydrokaempferol (*gray bars*), DMSO (*white bars*) (**Note 9**)

2–10 min (5–50% B), 13–16 min (50–5% B), and 16–20 min (5% B) (*see* **Note 7**).

3. Load 10 µl of your extracted sample.
4. Use a calibration curve of pure kaempferol for quantification of the product (Fig. 3).

4 Notes

1. In many of the described functional metabolite sensors, the transcription factor is encoded in direct proximity of their regulated genes. For construction of new biosensors, this setup is favorable.
2. The vector should be purified by Miniprep Kit. The pSEVA441 concentration is commonly 300 ng/µl in 50 µl.
3. Plasmid pRSF-FLS1 (pR-*Atfls*1) for the expression of the flavonol synthase from *Arabidopsis thaliana* was kindly provided by Prof. Byung-Gee Kim from the School of Chemical and Biological Engineering, Seoul National University, South Korea.
4. A critical parameter is the promoter and ribosome binding site (RBS), which greatly affects the expression level of different organisms. These factors should be optimized for *E. coli* in terms of GFP expression, but the expression of the transcriptional repressor needs to be adjusted. In this study, the native regions from *Bacillus subtilis* were functional in *E. coli*.

5. Fluorescence output will typically increase with copy numbers. However, too high copy numbers can cause a metabolic burden to the cell, which will drastically decrease your signal. In this study, we have used a medium to high copy number plasmid (~50–70), whereas higher copy numbers decreased the signal (>100).
6. The solubility of flavonoids which is low in aqueous solutions does not extend 0.1 mM.
7. Under these conditions, dihydrokaempferol and kaempferol were detected at 10.7 and 12.5 min of retention time, respectively.
8. To check for stochastic effects in reporter expression, you can analyze the sample by fluorescence-activated cell sorting (FACS). With the described sensor, there was a clear difference between the nonproducer and the producer and the latter could be enriched by FACS.
9. The maximal fluorescence of ~10.000 a.u. is lower than after the direct addition of 50 μM kaempferol (23.000 a.u. *see* Fig. 2c). This is most likely due to the fact that the production of kaempferol is slow and the sensor is responding to lower concentrations in the beginning.

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Oxyfunctionalization of Linear Alkanes with a Biosynthetic, Self-Sufficient, Selective, and Soluble Hydroxylase

Mélanie Bordeaux and Jullien Drone

Abstract

Selective oxyfunctionalization of C–H bonds carried by hydrophobic molecules is a challenging reaction. Numerous chemical and biological catalysts have been developed during the past decades to perform this difficult task. Chemical methods generally involve toxic or dangerous reagents and are not very selective. Thus, they are far from ideal and not fully compatible with the principles of green chemistry. On the road to biocatalytic and selective oxyfunctionalization of C–H bonds, we have produced and fully characterized a biosynthetic self-sufficient biocatalyst for alkane activation. To do so, we have fused a cytochrome P450 from *Alcanivorax borkumensis* SK2 with a Fe_2S_2/FMN reductase from *Rhodococcus* sp. NCIMB 9784. With this original enzyme, thereafter referred to as "A13-Red," the highly selective and efficient hydroxylation of alkanes from *n*-hexane to *n*-decane on the terminal methylic position was enabled under mild conditions. This enzyme was the first member of a novel family of biosynthetic omega hydroxylases. Detailed protocols for A13-Red construction, purification, and optimized reaction conditions for the in vitro hydroxylation of its preferred substrate (*n*-octane) are described in detail into this chapter.

Keywords: Biosynthetic self-sufficient hydroxylase, C–H bond activation, Cytochrome P450, Green chemistry, Linear alkanes

1 Introduction

Nature provides a large variety of hydrocarbons [1], including paraffins (alkanes, iso-alkanes, and alkenes), cycloparaffins (cycloalkanes, naphthenes), and aromatics, as a result of the metabolic activities of animals, plants, and microorganisms and by the physicochemical action of decay of organic matter. They are most evident in the form of crude oil and gas deposits. There is a body of opinion that petroleum (crude oil) deposits are too valuable to expend as fuel and should be conserved for the production of chemicals alone. Considering their numerous advantages (i.e., versatility, regio-, chemo-, enantioselectivity, environmental-friendly processes, etc.) [2], the role of microorganisms in a scenario of optimal use of such valuable raw materials would be central.

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Indeed, a very large number of microorganisms have the ability to convert hydrocarbons from crude oil into biomass and useful intermediates or products such as polyhydroxyalkanoates, wax esters, or triacylglycerols [3, 4]. These microbes are the foundations of hydrocarbon biotechnology. Understanding the underlying biological mechanisms of these conversions is of fundamental and practical interest. Several groups of researchers have worked to elucidate these biochemical mechanisms for several decades [5, 6], but current economic uncertainties limit the amount of research activity and the implementation of emerging technologies.

One of the most widespread catabolic pathways in hydrocarbon metabolism among microorganisms is initiated by the primary enzymatic attack of the hydrocarbon molecule. This is generally an oxyfunctionalization yielding an alcohol. For alkanes in particular, Nature has developed a great diversity of enzymatic hydroxylation systems [2]. Most of them share common features: they use dioxygen from air as the oxidizing agent, they use reduced nicotinamide dinucleotides (NAD(P)H) as electron suppliers, they are metalloenzymes based on copper and/or iron, and they operate under mild conditions [2, 6].

Among all these enzymatic systems involved into alkane hydroxylation, cytochromes P450 (P450) from the CYP153 family attracted our attention because they are monomeric soluble enzymes that hydroxylate alkanes [7–11]. Despite their attractiveness, every CYP153 enzyme needs at least one supplementary protein (also called a redox partner) to shuttle electrons from NAD(P)H to the active center of the P450 responsible for the oxidation reaction [12–17]. The requirement for these redox partners could be seen as a disadvantage, but in fact, it has been shown through different examples that P450s can be artificially fused with either their natural partner or with a foreign partner [18–20]. The resulting biosynthetic proteins are usually enzymatically active and self-sufficient. Using this strategy, we have fused a cytochrome P450 from *A. borkumensis* SK2 (CYP153A13a) with a Fe_2S_2/FMN reductase from *Rhodococcus* sp. NCIMB 9784 (RhFred) [21, 22]. The resulting polypeptide (thereafter A13-Red) was reliably expressed in *Escherichia coli* using high-cell-density cultivation (up to 100 g of dry-cell weight per liter of culture medium) [23]. In this chapter, we detail the construction of the gene encoding for A13-Red, our two-step purification strategy to yield A13-Red approximately 90% homogeneous and its use for the regioselective hydroxylation of *n*-octane into optimized conditions.

2 Materials

2.1 Construction of pA13-Red

1. Authentic sample of *Alcanivorax borkumensis* SK2 strain (DSM 11573) (www.dsmz.de)
2. Medium 809, composition for 1 L: NaCl 23.00 g, $MgSO_4 \times 7 H_2O$ 5.80 g, $MgCl_2 \times 2 H_2O$ 6.16 g, $CaCl_2 \times 2 H_2O$ 1.47 g, $Na_2HPO_4 \times 7 H_2O$ 0.89 g, $NaNO_3$ 5.00 g, $FeSO_4 \times 7 H_2O$ 0.03 g, sodium pyruvate 10.00 g, pH 7.0–7.5
3. Qiagen Gentra PureGene Yeast/Bacteria kit (www.qiagen.com)
4. pET28b(+) plasmid, Novagen (www.merckmillipore.com) previously digested by *EcoRI* and *NotI* enzymes
5. pET28b(+)-pikC-RhFred plasmid [19] (generous gift from Dr D. Sherman, University of Michigan, USA)
6. KOD Hot Start Master Mix, Novagen
7. Qiagen PCR Purification kit
8. Primers 153A13a-fw and 153A13a-rv for amplification of CYP153A13a, primers Eco887-fw and Eco887-rv for removal of the *EcoRI* restriction site at position 887 of CYP153A13a, and primers RhFred-fw and RhFred-rv for amplification of RhFred (*see* Table 1 for primer sequences)
9. Restriction enzymes: *DpnI*, *NdeI*, *EcoRI*, and *NotI*, New England Biolabs (www.neb.com)
10. T4 DNA ligase, New England Biolabs
11. *Escherichia coli* strains: DH5α and BL21 Star™ (DE3), Life Technologies (www.lifetechnologies.com)
12. Kanamycin sulfate antibiotic, Sigma-Aldrich (www.sigmaaldrich.com)

Table 1
Sequences of the primers used for the amplification of CYP153A13a and RhFred and for the removal of *EcoRI* restriction site at position 887 in CYP153A13a

Primer	Sequence
153A13a-fw	GTAGCTCATATGTCAACGAGTTCAGT
153A13a-rv	TACAGCGAATTCTTTTTTAGCCGTCAA
Eco887-fw	ATCAATCGTCCTTTGGAGTTCATTGGTAATCT
Eco887-rv	GAGATTACCAATGAACTCCAAAGGACGATTG
RhFred-fw	ACGGCTAAAAAAGAATTCGTGCTGCACCGG
RhFred-rv	TAATGCGGCCGCTCAGAGTCGCA

13. Growth media: Luria Bertani (LB) and Terrific Broth (TB), Sigma-Aldrich
14. Qiagen Plasmid Purification kit

2.2 Purification of A13-Red on Nickel-NTA Column

1. *E. coli* strain BL21 Star™ (DE3)/pA13-Red (fresh or frozen wet-cell pellet)
2. Lysis buffer: Tris–HCl 25 mM, NaCl 500 mM, glycerol 10% (v/v), triton X-100 1% (v/v), lysozyme 0.25 mg/mL, PMSF 1 mM, DNAse I 5 mg/mL, pH 7.5
3. Binding buffer: NaCl 800 mM, Tris–HCl 50 mM, pH 7.5
4. Washing buffer: NaCl 800 mM, Tris–HCl 50 mM, glycine 250 mM, pH 7.5
5. Elution buffer: NaCl 800 mM, Tris–HCl 50 mM, L-histidine 80 mM, pH 7.5
6. HisPrep FF 16/10 prepacked column (GE Healthcare)
7. Amicon Centrifugal Ultrafiltration Units (50-kDa cutoff)
8. Conservation buffer: Tris–HCl 25 mM, NaCl 500 mM, glycerol 20%, pH 7.5

2.3 Desalting of A13-Red Fractions After Ni-NTA Column

1. Desalting buffer: Tris–HCl 25 mM, NaCl 25 mM, glycerol 20%, pH 7.5
2. HiTrap desalting 5-mL prepacked columns (GE Healthcare)

2.4 Purification of A13-Red on Q Sepharose Column

1. Buffer A: Tris–HCl 25 mM, NaCl 80-mM glycerol 20%, pH 7.5
2. Buffer B: Tris–HCl 25 mM, NaCl 200 mM, glycerol 20%, pH 7.5
3. Q Sepharose XL prepacked column (1 mL) (GE Healthcare)

2.5 Enzymatic Hydroxylation of n-Octane

1. Wheaton 5-mL V-Vials with a magnetic stirrer
2. Reaction buffer: potassium phosphate buffer (100 mM, pH 7.4), glycerol 10%
3. *n*-Octane (Sigma-Aldrich; puriss. p.a., ≥99.0% GC)
4. Reduced nicotinamide adenine dinucleotide phosphate (NADPH) (Sigma-Aldrich, 25-mM stock solution into reaction buffer)
5. Bovine serum albumin (BSA) (Sigma-Aldrich, 100 g/L stock solution into reaction buffer)
6. NADP-dependent isocitrate dehydrogenase from *Bacillus subtilis* (BS-iDH, Sigma-Aldrich, 186 U/mL)
7. D/L-isocitrate (Sigma-Aldrich, 100-mM stock solution into reaction buffer)

8. $MgCl_2$ (Sigma-Aldrich, 500-mM stock solution into reaction buffer)
9. Catalase from bovine liver (Sigma-Aldrich, stock solution at 50 U/mL into reaction buffer)
10. Purified A13-Red (11.6-μM solution into Buffer B)
11. Dodecane (Sigma-Aldrich) 400-mM solution into absolute ethanol (Sigma-Aldrich)

3 Methods

The expression vector was designed for generating a fusion between the respective genes encoding for a 6×His-tag (N-Term), CYP153A13a from *A. borkumensis* SK2 and the P450 reductase domain (RhFred) of P450 RhF from *Rhodococcus* sp. NCIMB 9784 (C-Term) (Fig. 1) [22]. This construction enables the expression of A13-Red protein which can be captured by

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        10         20         30         40         50         60
MGSSHHHHHH SSGLVPRGSH MMSTSSSTSN DIQAKIINAT SKVVPMHLQI KALKNLMKVK
        70         80         90        100        110        120
RKTIGTSRPQ VHFVETDLPD VNDLAIEDID TSNPFLYRQG KANAYFKRLR DEAPVHYQKN
       130        140        150        160        170        180
SAFGPFWSVT RYEDIVFVDK SHDLFSAEPQ IILGDPPEGL SVEMFIAMDP PKHDVQRRAV
       190        200        210        220        230        240
QGVVAPKNLK EMEGLIRKRT GDVLDSLPLD TPFNWVPVVS KELTGRMLAS LLDFPYDERE
       250        260        270        280        290        300
KLVGWSDRLS GASSATGGEF TNEDVFFDDA ADMAWAFSKL WRDKEARQKA GEEPGFDLIS
       310        320        330        340        350        360
MLQSNEDTKD LINRPLEFIG NLALLIVGGN DTTRNSMSGG VLALNQFPEQ FEKLKANPKL
       370        380        390        400        410        420
IPNWSLKYSL ATPLAYMRRV AKQDVELNGQ TIKKGDRVLM WYASGNQDER KFENPEQFII
       430        440        450        460        470        480
DRKDTRNHVS FGYGVHRCMG NRLAELQLRI LWEELLPRFE NIEVIGEPER VQSNFVRGYS
       490        500        510        520        530        540
KMMVKLTAKK EFVLHRHQPV TIGEPAARAV SRTVTVERLD RIADDVLRLV LRDAGGKTLP
       550        560        570        580        590        600
TWTPGAHIDL DLGALSRQYS LCGAPDAPSY EIAVHLDPES RGGSRYIHEQ LEVGSPLRMR
       610        620        630        640        650        660
GPRNHFALDP GAEHYVFVAG GIGITPVLAM ADHARARGWS YELHYCGRNR SGMAYLERVA
       670        680        690        700        710        720
GHGDRAALHV SEEGTRIDLA ALLAEPAPGV QIYACGPGRL LAGLEDASRN WPDGALHVEH
       730        740        750        760        770        780
FTSSLAALDP DVEHAFDLEL RDSGLTVRVE PTQTVLDALR ANNIDVPSDC EEGLCGSCEV
       790        800        810        820
AVLDGEVDHR DTVLTKAERA ANRQMMTCCS RACGDRLALR L
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Fig. 1 Complete protein sequence of A13-Red. The construction was designed to obtain a 6×His-tag (residues from 5 to 10) merged with CYP153A13a from *A. borkumensis* SK2 (residues from 21 to 490) and the P450 reductase domain (RhFred) of P450 RhF from *Rhodococcus* sp. NCIMB 9784 (residues from 509 to 821). P450 and reductase domains are separated by the native peptidic linker (residues from 491 to 508) found in P450 RhF from *Rhodococcus* sp. NCIMB 9784. The *underlined sequence* corresponds to a characteristic peptide identified by nanoLC/MS/MS analysis

immobilized metal affinity chromatography (IMAC). After high-cell-density cultivation, cells of BL21 Star™ (DE3)/pA13-Red can be harvested by centrifugation and frozen at −20°C until the purification process described below.

3.1 Construction of pA13-Red

Construction of pA13-Red was performed in three steps. The first step is the cloning of *rhfred* gene (accession number AAM67416) into pET28b(+) between *EcoRI* and *NotI* to yield the intermediate plasmid pETRed. The second step is the amplification of *cyp153a13a* gene (accession number AY505118) from *A. borkumensis* SK2 genomic DNA and its modification in order to remove the *EcoRI* restriction site. Finally, the third step is the cloning of this modified gene into pETRed to yield pA13-Red.

(a) Step 1: Cloning of *rhfred* gene into pET28b(+)

1. The RhFred part of the construction should be amplified using RhFred-fw and RhFred-rv primers. The PCR reaction conditions are 95°C for 5 min; 30 cycles of 95°C for 20 s, 60°C for 10 s, and 70°C for 30s; and a final extension at 72°C for 5 min. The final reaction volume is 50 μL.
2. Purify the PCR product using the Qiagen cleanup kit.
3. Digest the PCR fragment with *EcoRI* and *NotI* enzymes for 2 h at 37°C followed by gel extraction or PCR cleanup.
4. Clone the digested RhFred insert into pET28b(+) plasmid previously digested by *EcoRI* and *NotI* enzymes by standard ligation procedure and transform it into *E. coli* DH5α competent cells following manufacturer's protocol.
5. The success of the cloning should be verified by colony PCR using RhFred-fw and RhFred-rv primers and sequencing.
6. Prepare plasmid pETRed using Qiagen MiniPrep kit and following manufacturer's instructions for cultivation.

(b) Step 2: Amplification and modification of *cyp153a13a* gene

1. Freeze-dried sample of *A. borkumensis* SK2 (DSM 11573) is reactivated following provider instructions and cultivated at 28°C into Medium 809 for 48 h.
2. After cultivation, cells are harvested by centrifugation and genomic DNA is extracted using Qiagen Gentra PureGene kit and following manufacturer's instructions.
3. For removal of *EcoRI* site at position 887 of CYP153A13a, overlap extension strategy is performed. Extracted genomic DNA was used as a template for fragment A and fragment B amplifications. Fragment A is amplified using 153A13a-fw and Eco887-rv primers. The PCR reaction conditions are 95°C for 2 min; 30 cycles of 95°C for 20 s, 50°C for 10 s, and 70°C for 20 s; and a final extension at 72°C for 5 min. Fragment B is amplified using Eco887-fw and 153A13a-rv

primers under the same PCR conditions. The final reaction volumes are both 50 μL.

4. The crude PCR mixtures are digested by *DpnI* and PCR products purified with Qiagen PCR Purification Cleanup kit.
5. Fragments A and B are reassembled using 153A13a-fw and 153A13a-rv primers. The PCR reaction conditions are 95°C for 2 min; 30 cycles of 95°C for 20 s, 50°C for 10 s, and 70°C for 30 s; and a final extension at 72°C for 5 min. The final reaction volume is 50 μL.
6. Purify the PCR product using the Qiagen cleanup kit.

(c) Step 3: Cloning of *cyp153A13a* gene into pETRed

1. Digest the PCR fragment with *NdeI* and *EcoRI* enzymes for 2 h at 37°C followed by gel extraction or PCR cleanup.
2. Clone the digested CYPA53A13a insert into pETRed plasmid previously digested by *NdeI* and *EcoRI* enzymes by standard ligation procedure and transform it into *E. coli* DH5α competent cells.
3. The success of the cloning should be verified by colony PCR using 153A13a-fw and RhFred-rv primers and sequencing.
4. Prepare plasmid pA13-Red using Qiagen MiniPrep kit and following manufacturer's instructions for cultivation (*see* **Note 1**).
5. Transform pA13-Red into BL21 Star™ (DE3) competent cells following manufacturer's protocol (*see* **Note 2**).

3.2 Purification of A13-Red on Nickel-NTA Column

1. Thoroughly resuspend the cell pellet in cold (4°C) lysis buffer (use approximately 2 mL for 1 g of pellet).
2. Incubate at 4°C for 30 min.
3. Centrifuge at 10,000 rpm, 30 min, 4°C.
4. Filter the supernatant through a 0.45-μm membrane.
5. Before loading, equilibrate the HisPrep FF 16/10 column with 10 column volumes (CV) of binding buffer.
6. Load the cell-free extract on the column (2.5 mL/min).
7. Wash the column with binding buffer (5 mL/min) until optical density at 280 nm (OD280) reaches baseline.
8. Wash the column with washing buffer (5 mL/min) until OD280 reaches baseline.
9. Elute the protein with elution buffer (2.5 mL/min). Collect 1-mL fractions and pool protein containing fractions.
10. Analyze the captured A13-Red by SDS-PAGE (Fig. 2) (*see* **Note 3**).
11. Elution buffer can be exchanged by ultrafiltration against conservation buffer and samples stored at −20°C.

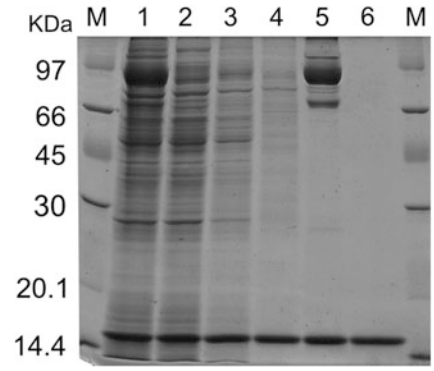

Fig. 2 SDS-PAGE (12% polyacrylamide) analysis of ion metal affinity chromatography fractions. The soluble fraction after lysis (*1*) was applied to HisPrep 16/10 column at a 2.5 mL/min flow rate. The flowthrough fraction containing unbound proteins (*2*) was collected. Two steps of washing were performed: the first one is carried out with binding buffer and the second one with washing buffer. Both fractions (respectively, *3* and *4*) were collected. Elution of A13-Red was performed using histidine (*5*). A final elution with 2-M imidazole (*6*) was performed as a control

3.3 Desalting of A13-Red Fractions After Ni-NTA Column

After Ni-NTA capture, A13-Red samples are contained into a salt-rich buffer (elution buffer). Prior to ion exchange chromatography, this salt-rich buffer has to be exchanged against desalting buffer for an optimal interaction between A13-Red and the stationary phase:

1. Before loading, two HiTrap desalting columns of 5 mL are connected in series and equilibrated with 10 CV of desalting buffer (*see* **Note 4**).
2. Load 1 mL of Ni-NTA purified A13-Red (1 mL/min).
3. Elute the protein with desalting buffer (1 mL/min). Collect 0.5-mL fractions; pool those containing the protein and having the desired conductivity.

3.4 Purification of A13-Red on Q Sepharose XL Column

A second purification step for removing the 70-kDa impurity was set up based on ion exchange. Since A13-Red has a calculated pI of 6.01, Q Sepharose stationary phase (strong cation) was preferred (*see* **Note 5**). The optimized protocol is described below:

1. Before loading, equilibrate the Q Sepharose XL 1-mL pre-packed column with Buffer A (10 CV).
2. Load the desalted A13-Red sample (up to 25 mL, 1 mL/min).
3. Wash the column with Buffer A (1 mL/min) until OD280 reaches baseline.
4. Elute A13-Red with Buffer B (1 mL/min). Collect 1-mL fractions and pool A13-Red containing fractions.

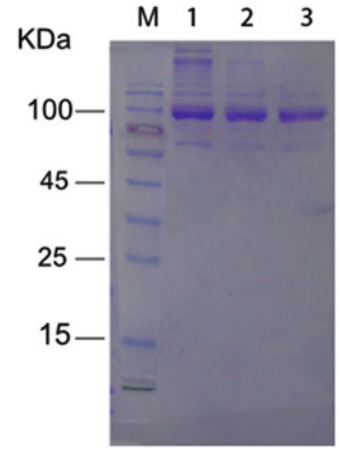

Fig. 3 SDS-PAGE (12% polyacrylamide) analysis after ion exchange and size exclusion chromatography. Lane 1: A13-Red sample after Q Sepharose XL (strong cation) chromatography. Lanes 2 and 3: A13-Red samples after Superdex 75 16/60 chromatography (not described in this chapter)

5. Analyze the purified A13-Red by SDS-PAGE (Fig. 3) (*see* **Notes 6** and **7**).
6. Store the protein at 4°C. For prolonged storage (more than 1 week), it is preferable to store A13-Red in small aliquots at −80°C.

A13-Red from this two-step purification protocol can be used without further purification in hydroxylation reactions.

3.5 Enzymatic Hydroxylation of n-Octane

Every reaction was performed into Wheaton 5-mL V-Vials at 25°C under gentle magnetic stirring, repeated thrice, and errors were estimated to be 5–10%. The protocol described below is optimized for a 1-mL total reaction volume (aqueous phase 500 μL/organic phase 500 μL).

1. Add 279 μL of reaction buffer into a Wheaton 5-mL V-Vial.
2. Add 50 μL of BSA 100 g/L (*see* **Note 8**).
3. Add 5 U (25 μL in our case) of BS-iDH stock solution (*see* **Note 9**).
4. Add 100 μL of D/L-isocitrate 100 mM.
5. Add 5 μL of $MgCl_2$ 500 mM.
6. Add 0.5 U of catalase stock solution (10 μL in our case) (*see* **Note 10**).
7. Add *n*-octane (500 μL) drop wise under gentle magnetic stirring.
8. Add 21 μL of A13-Red stock solution (final concentration 500 nM) and incubate for 1 min.

9. Add 10 μL of NADPH 25 mM to trigger the hydroxylation reaction.
10. Tightly screw the V-Vial cap and seal it with parafilm.

For analysis, the emulsified solution was transferred into a 1.5-mL tube and dodecane was added (5 μL, final concentration into the organic phase 4 mM, neglecting dodecane solubility into the aqueous phase). The sample was vortexed for 1 min and subjected to centrifugation (2 min, 10,000*g*, room temperature), and the organic layer was collected and analyzed by GC–MS (Shimadzu GC-2010 instrument) with an SLB-5MS column (30-m length; 0.25-mm diameter; Supelco). The injection and detection temperatures were both 250°C. For reactions with octane, the method used was an isotherm at 35°C for 5 min, followed by a temperature raise to 190°C at a rate of 10°C/min. The products were quantified by selected ion monitoring (SIM) program, and their concentrations were calculated from the corresponding area using dodecane as the internal standard (*see* **Note 11**).

4 Notes

1. The resulting plasmid pA13-Red and the strain BL21 Star™(DE3)/pA13-Red are freely available for academic and collaborative research under request.
2. Cultivation of microorganisms in flasks with a rich medium (LB, TB, or 2×YT) is the most simple approach, but important parameters for reproducibility such as medium composition, aeration, or pH cannot be controlled tightly. Expression of A13-Red in recombinant *E. coli* using this simple approach yielded in average 35 ± 25 mg of functional CYP per liter of culture with TB as described in references [22, 23]. We even witnessed unproductive cultivations. These large variations from batch to batch prompted us to develop a more reliable protocol to express A13-Red using high-cell-density cultivation (HCDC) with instrumented fermenters as described in details in reference [23]. Using this protocol, up to 5,000 nmol of functional A13-Red can be obtained per liter of HCDC.
3. IMAC can be sufficient to obtain A13-Red with 95% purity after flask cultivation, but it was not true for A13-Red produced by HCDC where only 70–75% purity was reached by performing IMAC only (Fig. 2). Since A13-Red is irreversibly inhibited by imidazole, elution was performed using histidine. One of the most abundant contaminating proteins (approx. 70 kDa) was identified to be CYP153A13a by acrylamide gel extraction and trypsin digestion of the extract followed by NanoLC/MS/MS analysis (Fig. 1). We assumed that a portion

of A13-Red was subjected to proteolysis into the cytoplasm of the bacteria during expression.

4. For scaling up the desalting step on A13-Red samples, up to five columns can be connected in series. For sample volumes up to 15 mL, HiPrep 26/10 Desalting is available. Up to four HiPrep 26/10 Desalting columns can be connected in series without increased backpressure (up to 60-mL sample volume).
5. A first series of experiments with a linear gradient of NaCl showed that the 70-kDa impurity was eluted at 80-mM NaCl while A13-Red at 200 mM.
6. High molecular weight impurities (above 200 kDa) still remained present after this step (Fig. 3). It is likely that these contaminants are polymers of A13-Red since their concentration progressively increased over time into the conservation buffer at −80°C into pure A13-Red samples. They can be eliminated by size exclusion chromatography using a Superdex 75 16/60 column (Fig. 3), but their presence had no significant impact on enzymatic hydroxylation activity.
7. For each purification step, functional P450 concentration is determined from the carbon monoxide difference spectra as previously described [24], using an extinction coefficient of 91 mM^{-1} cm^{-1} for the 450 minus 490-nm peak. Compared to the Bradford protein assay, CO-binding is a selective assay for P450. Moreover, it allows to determine the ration between functional (450 nm) and nonfunctional P450 (420 nm).
8. Stock solutions for the different additives should be prepared with reaction buffer (potassium phosphate buffer (100 mM, pH 7.4), glycerol 10%). For example, to prepare $MgCl_2$ 500-mM stock solution, solid $MgCl_2$ should be dissolved into reaction buffer.
9. By definition, for BS-iDH, one unit (U) corresponds to the amount of enzyme which converts 1-μmol D-isocitrate to α-ketoglutarate per minute at pH 7.5 and 37°C (NADP as cofactor).
10. By definition, one unit of catalase will decompose 1.0 μmole of H_2O_2 per min at pH 7.0 at 25°C, while the H_2O_2 concentration falls from 10.3 to 9.2 mM, measured by the rate of decrease of OD240.
11. Under these conditions, A13-Red remains active up to 78 h, and 1.6 mM of *n*-octanol is formed corresponding to a total turnover number about 3,250. No other regio-isomer or over-oxidation product was detected confirming the remarkable regio- and chemoselectivity of A13-Red.

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