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Bioprocess Engineering for Microbial Synthesis and Conversion of Isoprenoids

Hendrik Schewe, Marco Antonio Mirata and Jens Schrader

Abstract Isoprenoids represent a natural product class essential to living organisms. Moreover, industrially relevant isoprenoid molecules cover a wide range of products such as pharmaceuticals, flavors and fragrances, or even biofuels. Their often complex structure makes chemical synthesis a difficult and expensive task and extraction from natural sources is typically low yielding. This has led to intense research for biotechnological production of isoprenoids by microbial de novo synthesis or biotransformation. Here, metabolic engineering, including synthetic biology approaches, is the key technology to develop efficient production strains in the first place. Bioprocess engineering, particularly in situ product removal (ISPR), is the second essential technology for the development of industrial-scale bioprocesses. A number of elaborate bioreactor and ISPR designs have been published to target the problems of isoprenoid synthesis and conversion, such as toxicity and product inhibition. However, despite the many exciting applications of isoprenoids, research on isoprenoid-specific bioprocesses has mostly been, and still is, limited to small-scale proof-of-concept approaches. This review presents and categorizes different ISPR solutions for biotechnological isoprenoid production and also addresses the main challenges en route towards industrial application.

Keywords Bioprocess · In situ product removal · Isoprenoids · Solvent tolerance · Terpenoids

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1 Introduction

Isoprenoids (the terms "isoprenoid", "terpene", and "terpenoid" are used synonymously throughout this work) are a class of natural products present in all living organisms. The great diversity of structures accompanied by many varied biological functions lets isoprenoids play a vital role in basic intra- and intercellular processes, for example, as components of cell membranes, respiratory chains, photosynthetic light harvesting systems, or as hormones. All isoprenoids are derived from prenyl diphosphate precursors and consist of one or more C_5 isoprene units which are condensed to different isoprenoid classes (C_5: hemiterpenoids, C_{10}: monoterpenoids, C_{15}: sesquiterpenoids, C_{20}: diterpenoids, C_{25}: sesterterpenoids, C_{30}: triterpenoids, C_{40}: tetraterpenoids and $> C_{45}$: polyterpenoids). Terpenoids are major components of plant essential oils and extracts whose antiseptic and medicinal effects and in particular their sensory properties and value for food preservation have been used since the Middle Ages [8]. Nowadays, industrially relevant isoprenoid molecules cover a wide range of products such as flavors and fragrances [83], pharmaceuticals [20], and biofuels [66] corresponding to multibillion US$ markets.

The prime examples illustrating the tremendous progress made in isoprenoid biotechnology are the microbial de novo synthesis of farnesene as both fragrance and biofuel precursor [102], of amorphadiene and artemisinic acid as antimalaria drug artemisinin precursors [65, 103], and of isoprene as a chemical commodity and monomer for synthetic rubber synthesis [105]. Also, the plant cell culture based biosynthesis of precursors of the anticancer blockbuster drug Taxol [51] represents an important industrially applied isoprenoid biotechnology. Finally, in the area of

microbial isoprenoid conversion, a number of high-yielding approaches have been published as well, for example, for the biotransformations of limonene to perillyl alcohol [21, 22], to perillic acid [54], and to α-terpineol [9] or of α-pinene oxide to isonovalal [34], to name only a few. The reader is kindly referred to the respective chapters in the same volume series addressing the biotechnology of specific isoprenoid products.

Due to their often complex structure, chemical synthesis of isoprenoids is usually difficult and expensive, and isolation from their natural sources is also typically low yielding. In contrast, biotechnological strategies take advantage of the pronounced regio- and stereo selectivities of enzymatic reactions (usually by use of microbial cells) and the possibility of overproducing isoprenoids in tailored bioprocesses. One strategy is whole-cell biocatalysis, which is usually favored if structurally closely related precursor molecules are abundantly available, for example, the monoterpenes limonene or pinene for carvone or verbenone production, respectively [44, 46], and therefore represent cheap starting materials. The target products are often obtained by selective oxyfunctionalization reactions catalyzed by only one or a few enzymes, such as P450 monooxygenases and alcohol dehydrogenases. The other strategy follows the cell factory notion, that is, the de novo biosynthesis with microorganisms, whose metabolism has to be comprehensively re-engineered for isoprenoid production starting from inexpensive carbon sources, such as sugar [20]. Metabolic engineering typically involves optimization of the flux through isoprenoid biosynthetic pathways, that is, the methylerythritol phosphate or MEP pathway, also referred to as the 1-deoxy-D-xylulose 5-phosphate or DXP pathway, and the mevalonate (MVA) pathway, depending on the host strain or strategy used. Both pathways lead to the key C_5 isoprenoid intermediate isopentenyl diphosphate, from which isoprenoid synthesis is usually continued towards the final product via a cascade comprising prenyltransferase(s), a terpene synthase, and often further terpene decorating enzymes [19].

The question remains why, despite the many economically interesting applications of isoprenoids and the intrinsic advantages of enzymes as highly selective biological catalysts for isoprenoid formation, only few industrial-scale bioprocesses have been established to date. In addition to the obvious challenges associated with a comprehensive genetic reprogramming of cellular metabolism, research is also consistently hampered by a couple of physicochemical properties of isoprenoids. A recurring phenomenon in bioconversion or de novo synthesis of isoprenoids is the hydrophobicity and volatility of the substrate and/or product, and the fact that these properties are often accompanied by a pronounced cytotoxicity. However, hydrophobicity and volatility of isoprenoids can be exploited for developing selective in situ product removal (ISPR) techniques which are usually also mandatory if product toxicity is a major issue. Alternatives might be the improvement of microbial resilience by genetic engineering or the use of solvent-tolerant microbes. For this review chapter we compiled bioprocess engineering approaches for microbial (bacterial and fungal) synthesis and conversion of isoprenoids with special focus on technical solutions for ISPR. We present laboratory- and industrial-scale bioprocesses and discuss the major hurdles towards industrial implementation.

Microbial processes for the production of carotenoids and steroids as well as isoprenoid production by plant cell cultures and by engineering in vivo plant isoprenoid biosynthesis are outside the scope of this review.

1.1 In situ Product Removal (ISPR)

Considering the physicochemical and biochemical properties of isoprenoids (hydrophobicity, volatility, and toxicity, all of which are discussed in later chapters of this review), a successful biotechnological production of isoprenoids has to rely on both metabolic engineering and process engineering in order to overcome inhibitory (reversible loss of activity) or toxic (irreversible loss of activity) effects of isoprenoid precursors or products. Due to the characteristics of isoprenoids, bioprocess development needs to focus on the integration of ISPR techniques. ISPR techniques have already been thoroughly investigated since the early 1980s due to a recurring problem of bioprocessing: the productivity of biocatalytic processes is frequently limited by the need to operate the reaction under conditions unsuited to the biocatalyst [49, 89]. Although chemical synthesis can be relatively flexible in matters of reaction conditions, biological systems have evolved over hundreds of millions of years to function under relatively limited conditions, that is, in a comparably narrow "process window". Applying the same economical necessities that guide optimization of industrial chemical production processes to biological systems and force biocatalysts to function outside the environment for which they have evolved leads to compromises over bioreactor design and operation. Because inhibition of enzymatic reactions or microbial growth is usually observed at elevated product concentrations, dilute product streams and low productivities are characteristics of biotechnological processes [84, 89]. A technical approach to overcome the problem of low productivities is the application of ISPR for the immediate separation of a product from the producing biocatalyst [35]. The separation is achieved by compartmentalization of biocatalyst and substrate and/or product thereby increasing the productivity or yield of a biotechnological process by

- Overcoming inhibitory toxic effects of the product or the substrate
- Minimizing product loss due to degradation or uncontrolled release
- Reduction of liquid volume by concentration of the product in a separate phase
- Reducing the total number of downstream processing steps.

Although the compartmentalization displays the aforementioned beneficial effects it also introduces mass transfer fluxes of the product and/or substrate over interfaces thereby creating new possible bottlenecks influencing process productivity and yield. In order to minimize adverse effects by limited mass transfer the choice of the appropriate ISPR technique is as important as it is challenging.

Several techniques are available to separate inhibitory or toxic substances from biotechnological processes depending on their physicochemical properties. Molecular weight, hydrophobicity, volatility, charge, and specific binding properties of a

compound can help to decide the appropriate ISPR technique [35]. Five main techniques are available to remove a product from the vicinity of the biocatalyst [89]. Extraction into another phase makes use of differences in hydrophobicity of substrate or product and biocatalyst. The receiving second phase may be an organic solvent, a supercritical fluid, an ionic liquid, or another aqueous phase. ISPR by perstraction involves the separation of the extractive phase from the biocatalyst-containing phase by a membrane. High volatility favors evaporation as a separation technique. Evaporation is accomplished by stripping or vacuum distillation. Pervaporation and transmembrane distillation are applied as membrane-supported evaporation techniques. Separation by size is achieved by dialysis, electrodialysis, reverse osmosis, or nanofiltration. Depending on the charge of the target molecules, adsorption to hydrophobic carriers, ion-exchange resins and affinity adsorption can be used. Finally, a charged product may be precipitated by the addition of a counter-ion.

Figure 1 depicts possible configurations of bioreactor and separation units and different modes of operation for the five principal ISPR techniques described above.

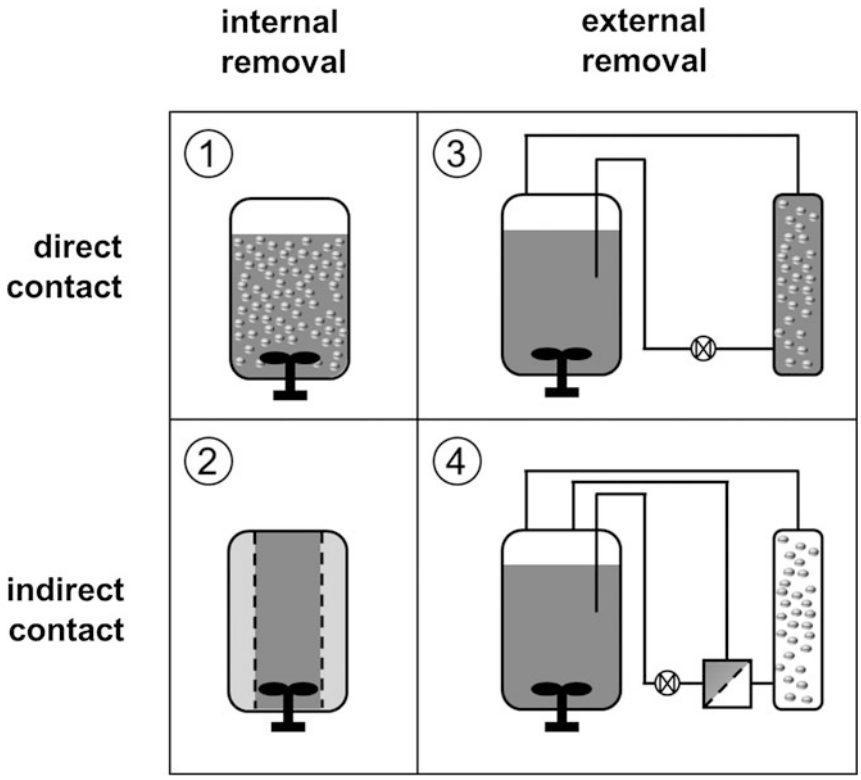

Fig. 1 Options of in situ product removal (ISPR) from a bioreactor. The ISPR can take place inside the reactor (internal) or in an external loop. The biocatalyst can be in direct contact with the extractive phase or may be separated by various techniques, such as a membrane (shown as *dotted lines*) or a gel matrix. The biocatalyst-containing phase is shown in *dark gray*, the extractive phase is shown in *light gray*. The complexity of the setup increases from 1 to 4. Modified after Woodley et al. [108]

The physicochemical properties of isoprenoid compounds are reflected in the chosen ISPR techniques listed in Tables 1 and 2. The hydrophobicity and volatility of isoprenoid compounds favor extraction, hydrophobic adsorption, or evaporation. Water solubilities of terpenes are generally low, with those for terpene hydrocarbons far lower than those for oxyfunctionalized terpenes. As an example, the saturation concentration of limonene in water at 25 °C is around 0.15 mM, that of carveol 19 mM [33]. The low water solubility of many terpenes and their high activity coefficients in dilute aqueous solution results in their excessive accumulation in the gas phase over the liquid phase, as compared to an ideal system according to Raoult's law. This leads to relatively quick stripping from an aqueous phase under typical bioreactor conditions. For instance, limonene is completely removed from an aqueous solution of 7.5 mg/L in a 3-L reactor, which is stirred at 1000 rpm and aerated with 0.9 L/min, within only 30 min [33]. This makes it comprehensible, that even when applying certain ISPR methods substrate and/or product loss may occur in significant amounts [15, 21, 64]. The following chapters give examples of application of ISPR techniques in bioprocess engineering for synthesis and conversion of isoprenoids using the categories depicted in Fig. 1.

2 Internal and External Removal of Isoprenoid Products by Direct Contact

Bioprocesses with a direct contact ISPR technique according to numbers 1 and 3 in Fig. 1 usually consist of a biocatalyst-containing aqueous phase and an immiscible second phase. The second phase may be located inside the bioreactor (internal removal) or in an external loop (external removal). The second phase functions typically as either a product sink removing potentially inhibitory products from the aqueous phase or a substrate reservoir delivering substrates at low concentrations to the aqueous phase. This combination of substrate release and product removal is often referred to as substrate feeding and product removal (SFPR) and can be seen as a method to avoid the toxic effect of substrate and product. The second phase has a larger affinity for the target compounds than the aqueous phase, allowing in the case of substrate delivery the loading of large amounts of poorly water-soluble substrate into the second phase. This extractive bioprocess concept is often referred to as a two-phase partitioning bioreactor (TPPB) [24, 52]. In two-liquid phase partitioning bioreactors for microbial processes the extractive second phase consists of a hydrophobic liquid phase [28]. As an alternative to a liquid second phase a solid extractive phase (e.g., a solid adsorbent material) can be used. Whether a liquid or solid phase is used, the fundamental intention is to enhance the mass transfer rate of apolar toxic compounds with low bioavailability and to control their delivery or removal. The considerations involved in the selection of an appropriate extractive phase, and the advantages and drawbacks of the application of a liquid or a solid phase are discussed in the next two sections.

Table 1 Bioprocesses for synthesis and conversion of isoprenoids with internal and external in situ product removal by direct contact using aqueous–organic or aqueous–solid two-phase systems. Only literature describing cultivations in bioreactors is cited

ISPR details	Organism	Substrate	Product	t (h)	V_{tot} (L)	Cell condition	X (g L^{-1})	c (g L^{-1})	$Y_{P/X}$ (g g^{-1})	STY (mg L^{-1} h^{-1})	Reference
Internal SFPR STR extractive phase hexadecane (log P_{Oct} = 8.9) V_{Aq} = 0.5 L V_{Org} = 0.5 L	*Pseudomonas rhodesiae CIP* 107491	α-pinene oxide	Isonovalal	20	1	Permeabilized	–	266	–	13,300	Fontanille and Larroche [34]
Internal SFPR STR extractive phase sunflower oil V_{Aq} = 0.15 L V_{Org} = 0.15 L	*Sphingobium* sp.	Limonene	α-terpineol	144	0.3	Resting	4.6	23	5	160	Bicas et al. [9]
Internal SFPR STR extractive phase bis (2-ethylhexyl) phthalate (log P_{Oct} = 7.5) V_{Aq} = 0.5 L V_{Org} = 1 L	Recombinant *Pseudomonas putida* KT2440	Limonene	Perillyl alcohol	24	1.5	Growing	20.1	4.37	0.22	182	Cornelissen et al. [22]
Internal SFPR STR extractive phase bis (2-ethylhexyl) phthalate (log P_{Oct} = 7.5) V_{Aq} = 0.5 L V_{Org} = 1 L	Recombinant *Escherichia coli*	Limonene	Perillyl alcohol	26	1.5	Growing	29.3	5.96	0.20	229	Cornelissen et al. [21]

(continued)

Table 1 (continued)

ISPR details	Organism	Substrate	Product	t (h)	V_{tot} (L)	Cell condition	X (g L^{-1})	c (g L^{-1})	$Y_{P/X}$ (g g^{-1})	STY (mg L^{-1} h^{-1})	Reference
Internal SFPR STR extractive phase bis (2-ethylhexyl) phthalate (log P_{Oct} = 7.5) V_{Aq} = 1 L V_{Org} = 0.55 L	Recombinant *Pseudomonas putida*	Limonene	Perillyl alcohol	75	1.55	Growing	15	2.3	0.15	31	van Beilen et al. [97]
Internal SFPR STR extractive phase diisononylphthalate (log P_{Oct} = 9.4) V_{Aq} = 0.15 L V_{Org} = 0.1 L	Recombinant *Escherichia coli*	α-pinene	α-pinene oxide verbenol myrtenol	5	0.25	Growing	10	0.61	0.061	122	Schewe et al. [80]
Internal SFPR STR extractive phase silicone oil V_{Aq} = 3.1 L V_{Org} = 0.5 L	*Rhodococcus erythropolis* DCL14	*Trans*-carveol	Carvone	28.75	3.6	Growing	0.5	0.823	1.65	29	Morrish et al. [56]
Internal SFPR STR extractive phase hexadecane (log P_{Oct} = 8.9) V_{Aq} = 1. 5 L V_{Org} = 0.15 L	*Saccharomyces cerevisiae*	Geraniol	Citronellol	60	1.65	Resting	–	4.9	–	82	Valadez-Blanco et al. [96]

(continued)

Table 1 (continued)

ISPR details	Organism	Substrate	Product	t (h)	V_{tot} (L)	Cell condition	X (g L^{-1})	c (g L^{-1})	$Y_{P/X}$ (g g^{-1})	STY (mg L^{-1} h^{-1})	Reference
Internal SFPR STR extractive phase hexadecane (log P_{Oct} = 8.9) V_{Aq} = 0.4 L V_{Org} = 0.4 L	*Pseudomonas rhodesiae* CIP107491	α-pinene oxide	Novalic acid	8	0.8	Resting	7	8	1.14	1,000	Linares et al. [48]
Internal SFPR STR extractive phase hexadecane (log P_{Oct} = 8.9) V_{Aq} = 1.6 L V_{Org} = 0.1 L	Recombinant *Saccharomyces cerevisiae*	Geraniol	Citronellol	11.5	1.7	Resting	6.25	0.176	0.028	15	Doig et al. [29]
Internal SFPR air-driven column reactor extractive phase dodecane (log P_{Oct} = 6.8) V_{Aq} = 0.07 L V_{Org} = 0.04 L	*Rhodococcus erythropolis* DCL14	*Trans*-carveol	Carvone	460	0.11	Growing	–	38.9	–	85	de Carvalho and da Fonseca [27]
Internal extraction by dodecane (log P_{Oct} = 6.8) STR V_{Aq} = 1.2 L V_{Org} = 0.12 L	Recombinant *Pichia pastoris*	De novo synthesis; glucose, methanol	Nootkatone	108	1.32	Growing	100	0.187	0.002	1.7	Wriessnegger et al. [109]

(continued)

Table 1 (continued)

ISPR details	Organism	Substrate	Product	t (h)	V_{tot} (L)	Cell condition	X (g L^{-1})	c (g L^{-1})	$Y_{P/X}$ (g g^{-1})	STY (mg L^{-1} h^{-1})	Reference
Internal extraction by dodecane (log P_{Oct} = 6.8) STR V_{Aq} = 1.2 L V_{Org} = 0.3 L	Recombinant *Escherichia coli*	De novo synthesis; LB-medium	Taxadiene	120	1.5	Growing	19	0.095	0.005	0.8	Boghigian et al. [12]
Internal extraction by dodecane (log P_{Oct} = 6.8) STR V_{Aq} = 1 L V_{Org} = 0.1 L	Recombinant *Escherichia coli*	De novo synthesis; glycerol, yeast extract	Sclareol	48	1.1	Growing	60	1.46	0.024	30	Schalk et al. [79]
Internal extraction by Diisononylphthalate (log P_{Oct} = 9.4) STR V_{Aq} = 1 L V_{Org} = 0.5 L	Recombinant *Escherichia coli*	De novo synthesis; glucose	Limonene	45	1.5	Growing	61.8	0.9	0.015	20	Willrodt et al. [107]
Internal extraction by dodecane (log P_{Oct} = 6.8) STR V_{Aq} = 6 L V_{Org} = 0.6 L	Recombinant *Escherichia coli*	De novo synthesis; TB-medium	Amorpha-4,11-diene	60	6.6	Growing	5.4	0.48	0.09	8	Newman et al. [60]
Internal extraction by dodecane (log P_{Oct} = 6.8) STR V_{Aq} = 1 L V_{Org} = 0.1 L	Recombinant *Saccharomyces cerevisiae*	De novo synthesis; glucose	α-santalene	62	1.1	Growing	–	0.0072	–	1.16×10^{-4}	Scalcinati et al. [77]

(continued)

Table 1 (continued)

ISPR details	Organism	Substrate	Product	t (h)	V_{tot} (L)	Cell condition	X (g L^{-1})	c (g L^{-1})	$Y_{P/X}$ (g g^{-1})	STY (mg L^{-1} h^{-1})	Reference
Internal extraction by dodecane (log P_{Oct} = 6.8) STR V_{Aq} = 1 L V_{Org} = 0.2 L	Recombinant *Escherichia coli*	De novo synthesis; complex medium	Taxadiene	120	1.2	Growing	10	0.17	0.017	1.4	Ajikumar et al. [1]
Internal extraction by dodecane (log P_{Oct} = 6.8) STR V_{Aq} = 1 L V_{Org} = 0.1 L	Recombinant *Escherichia coli*	De novo synthesis; glucose	Amorpha-4,11-diene	140	1.1	Growing	90	27.4	0.3	196	Tsuruta et al. [94]
Internal extraction by dodecane (log P_{Oct} = 6.8) STR V_{Aq} = 4 L V_{Org} = 0.3 L	Recombinant *Saccharomyces cerevisiae*	De novo synthesis; galactose	Cubebol	76	4.3	Growing	10	5.86×10^{-4}	5.86×10^{-5}	7.7×10^{-6}	Asadollahi et al. [7]
Internal extraction by methyl oleate (log P_{Oct} = 7.6) STR V_{Aq} = 1.8 L V_{Org} = 0.2 L	Recombinant *Saccharomyces cerevisiae*	De novo synthesis; ethanol	Amorpha-4,11-diene	116	2	Growing	–	41	–	353	Westfall et al. [103]
Internal extraction by isopropyl myristate (log P_{Oct} = 7.4) STR V_{Aq} = 0.8 L V_{Org} = 0.4 L	Recombinant *Saccharomyces cerevisiae*	De novo synthesis; ethanol	Artemisinic acid	140	1.2	Growing	–	25	–	179	Paddon et al. [65]

(continued)

Table 1 (continued)

ISPR details	Organism	Substrate	Product	t (h)	V_{tot} (L)	Cell condition	X (g L^{-1})	c (g L^{-1})	$Y_{P/X}$ (g g^{-1})	STY (mg L^{-1} h^{-1})	Reference
Internal SFPR, STR adsorption to hydrophobic polymer	*Corynespora cassiicola* DSM 62475	Racemic linanool	Linalool oxides	120	0.5	Growing	n.d.	4.6	–	38	Bormann et al. [15]
Internal SFPR, STR adsorption to hydrophobic polymer	*Rhodococcus erythropolis* DCL14	*Trans*-carveol	Carvone	48.75	3.83	Growing	0.7	4.83	6.9	99	Morrish and Daugulis [57]
External SFPR, STR adsorption to anion exchange polymer	*Pseudomonas putida* DSM 12264	Limonene	Perillic acid	168	0.16	Growing	9	31	3.44	185	Mirata et al. [54]
Internal SFPR, Pad-packed Interface bioreactor extractive phase decane (log P_{Oct} = 5.8)	*Hansenula saturnus* IF0 0809	Racemic citronellol	Citronellic acid	288	1.1	Biofilm	–	18	–	62.5	Oda et al. [61]
Internal SFPR, Multistory interface bioreactor extractive phase decane (log P_{Oct} = 5.8)	*Pichia kluyveri* IF0 1165	Racemic citronellol	Citronellyl acetate	432	7	Biofilm	–	14.5	–	33.6	Oda et al. [62]

t reaction time; V_{tot} reaction volume (organic and aqueous phase); X cell dry weight concentration; c product concentration in relation to total volume; $Y_{P/X}$ product yield coefficient; *STY* space time yield; *ISPR* in situ product removal; *SFPR* substrate feeding and product removal; *STR* Stirred tank reactor

Table 2 Bioprocesses for synthesis and conversion of isoprenoids with internal and external in situ product removal by indirect contact. Only literature describing cultivations in bioreactors is cited

ISPR details	Organism	Substrate	Product	t (h)	V_{tot} (L)	Cell condition	X (g L^{-1})	c (g L^{-1})	$Y_{P/X}$ (g g^{-1})	STY (mg L^{-1} h^{-1})	Reference
Stripping, absorption into hexadecane STR	*Saccharomyces cerevisiae*	Geraniol	Citronellol	45	5.2	Resting	10	0.09	0.009	2	Arifin et al. [4]
Stripping, absorption into hexadecane STR	*Saccharomyces cerevisiae*	Geraniol	Citronellol	36	5.2	Growing	3	0.045	0.015	1	Arifin et al. [5]
Stripping, adsorption to hydrophobic polymer STR	*Pleurotus ostreatus* DSMZ 1020	β-myrcene	Perillene	240	1.8	Growing	210 (wet biomass)	0.08		0.3	Krings and Berger [45]
Stripping, adsorption to hydrophobic polymer STR	*Streptomyces citreus* CBS 109.60	De novo synthesis; complex medium	Geosmin	4	2.5	Growing	2	2.6×10^{-3}	1.3×10^{-3}	0.7	Pollak and Berger [68]
Stripping, adsorption to hydrophobic polymer STR	*Cystoderma carcharias* CBS 157.47	Citronellol	3,7-dimethyl-1,6,7-octanetriol	240	2	Growing	15	0.866	0.058	3.6	Onken and Berger [64]
Pervaporation STR	*Ceratocystis moniliformis* ATCC 12861	De novo synthesis; glucose, potato–dextrose broth	Geraniol citronellol	260	3.5	Growing	2	0.04 0.02	0.02 0.01	0.2 0.1	Bluemke and Schrader [11]

(continued)

Table 2 (continued)

ISPR details	Organism	Substrate	Product	t (h)	V_{tot} (L)	Cell condition	X (g L^{-1})	c (g L^{-1})	$Y_{P/X}$ (g g^{-1})	STY (mg L^{-1} h^{-1})	Reference
Perstraction in membrane reactor, Extractive phase hexadecane STR	*Pseudomonas fluorescens* NCIMB 11671	α-pinene oxide	Isonovalal	380	2.4	Resting	60	45	0.75	118	Boontawan and Stuckey [14]
Perstraction in membrane reactor, Extractive phase hexadecane STR	*Saccharomyces cerevisiae*	Geraniol	Citronellol	170	0.66	Resting	–	4.25	–	25	Valadez-Blanco et al. [96]
Perstraction in membrane reactor, Extractive phase hexadecane STR	*Saccharomyces cerevisiae*	Geraniol	Citronellol	11.5	1.7	Resting	6.25	0.141	0.022	12	Doig et al. [29]

2.1 Two-Liquid Phase Bioprocesses

In two-liquid phase bioprocesses a biphasic reaction medium is created by the addition of a solvent with preferably low water-solubility to an aqueous phase containing the biocatalyst. Biphasic systems assure compartmentalization, thus avoiding inhibitory (reversible loss of activity) or toxic effects (irreversible loss of activity) on the biocatalyst and minimizing substrate or product loss by volatilization. The stirred-tank reactor is by far the most popular reactor type used in isoprenoid bioprocesses. Out of the 36 isoprenoid bioprocesses with ISPR presented in this review 33 used stirred-tank reactors (c.f. Tables 1 and 2). Another reactor type is the airlift or bubble column reactor where interface mixing is accomplished by gas bubbles [27]. Bioconversion or de novo synthesis of isoprenoids in liquid-impelled loop reactors [98] have not yet been described for isoprenoid bioprocesses. In a stirred-tank reactor the interfacial area available for mass transfer between the two immiscible phases is easily regulated via agitator speed [50]. The two phases form a fine emulsion under operational conditions. The average droplet diameter depends, among other parameters, on agitator speed, resulting in a direct relation of total specific surface area and agitator speed [99]. However, because the extractive phase needs to be hydrophobic in nature in order to be able to extract a hydrophobic product or deliver a hydrophobic substrate, it may convey toxic effects to the biocatalyst. Hence, biocompatibility is one of the criteria for the selection of a suitable organic phase. The toxic effect of an organic solvent or its biocompatibility can be correlated with its log P_{Oct} [47]. The log P_{Oct} value describes the logarithm of the partition coefficient of an organic solvent in a standard octanol–water system. Organic solvents with log P_{Oct} values greater than 4 are usually suitable for direct contact with biocatalysts whereas lower log P_{Oct} values generally result in loss of activity [18]. The log P_{Oct} value helps to limit the screening effort by reducing the amount of possible organic solvents to a manageable number. Sikkema and colleagues described why relatively low aqueous concentrations of hydrocarbons can have a devastating effect on cell membranes [87]. In their studies with liposomes prepared from *Escherichia coli* phospholipids a correlation between the membrane–buffer partition coefficients and the octanol–water partition coefficients (log P_{Oct}) of several hydrocarbons was found. According to this correlation, the sparingly water-soluble monoterpene limonene (log P_{Oct} = 4.46, water solubility 0.15 mmol/L) would partition into the cell membrane and accumulate to a membrane concentration of up to 728 mmol/L (99 g/L). Consequences of hydrocarbon accumulation into and interaction with the cell envelope, the entity of cell membranes and cell wall, are cell membrane swelling, destruction of proton motive force, alterations in enzyme activities, and loss of cell membrane or cell wall integrity or function [17, 28, 88].

In addition to biocompatibility, other desirable criteria for an organic solvent as extractive phase have been identified and phase volume ratio (organic volume/aqueous volume) effects have to be considered (Table 3). The phase volume ratio affects the amounts of water-soluble and poorly water-soluble organic components present in the bioreactor, the degree of reduction or elimination of substrate or product

Table 3 Desirable characteristics, selection criteria, and phase volume ratio considerations of an organic phase in two-liquid phase bioprocesses [18, 50]

Desirable solvent characteristics
Favorable distribution coefficient for product
High selectivity
Low emulsion-forming tendency
Low aqueous solubility
Chemical and thermal stability
Favorable properties for product recovery
Nonbiodegradability
Nonhazardous
Inexpensive
Available in bulk quantities
Biocompatibility
Factors to consider
Solubility limit of S and P in organic phase
Solvent recycling options
Environmental impact
Phase volume ratio effects
Ease of pH control
Nature of dispersed phase
Possibility of phase inversion
Ease of phase separation
Interfacial area available for mass transfer
Inter-droplet mixing

inhibition, and the absolute and specific interfacial areas available for mass transport [50]. The most common approach is to operate with the aqueous phase as the continuous phase with a phase volume ratio typically less than 0.3–0.4 (c.f. Table 1).

A couple of publications illustrate the challenges involved with solvent screening following the desired properties listed in Table 3. Usually a number of organic solvents with appropriate log P_{Oct} values are compiled and experiments conducted to determine biocompatibility, partition coefficients for substrate, and/or product and biodegradability [6, 22, 37, 56, 80]. To test for biocompatibility an activity profile can be created by plotting an organic solvent log P_{Oct} against biocatalyst activity. However, this activity profile is specific to a particular biocatalyst and the extent of its contact with the organic phase [50]. The extent of contact depends on many process-specific factors such as available interfacial area, power input by mixing or shaking, concentration of surface-active components, concentration of biocatalyst, and the amount of organic solvent, to name a few. These factors may change during the bioprocess or with bioprocess scale. Therefore, the selection of a suitable organic solvent remains empirical and is often limited to the investigated setup. For example, using recombinant *E. coli* for the biotransformation of α-pinene (log P_{Oct} = 4.5) presented as a separate organic phase in shaking flasks resulted in product formation [81]. In contrast, the application of stirred-tank bioreactors using the same biocatalyst, aqueous medium, and phase volume ratio led to a complete loss of biocatalyst activity due to extended contact of the biocatalyst with α-pinene. Activity could be regained by the use of a different organic phase with a higher log P_{Oct} value [80] as a reservoir for α-pinene, "masking" its detrimental effects.

Searching for a suitable organic phase for the biotransformation of carveol to carvone with *Rhodococcus erythropolis* DCL14, Morrish and colleagues determined the critical log P_{Oct} of *R. erythropolis* DCL14 by exposing the cells to a selection of 12 organic solvents with log P_{Oct} ranging from 1.25 to 9.04 [56]. The critical log P_{Oct} of *R. erythropolis* DCL14 was found to be 5 in shake flask experiments. After further shake flask studies on biodegradability and determination of partition coefficients three organic solvents remained for bioreactor testing. Bioconversions were now tested in a stirred-tank reactor at a phase volume ratio of 0.16. In the end silicone oil proved to be the most suitable solvent although 1-dodecene showed superior affinity for the product. In contrast to experimental data from shaking flask experiments strong emulsion formation was observed when 1-dodecene was used as the organic phase in the stirred-tank reactor. The aforementioned examples illustrate the setup-specific nature of solvent screening experiments. Screening at smaller scales is less laborious and time consuming, however, the results are often not transferable to larger scale, especially if shake flask experiments are intended to optimize aqueous–organic two-liquid phase bioprocesses. Here, parallel fermentation systems are far superior as they are run under realistic process conditions at small scale.

The formation of emulsions, although in some cases advantageous for creation of a large interfacial area, may create significant problems in separation of the organic phase for further product removal; furthermore, organic phase or cell recycling represents an important practical issue in downstream processing of industrial-scale bioprocesses [39]. Surface-active components, often produced by microorganisms themselves as a protective mechanism or to improve substrate availability, impede phase separation and promote the formation of stable emulsions. Phenomena associated with stable emulsions in isoprene-producing bioprocesses were described as difficulties in determination of cell, substrate, or product concentration [56]; formation of a third phase partitioning of cell mass in the organic phase [26]; or problems in recovery of organic solvent and product [96]. The tendency for emulsion formation can change with phase volume ratio. A phase volume ratio of 0.2 led to a complete emulsification of dodecane in a stirred-tank reactor bioconversion of carveol to carvone by *R. erythropolis* whereas at a phase volume ratio of 0.4 half of the solvent was emulsified [26]. Although cell migration into the organic phase and problems in phase separation were reported, the bioprocess with complete emulsification of the organic phase yielded the highest carvone production rates compared to other configurations such as a membrane reactor.

Although strong emulsion formation of hexadecane with the aqueous phase during the reduction of geraniol to citronellol by *Saccharomyces cerevisiae* caused the authors to eventually employ a membrane reactor (indirect contact, c.f. Fig. 1) which actually eliminated emulsification, the space–time yield and product concentration was higher when the direct contact ISPR technique in a stirred-tank reactor was used [29, 96]. Furthermore, the spontaneous formation of an oil-in-water emulsion was found advantageous in an aqueous–organic two-phase bioprocess for isonovalal production from α-pinene oxide by *Pseudomonas rhodesiae*

[34]. Considering that emulsion droplet sizes in aqueous–organic two-phase systems in stirred-tank reactors can be as small as 10 μm resulting in organic–aqueous interfacial areas of 130 m^2 dm^{-3} [82], mass transfer limited reactions can profit from emulsion formation. However, droplet-stabilizing surface-active components such as fermentation broth, proteins, biomass, and biosurfactants can also considerably reduce the overall mass transfer coefficient by adsorbing to the aqueous–organic interface thereby damping turbulence transfer across the interface or, in the case of biomass, probably by blockage of the available interfacial area by multilayers of biomass [69]. These examples demonstrate the mixed blessing of emulsion formation in aqueous–organic two-phase processes: that is, increased mass transfer rates versus problems in phase separation and product or cell recovery.

Additional aspects of solvent selection may involve the physicochemical nature of the organic phase. Vegetable oil was favored over hexadecane in the biotransformation of limonene to α-terpineol by *Sphingobium* sp. because a vegetable oil phase helped to achieve good anaerobic conditions that increased α-terpineol synthesis [9]. Other effects of the organic phase on biocatalyst or reaction can be: an influence on the enantiomeric specificity of the reaction accompanied by reduction of the e.e. value [96] or degradation of the organic phase by the microorganism [27].

In addition to instant removal from the vicinity of the biocatalyst the organic phase can serve as a protective environment for the product. Direct contact with an aqueous phase can cause autoxidation of terpenoid products and immediate extraction into an organic phase makes product accumulation possible in the first place such as in the case of nootkatol and nootkatone [37] or pinene oxide [80].

Long-chain alkanes (dodecane, hexadecane), silicone or vegetable oil, or diesters of phthalic acid [bis (2-ethylhexyl) phthalate, diisononyl phthalate] are examples for commonly used biocompatible organic solvents with high log P_{Oct} values in isoprenoid-producing bioprocesses (c.f. Table 1). Alternatively, a neat phase of substrate may be used for both substrate reservoir and extractive phase for the product, forming a SFPR system as published by Savithiry and colleagues who used undiluted limonene as the extractive phase during the bioconversion of limonene to α-terpineol by recombinant *E. coli* [76]. During the de novo production of biofuel sesquiterpenes farnesene or bisabolene by recombinant *S. cerevisiae* and *E. coli*, the insoluble product readily separates from the aqueous phase forming an aqueous–organic two-phase system in the course of the bioprocess which then acts as a product sink for further product extraction [67, 74]. Of course application of undiluted terpenoid substrate or product in an aqueous–organic two-phase system implies insignificant toxic and inhibitory effects on the biocatalyst.

Biofilm formation has been described as disadvantageous in terpene bioconversion studies when they interfered with substrate or product transport between the extractive phase and the biocatalyst [27, 57], however, biofilms can be used deliberately in biocatalytic approaches. Using catalytic biofilms allows for the exploitation of the advantages of a continuous reactor operation such as cell reuse, steady-state conditions with constant product qualities, and avoidance of downtimes, while at the same time the disadvantages of immobilization including reduced absolute activity and viability of the biocatalyst, additional costs, and lack

of universally applicable immobilization concepts are avoided [38]. For isoprenoids, the only example described thus far followed an alternative approach using biofilms in direct contact with an extractive phase. The microbes are grown on nutrient-soaked carrier materials and then brought into direct contact with the substrate via a biocompatible organic carrier solution [61, 62]. Oxygen is supplied through the organic phase which may exhibit higher oxygen solubility compared to water [63]. A major disadvantage, however, is that the nutrient and water content and accumulation of metabolic by-products in the hydrophilic carrier material cannot be controlled during the process.

Considering the isoprenoid bioprocesses with direct contact and internal removal listed in Table 1 it is notable that only laboratory-scale systems with total reaction volumes typically below 10 L have been published and that most publications describe a proof-of-concept without further investigating product purification from the receiving phase. Exceptions are the distillation of limonene produced by recombinant *E. coli* from diisononyl phthalate [107], the purification of artemisinic acid from isopropyl myristate [65], and a stirred-tank reactor operated in continuous cultivation mode with a continuous recovery of the product α-santalene and regeneration of the extractive solvent dodecane [78]. On the shaking flask scale the recovery of bisabolene from the organic phase and its purification and subsequent chemical treatment have been described [67]. The only example of an industrial-scale isoprenoid bioprocess with direct internal removal of the product remains the production of the sesquiterpene farnesene by Amyris Inc. [102]. However, technical details of this process were not published.

2.2 Solid Phase Extraction

An extractive liquid organic phase of a TPPB can be replaced by a solid phase. It was found that some thermoplastics have strong affinities for hydrophobic organic molecules and show partitioning behavior similar to organic solvents and can therefore function as second phase and product sink or substrate source [3]. Polymers display several advantages and disadvantages in comparison to a liquid phase (Table 4). Desirable polymer characteristics in solid–liquid TPPBs are also listed in Table 4.

Few researchers employed the adsorption of isoprenoid products to polymers directly added to the aqueous phase (c.f. Table 1). Usually dispersed organic solvent droplets of an oil in water emulsion in a two-liquid phase-partitioning bioreactor form a larger available surface area and allow higher mass transfer rates because the droplet size is significantly smaller (µm scale) compared to the typical size of solid polymer materials (mm scale). For example, with an estimated droplet size of 30 µm, 50 mL of organic solvent in 1 L of aqueous phase form a total specific interfacial area of 8.7 m^2 dm^{-3} [99]. In contrast, 50 g of solid spherical or cylindrical beads with diameter of 2–4 mm result in a total specific interfacial area of 0.07 m^2 dm^{-3} in 1 L of aqueous phase [72]. Furthermore, equilibrium in a

Table 4 Advantages, disadvantages, and desirable properties of polymer beads for direct contact ISPR [73]

Advantage
Biocompatible
No emulsion formation
Resistance to microbial degradation
Reusable
Ease of separation from aqueous phase
Polymer structure can be tailored to enhance selective absorption of the desired target molecule(s)
Disadvantage
Low capacity determined by surface area
Mass transfer limitations due to biofilm formation
Delayed equilibrium between solid and liquid phase due to low diffusion rates
Desirable properties
Commercially available at a low cost
Nonhazardous
Nontoxic to the employed organisms
Not consumed as carbon and energy source or otherwise biodegradable
Not promoting biofilm formation under operational conditions
Possessing desirable affinity for the target molecule(s)
Thermally stable for sterilization purposes
Stable in aqueous medium at the pH and electrolyte concentration of the employed culture medium
Stable in medium employed to load polymer with target compounds

liquid–liquid system is established almost instantaneously whereas equilibrium between a solid and liquid phase can be delayed due to diffusion into the solid polymer matrix [72]. Nevertheless, application of solid polymer materials as the extractive phase can lead to improved volumetric productivities compared to two-liquid phase-partitioning bioreactors. Utilizing two important advantages of solid polymers, the biocompatibility and low emulsion formation tendency, the volumetric productivity of the biotransformation of carveol to carvone by *R. erythropolis* DCL14 was improved by a factor of 3.5 in comparison to a two-liquid phase-partitioning bioreactor where strong emulsion formation was observed [57]. The fact that, in contrast to experiments in a two-liquid phase-partitioning bioreactor, no dramatic morphological changes to the cells were observed provided proof for superior biocompatibility of polymer beads. Selection criteria for a suitable polymer material were bioavailability, partition coefficients, thermal stability, and biofilm formation tendency. The ease of separation of solid polymer material from the aqueous phase and the straightforward approach for product recovery led to high product concentrations and purities in eluates [15, 57] or even a crystallized product of 98 % purity [54].

On a laboratory scale product extraction from polymer beads may be much easier than the removal of products from a liquid–organic extracting phase where distillation of the solvent or back extraction using another immiscible solvent is

required. However, on an industrial scale the additional step of polymer bead separation and subsequent product recovery may disproportionately increase process costs. Another problem arises when polymer beads are used in bioconversions where substrate and product share similar physicochemical properties. In these cases the polymer material may not be selective enough which can lead to decrease of conversion yields. Polymer beads were used to feed the substrate linalool and simultaneously remove the product linalool oxide during the bioconversion catalyzed by *Corynespora cassiicola* DSM 62475 [15]. Inasmuch as linalool and linalool oxide share similar adsorption characteristics to the polymer material used, 35 % of the substrate linalool was still eluted from polymer beads at the end of the process, thereby reducing the conversion yield. If the substrate and the product possess significantly different physicochemical properties polymer material can be successfully used in a SFPR approach. Anion exchange polymer beads (Amberlite IRA 410 Cl) were used to remove perillic acid efficiently from limonene bioconversion catalyzed by *Pseudomonas putida* GS1 [54]. The same adsorber was used by Alonso-Gutierrez et al. [2] to remove the product perillyl alcohol selectively from a de novo biosynthesis with a recombinant *E. coli* shake flask culture while avoiding detracting the immediate precursor in the isoprenoid pathway, limonene. The latter problem occurred when dodecane was used as an overlying organic phase instead, disabling perillyl alcohol formation.

3 Internal and External Removal of Isoprenoid Products by Indirect Contact

Bioprocesses with an indirect contact ISPR technique according to numbers 2 and 4 in Fig. 1 usually separate the biocatalyst from the extractive phase. The extractive phase may be located inside the bioreactor (internal removal) or in an external loop (external removal).

3.1 Stripping and Pervaporation

The high vapor pressure of some isoprenoids favors their transition from the liquid phase to the vapor phase at bioprocess temperatures. If the vapor phase is mobile as in the case of bioreactor off-gas the isoprenoid compound is stripped from the liquid phase. Stripping can effectively reduce the concentration of the isoprenoid product in the aqueous phase and avoid toxic effects on the biocatalyst. A straightforward way to remove isoprenoid volatile products from bioprocesses is their separation from bioreactor off-gas streams by adsorption to a solid-phase material such as activated carbon [105] or thermoplastics [45, 64, 68], or absorption into a hydrophobic liquid [4, 5]. The receiving phase has a certain affinity and capacity for the

product: that is, at certain product loadings breakthrough of product leading to product loss occurs if no technical countermeasures (gas sensor, change of reservoir) have been installed [64, 105]. In addition to affinity and capacity of the receiving phase variables determining the efficiency of stripping are temperature and abundance of other gases such as CO_2, low molecular weight contaminants, or water vapor [105]. Desorption from adsorber materials can be achieved with organic solvents or, in the case of isoprene, by steam or nitrogen followed by condensation of the product vapor into the concentrated liquid product. Purification from absorbing liquids is usually achieved by distillation or back extraction using another immiscible solvent. Provided that the physicochemical properties allow the application of this ISPR technique stripping can be seen as a flexible and relatively easily scalable method.

If the product first diffuses through a membrane before it is evaporated into the vapor phase this is called pervaporation or in case of a hydrophobic membrane, organophilic pervaporation. The membrane forms a selective barrier between the aqueous phase (feed) and the vapor phase (permeate). Because pervaporation selectivity is primarily governed by differences in transport rate of components through the membrane and not by vapor pressure it can also be applied to less volatile compounds. Although organophilic pervaporation has always been considered a membrane-based technique that has great potential for mild, natural aroma recovery, industrial application remains at pilot-scale operations at the most [106] in contrast to hydrophilic pervaporation, which is industrially used for dewatering of organic solvents. Advantages of organophilic pervaporation are that available organophilic pervaporation membranes act as both sterile barrier and separation medium, rendering cell separation unnecessary. The membranes are biocompatible, incur negligible fouling tendency, and are steam sterilizable. Despite the many advantages of the combination of organophilic pervaporation with biosynthesis or conversion of isoprenoids in a bioreactor only a few such processes have been described in the literature thus far. During cultivation of *Ceratocystis moniliformis* ATCC 12861 ethyl acetate, propyl acetate, isobutyl acetate, isoamyl acetate, citronellol, and geraniol were isolated by organophilic pervaporation [11]. The choice of membrane material influenced product composition. Using a polyoctylmethylsiloxane membrane the process yielded higher concentrations of esters; using a polyetheramide-block–copolymer membrane the permeate contained higher concentrations of the terpenoids citronellol and geraniol. Another approach also used a flat-sheet organophilic pervaporation membrane to demonstrate proof-of-principle for in situ separation of the C13-norisoprenoid β-ionone from enzymatic β-carotene cleavage [59]. The biggest challenge still hampering a broader application of organophilic pervaporation for in situ volatiles separation is the limited transmembrane flux of the product. Here, membrane modules arranged as tube bundles with much larger interfacial membrane surface areas would most probably help in significantly improving overall performance.

3.2 Perstraction in Membrane Reactors

In a two-liquid phase bioreactor setup the biocatalyst-containing phase and the extractive phase can be separated by a membrane. The aim of such a membrane reactor is to avoid the consequences of direct contact between the aqueous and the organic phases such as emulsion formation and toxic effects on the biocatalyst. The biocatalyst and organic phases are separated by the membrane, therefore the range of solvents that can be used to assist biosynthesis or bioconversions can be expanded to solvents with high product affinity but low biocompatibility [96]. On the downside, a membrane may exhibit a considerable resistance to mass transfer between the aqueous and the organic phase [13, 14, 96]. For instance, according to the transport mechanism of a solute from the bulk aqueous phase to the bulk organic phase through a flat-sheet, hydrophobic, asymmetric, polyimide, organic solvent nanofiltration membrane there are five possible bottlenecks for solute transfer [85, 96]: transfer from the aqueous solution bulk to the aqueous/membrane interface through the aqueous liquid film, partition into the membrane, diffusion across the membrane, partition from the membrane into the organic liquid, transfer from the membrane/organic–liquid interface to the bulk of the organic liquid through the organic–liquid film. Because of these mass transfer constraints membrane bioreactors often show inferior performance when compared to an aqueous–organic two-phase system with direct contact [29, 96]. An integral part of a membrane reactor layout is the determination of the overall mass transfer coefficient of the solute of interest [13]. Together with the specific activity of the biocatalyst a suitable membrane surface area to biomass ratio can be calculated [29, 104]. By selecting a suitable membrane surface area to biomass ratio mass transfer limitations and the associated reduction of biocatalyst specific yields can be avoided.

Three types of membranes have been used in membrane reactors for isoprenoid synthesis and conversion: nonporous, microporous, and nanoporous. Silicone rubber is a nonporous membrane material displaying high permeability and selectivity to small hydrophobic molecules and prevents aqueous breakthrough and is impermeable to ionic species and macromolecules. The high permeability of silicone rubber is due to extensive swelling in the presence of organic solvents [30]. The silicone membrane swelling must be considered in membrane bioreactor design to prevent membrane bursting or solvent breakthrough. Silicone rubber tubing submerged into a liquid phase of a stirred-tank reactor is a practical and straightforward implementation of a membrane reactor [14, 27]. The organic phase may be pumped through the silicone rubber tubing which is submerged in the aqueous phase such as during the bioconversion of geraniol to citronellol by *S. cerevisiae* [29] or the bioconversion of α-pinene oxide to isonovalal by *Pseudomonas fluorescens* NCIMB 11671 [14]. Alternatively, the aqueous phase containing the biocatalyst *R. erythropolis* DCL14 was circulated inside a silicone tube that was immersed in dodecane containing the substrate carveol and the accumulated

product carvone [27]. However, this mode led to biofilm formation inside the silicone tubing along with a reduction in mass transfer of substrate and product across the membrane, a problem often observed in membrane bioreactor setups.

A relatively hydrophilic microporous nylon membrane was used in a membrane reactor for the kinetic resolution of racemic menthyl acetate by *Bacillus subtilis* NCIMB 11705 [104]. A crucial point in process operation of microporous membranes is the careful control of the transmembrane pressure to avoid phase breakthrough, which leads to mixing of the two phases and to the same emulsification problems experienced in direct contact systems [29]. Surface-active components of the aqueous phase such as biosurfactants, proteins, or the cells themselves can cause a considerable reduction in the membrane breakthrough pressure [95]. However, in the case of kinetic resolution of racemic menthyl acetate the cells were placed on the same side of the membrane as the organic phase, pure racemic menthyl acetate, and the aqueous phase removed acetate from the vicinity of the cells. No breakthrough of either phase was observed provided the right pressure was maintained. Although cell stability was lower in the membrane reactor setup compared to an aqueous–organic two-phase system, probably due to better contact with the phase interface, the specific activity obtained in the membrane reactor was about fivefold that in the emulsion system with direct contact [104].

A nanofiltration membrane was used in a membrane bioreactor with the bioconversion of geraniol to citronellol by *S. cerevisiae* as the model reaction [96]. Measurements of partition coefficients and overall mass transfer coefficients identified toluene (log P_{Oct} = 2.4) as the superior extractive phase in comparison to hexadecane (log P_{Oct} = 8.8). As expected, bioconversion experiments with direct internal contact in an aqueous–organic two-phase system yielded no product when toluene was used whereas hexadecane proved to be biocompatible. Applying the nanofiltration membrane to separate the aqueous and organic phases showed higher space–time yields but at the same time lower e.e. values when toluene was used instead of hexadecane. Due to mass transfer limitations even the membrane reactor with toluene was outperformed by the direct contact ISPR system using the aqueous–organic two-phase system. The mass transfer resistance could be located in the membrane for the hexadecane system whereas for the toluene system the contribution of the aqueous liquid film mass transfer resistance became predominant [96].

The immobilization of cells in carrier materials can be seen as a compartmentalization of the biocatalyst and product. Fungal mycelia of *Penicillium digitatum* immobilized in calcium alginate were used to convert limonene to α-terpineol [92]. Alginate beads could be reused in batch conversions or applied in a continuous bioreactor setup. However, the alginate matrix formed a diffusional barrier for oxygen and limonene and decreased the substrate affinity by increasing the apparent K_M of the bioconversion.

4 Towards Industrial Isoprenoid Processes

Few microbial bioprocesses involving isoprenoids have been developed to industrial scale. Notable examples are the de novo productions of isoprene realized by the joint research of Genencor and Goodyear [105], of farnesene by Amyris Inc. [102], and of artemisinic acid for artemisinin by Amyris/Sanofi [65]. The basis of an industrial bioprocess is always a suitable biocatalyst able to accumulate high product titers in a short time. For this, whole-cell biocatalysts or microbial cell factories have to be optimized in iterative cycles by the tools of systems biology and metabolic engineering, but they also have to be integrated successfully into the setting of an industrial process. A simple constraint might be that this process requires integration of biotechnology and chemical engineering at a skill level not usually present in a single company [105].

4.1 Challenges of ISPR Implementation

Focusing on the technical side of bioprocess development, realization of ISPR techniques in industrial processes generally faces a number of challenges. A respective ISPR technique usually involves high investment costs for research and development and the construction of rather complex facilities. These measures have been usually dedicated to a single process, thereby reducing the flexibility of plant setup and increasing the pressure of efficient capital utilization [89]. However, nowadays with the concept of microbial platform strains, an array of different terpenoid products may be produced by the same microbe under almost identical fermentation conditions, which makes the use of process settings with ISPR more affordable ("one process—many products"). ISPR research and development are further hampered by the fact that ISPR techniques are either not easily scalable from laboratory conditions to production scale or that the suitable ISPR technique itself may change with process scale. Further consideration has to be put into the required product purity for subsequent process steps. Depending on the final application of the product of a bioprocess, legal limitations, such as regulations of product, process, work, and environmental safety, may further affect the choice of the ISPR technique. Adequately addressing all these points may lead to prolonged process development times and an extended time-to-market for a new product. In consequence, a less elaborated but faster process may be preferred [91].

As one of the few publications on industrial-scale isoprenoid bioprocesses the biological production of isoprene illustrates the aforementioned challenges [105]. It also highlights a central key to successful implementation of a large-scale bioprocess: an integrated approach that, from the choice of host strain, to the design and engineering of an isoprene cell factory, to its cultivation/fermentation, and finally to product recovery and purification, never loses the focus on large-scale applicability.

4.2 Industrial Production Strain Prerequisites

Most proof-of-concept research projects focus on only one or a couple of aspects of a bioprocess, thereby separating the microorganism and the isoprenoid synthesis or the conversion from the rest of the process design and neglecting demands of large-scale operations for certain organism properties [23]. In addition to those microorganism properties generally desirable from a bioprocess-oriented viewpoint, such as the ability to use low-cost feedstocks, tolerance to extreme conditions, or resistance to inhibitors, isoprenoid bioprocesses would benefit from biocatalysts with low-emulsion–forming tendency and tolerance against organic compounds. This would dramatically increase the ease of product recovery in aqueous–organic two-phase systems simply by gravity separation without the need for centrifugation or de-emulsification techniques which further raise process costs [39]. For price competitive "low-cost high-volume" products such as biofuels the possibility of operating a bioprocess in a continuous mode including the possibility of cell reuse is a valuable measure to increase productivity and lower capital cost [23]. A closed bioreactor infrastructure to prevent contaminations operating in batch or fed-batch mode, as is usually the case in laboratory-scale experiments, can significantly increase infrastructure costs and will most likely not be cost-competitive enough at the required scale [102]. Continuous operation demands a robust biocatalyst able to tolerate conditions unfavorable to competing contaminating organisms, such as extreme pH, salt concentrations, or temperatures in order to maintain monoseptic conditions. Continuous cultures require a genetically stable strain with constantly high productivity. Unfortunately, production strains may change their phenotype during production conditions. The overproduction of a molecule in a fermentation process is usually an energy burden for the microorganism. Any mutation that leads to a decrease in that metabolic burden yields faster-growing mutants with decreased productivity that eventually overgrow the initial production host. Therefore, production organisms must be maintained under strong selection so that they do not lose their beneficial feature. Production processes are situations of prolonged cultivation where selection pressure can hardly be maintained. Once this selection process is interrupted by prolonged cultivation or repeated inoculation of new media, productivity may be lost [111]. However, commonly used techniques to maintain selection pressure such as antibiotic resistance markers (e.g., ampicillin or kanamycin) may not be applicable at large scale because of concerns of drug-resistant isolates being released into the environment [102]. The commonly used laboratory genetic tool to transfer genes, the plasmid, establishes a further potential route for horizontal gene transfer [93] and introduces a certain degree of genetic instability [90]. For the de novo production of isoprenoids from sugar-based raw materials cells need to reduce the carbohydrate and get rid of the carbohydrate oxygen. In biological systems this is achieved by the use of reducing equivalents [$NAD(P)H_2$] and rejection of excess oxygen as water. Redox imbalances, i.e., the excess formation of NAD(P)H2, which is often observed in production hosts, need to be removed aerobically (by oxidation to water). Therefore, bioreactor aeration

must be carefully adjusted to avoid excessive NAD(P)H_2 oxidation and, thus, a decrease of carbohydrate-to-hydrocarbon yields below theoretical maximum values corresponding to reduced overall process productivity [10]. Another way to improve productivity is to divide a bioprocess into a growth phase, where nutrients are employed to build up biomass rapidly, and a production phase, where nutrients are exclusively used for product formation. In laboratory environments this is accomplished by the application of induction systems including IPTG in *E. coli* or galactose in *S. cerevisiae*. On an industrial scale these induction systems would not be cost-competitive and alternative routes to limit growth while allowing production are necessary [102]. An easy and well-scalable way to achieve such a "metabolic switch," that is, separation of growth and production phases, can be carbon or nitrogen restriction [94]. These examples of industrial-scale concerns on the one side and laboratory routine on the other side demonstrate a certain discrepancy caused by the different driving forces of scientific research and economic feasibility.

4.3 Terpene Toxicity

It is obvious that the choice of the host organism may ultimately determine the success or failure of an isoprenoid production process [102]. Each organism utilized in isoprenoid bioprocesses has its advantages and disadvantages that need to be weighed up against each other. Isoprenoids are essential components of living cells. However, their biotechnological overproduction in bioreactor systems may cause significant stress to exposed organisms. The toxicity of isoprenoids towards single cells varies with their structure. Due to their presumed natural function as defense molecules, toxins, growth inhibitors, or repellents, monoterpenoids are generally highly toxic to microorganisms as are some sesqui- and diterpenoids as well [36]; however, prominent examples of biotechnologically synthesized sesquiterpenes, such as farnesene or bisabolene are tolerated by the producing microbe in high concentrations [16, 67, 74]. Toxicity of hydrophobic compounds to microbial cells is often explained by accumulation of hydrophobic molecules in the lipid bilayer of the cell membrane ultimately resulting in a loss of its function as a permeability barrier, as a protein and reaction matrix, and as an energy transducer [41]; additionally, effects on other components of the cell envelope, for example, the cell wall have been described [17].

4.4 Microbial Solvent Tolerance

Microbial cells have developed different mechanisms to adapt to the presence of toxic solvents. In short, these mechanisms involve morphological adaptions, changes of the energy status, modification of the cell membrane fluidity, changes in the cell wall and outer membrane, modifications of surface properties such as

charge and hydrophobicity, transformation, and degradation of the solvent and active transport of solvents from the membrane into the environment by an energy-dependent efflux system [40]. The reader is kindly referred to one of the many detailed reviews on this subject [25, 41, 70, 71, 75, 86]. The tolerance of microorganisms towards organic and toxic compounds varies from organism to organism. Two different strategies to benefit from microbial solvent tolerance in bioprocesses with hydrophobic substrates or products are the use of well-known solvent-tolerant strains as production hosts or, alternatively, to transfer solvent-tolerance–conveying elements to well-established production hosts.

4.5 Solvent-Tolerant Hosts

Considering the usually hydrophobic and often toxic nature of isoprenoids, the choice of highly solvent-tolerant microbes, such as the gram-negative *P. putida* or the gram-positive *R. erythropolis* (c.f. Table 1) as whole-cell biocatalysts for the biotransformation of monoterpenes seems obvious. However, solvent-tolerance mechanisms are a consequence of natural selection on stress conveyed by hydrophobic substances. Some of the protective mechanisms of solvent tolerance may prevent substrate access to or product release from the biocatalyst thereby reducing product yields. For example, the outer membrane of gram-negative bacteria, in particular the lipopolysaccharide layer, was found to be an effective permeability barrier for monoterpenes [34]. Permeabilization by freeze drying or chemicals enhanced reaction rates. However, permeabilization is only applicable when the respective catalytic reaction is cofactor independent and will most likely prove to be too expensive to be applied in industrial-scale processes. Its metabolic versatility and its solvent tolerance make *P. putida* a promising biocatalyst for the bioconversion of terpenes [55]. A convincing example for the robustness of *P. putida* in the presence of monoterpenes is the conversion of (+)-limonene to (+)-perillic acid, a natural monoterpenoic acid with high potential as an antimicrobial cosmetic ingredient [54]. The microbe efficiently produces the target terpenoid without by-product formation in a fed-batch process where the precursor limonene can reach levels in the gram per liter range forming a separate organic phase in the bioreactor. However, the metabolic versatility of pseudomonads may also be a hindrance for establishing a bioprocess as it can result in unwanted side reactions and decrease product yields. Recombinant *P. putida* GPo12 was first described as a biocatalyst for the bioconversion of limonene to perillyl alcohol, a potential anticancer compound, catalyzed by a P450 monooxygenase from *Mycobacterium* sp. strain HXN-1500 in an aqueous–organic two-phase system [97]. Further research increased productivity sixfold by switching to *P. putida* KT2440 and conducting a careful carbon source selection and optimization of cell physiology [22].

However, additional research revealed that unknown enzymatic activities of *P. putida* KT2440 oxidized the product perillyl alcohol to the unwanted side products perillyl aldehyde and perillic acid. These side products constituted up to

26 % of the total amount of oxidized terpenes [21]. Another example for host-mediated unwanted side reactions is the reduction of geraniol to citronellol by unspecific dehydrogenases of the whole-cell biocatalyst *S. cerevisiae* [96]. Concerning tolerance to hydrophobic and toxic molecules such as the monoterpene limonene, which, for example, may serve as a precursor for jet biofuels in the future [10, 66], *P. putida* should have a clear advantage over monoterpene-sensitive *S. cerevisiae* [16, 17, 54]. However, the first de novo production of a monoterpene with *P. putida* has been described only very recently [53]. By introducing a heterologous bacterial mevalonate pathway from *Myxococcus xanthus* together with a plant geraniol synthase, *P. putida* GS1 produced about 200 mg/L geranic acid in a bioreactor. The monoterpenoic acid showed high efficiencies as a fungicide for corn protection [110]. The acid is produced due to endogenous oxidative enzymes converting geraniol, the direct product of the terpene synthase reaction, to the corresponding acid without accumulation of intermediates. If it were possible to narrow down the pronounced oxidative capacity of *P. putida* (e.g., by selective gene deletions or by running an appropriate process regime), this solvent-tolerant bacterium could be an interesting host for de novo production of monoterpene alcohols or hydrocarbons as well.

In an industrial process population heterogeneity is an unwanted side-effect that leads to incalculable productivities. Population heterogeneity can often be observed in *P. putida* cultivations [42]. Population heterogeneity functions as a safeguard of the respective population against fast-changing environmental conditions such as hydrophobicity. For example, direct contact of *P. putida* S12 with a toluene phase initiates a mutator element leading to the formation of a subpopulation with increased survival rates [101]. In this case population heterogeneity can be seen as another mechanism of solvent tolerance. Other sources of population heterogeneity are epigenetic modifications, asymmetric cell division, different growth and cell cycle states, microenvironmental conditions, and permanently changing levels of transcripts and proteins [58]. Inasmuch as transformation and degradation are mechanisms of solvent tolerance (see above), and solvent tolerance is often achieved by a combination of several protective mechanisms, metabolic versatility, population heterogeneity, possible side reactions, and an increased tendency to create stable emulsions are the flipside of solvent-tolerant strains.

4.6 Transfer of Solvent Tolerance

Taking a look at renewable diesel platforms, *S. cerevisiae* or other similar fungal systems are, in addition to *E. coli*, another popular choice for a development platform. Its genetic accessibility, robust and well-studied genetics, tolerance to low pH, resistance to osmotic stress, and immunity to viral contaminations make *S. cerevisiae* a production host with many of the requested properties beneficial in industrial bioprocesses [43]. In addition, problems with certification of *S. cerevisiae* genetically modified organisms on the government level are unlikely due to its

well-established reputation as a nonhazardous organism. However, biofuel toxicity still limits production and it will be necessary to improve tolerance to further increase yields [31]. An approach to benefit from increased solvent tolerance without the drawback of unwanted side reactions can be the expression of efflux pumps, heat shock proteins, membrane modifying proteins, and activation of general stress response genes in suitable expression hosts [31]. Recently, heterologous expression of a specialized ABC efflux transporter from the fungal pathogen *Grosmannia clavigera* enhanced survival of *S. cerevisiae* in the presence of a mixture of the monoterpenes (+)-limonene, (+)-3-carene, racemic α-pinene, and (−)-β-pinene [100]. A couple of energy-dependent efflux systems have been described to increase tolerance against terpenoids in well-established production hosts and thereby sometimes improving process parameters. By first screening a library of primarily uncharacterized hydrophobe/amphiphile efflux pumps of the family of resistance-nodulation–division (RND) pumps from gram-negative bacteria and then heterologously expressing an efflux pump from *Alcanivorax borkumensis* limonene tolerance of *E. coli* was increased resulting in a 64 % improvement in limonene yield of a limonene production strain [32].

The outer-membrane protein alkL has been shown to increase transmembrane transport of monoterpene substrate limonene and product perillyl alcohol [21], thereby decreasing the reduction of mass transfer across the outer membrane of gram-negative *E. coli* and increasing reaction rates [21].

5 Conclusions

Despite the many exciting applications of isoprenoids as biofuels, pharmaceuticals, flavors and fragrances, and the advantages of microbial isoprenoid production, research on isoprenoid bioprocesses has mostly been and still is limited to proof-of-concept approaches with a focus on enhancing product concentrations and yields. Often no further considerations are given on critical targets for process technology and process scale implementation such as suitability of the strain employed, ease of product separation, purification, or comparison of different ISPR techniques. This can be attributed to the fact that most of the published research is conducted in nonprofit environments under idealized laboratory conditions where the industrial relevant considerations such as time-to-market, GMO certification, strain robustness, efficient capital utilization, or lot-to-lot variations of feedstock sources are not comparably relevant. In light of the physicochemical and biochemical properties of isoprenoids an industrial-scale isoprenoid bioprocess without ISPR, that is, compartmentalization of biocatalyst and substrate and/or product to avoid inhibitory or toxic effects on the biocatalyst or to avoid product loss by evaporation or degradation, is hardly imaginable. Because the implementation of a suitable ISPR technique is a laborious, time-consuming, expensive, and often an empirical process that needs to be verified on every new process scale, only few industrial-size microbial isoprenoid production processes have been published to date.

Experiences from the implementation of large-scale biofuel processes suggest that large-scale microbial isoprenoid bioprocesses would also benefit from an integrated approach of systems biology, metabolic and process engineering. The focus on process design and large-scale applicability during the whole development of a production strain has proven to be a challenging but ultimately rewarding necessity for a successful upscaling of an isoprenoid bioprocess. Considering the final process, the choice of a solvent-tolerant microbe such as *P. putida* instead of baker's yeast or *E. coli* as the host may be an alternative if the production of toxic compounds such as monoterpenoids is aimed at and/or technical substrates containing inhibitors have to be used. However, the genetic tools available still lag behind those developed for yeast and *E. coli* and genetic instability and metabolic diversity might be further disadvantages of *P. putida* as the host strain. Nevertheless, for the time being it cannot be foreseen whether the transfer of solvent-tolerance mechanisms to conventional hosts by metabolic and cell engineering will be a successful endeavor; thus both strategies are worth being followed in this segment of isoprenoid products.

References

1. Ajikumar PK, Xiao W-H, Tyo KEJ, Wang Y, Simeon F, Leonard E, Mucha O, Phon TH, Pfeifer B, Stephanopoulos G (2010) Isoprenoid pathway optimization for taxol precursor overproduction in *Escherichia coli*. Science 330(6000):70–74
2. Alonso-Gutierrez J, Chan R, Batth TS, Adams PD, Keasling JD, Petzold CJ, Lee TS (2013) Metabolic engineering of *Escherichia coli* for limonene and perillyl alcohol production. Metab Eng 19:33–41
3. Amsden BG, Bochanysz J, Daugulis AJ (2003) Degradation of xenobiotics in a partitioning bioreactor in which the partitioning phase is a polymer. Biotechnol Bioeng 84(4):399–405
4. Arifin AA, Don MM, Uzir MH (2011) Baker's yeast mediated biotransformation of geraniol into citronellol using a continuous-closed-gas-loop bioreactor (CCGLB) system. Biochem Eng J 56(3):219–224
5. Arifin AA, Don MM, Uzir MH (2011) The feasibility of growing cells of Saccharomyces cerevisiae for citronellol production in a continuous-closed-gas-loop bioreactor (CCGLB). Bioresour Technol 102(19):9318–9320
6. Asadollahi MA, Maury J, Møller K, Nielsen KF, Schalk M, Clark A, Nielsen J (2008) Production of plant sesquiterpenes in Saccharomyces cerevisiae: effect of ERG9 repression on sesquiterpene biosynthesis. Biotechnol Bioeng 99(3):666–677
7. Asadollahi MA, Maury J, Patil KR, Schalk M, Clark A, Nielsen J (2009) Enhancing sesquiterpene production in Saccharomyces cerevisiae through in silico driven metabolic engineering. Metab Eng 11(6):328–334
8. Bakkali F, Averbeck S, Averbeck D, Idaomar M (2008) Biological effects of essential oils—a review. Food Chem Toxicol 46(2):446–475
9. Bicas JL, Fontanille P, Pastore GM, Larroche C (2010) A bioprocess for the production of high concentrations of R-(+)-α-terpineol from R-(+)-limonene. Process Biochem 45(4):481–486
10. Blanch HW (2012) Bioprocessing for biofuels. Curr Opin Biotechnol 23(3):390–395
11. Bluemke W, Schrader J (2001) Integrated bioprocess for enhanced production of natural flavors and fragrances by *Ceratocystis moniliformis*. Biomol Eng 17(4–5):137–142

12. Boghigian B, Myint M, Wu J, Pfeifer B (2011) Simultaneous production and partitioning of heterologous polyketide and isoprenoid natural products in an *Escherichia coli* two-phase bioprocess. J Ind Microbiol Biotechnol 38(11):1809–1820
13. Boontawan A, Stuckey DC (2005) Mass Transfer of Terpenes through a Silicone Rubber Membrane in a Liquid-Liquid Contacting System. Biotechnol Prog 21(6):1680–1687
14. Boontawan A, Stuckey DC (2006) A membrane bioreactor for the biotransformation of α-pinene oxide to isonovalal by *Pseudomonas fluorescens* NCIMB 11671. Appl Microbiol Biotechnol 69(6):643–649
15. Bormann S, Etschmann M, Mirata M-A, Schrader J (2012) Integrated bioprocess for the stereospecific production of linalool oxides from linalool with *Corynespora cassiicola* DSM 62475. J Ind Microbiol Biotechnol 39(12):1761–1769
16. Brennan TC, Turner CD, Kromer JO, Nielsen LK (2012) Alleviating monoterpene toxicity using a two-phase extractive fermentation for the bioproduction of jet fuel mixtures in *Saccharomyces cerevisiae*. Biotechnol Bioeng 109(10):2513–2522
17. Brennan TCR, Krömer JO, Nielsen LK (2013) Physiological and transcriptional responses of *Saccharomyces cerevisiae* to d-Limonene show changes to the cell wall but not to the plasma membrane. Appl Environ Microbiol 79(12):3590–3600
18. Bruce LJ, Daugulis AJ (1991) Solvent selection strategies for extractive biocatalysis. Biotechnol Prog 7(2):116–124
19. Chandran SS, Kealey JT, Reeves CD (2011) Microbial production of isoprenoids. Process Biochem 46(9):1703–1710
20. Chang MCY, Keasling JD (2006) Production of isoprenoid pharmaceuticals by engineered microbes. Nat Chem Biol 2(12):674–681
21. Cornelissen S, Julsing MK, Volmer J, Riechert O, Schmid A, Bühler B (2013) Whole-cell-based CYP153A6-catalyzed (*S*)-limonene hydroxylation efficiency depends on host background and profits from monoterpene uptake via AlkL. Biotechnol Bioeng 110 (5):1282–1292
22. Cornelissen S, Liu S, Deshmukh AT, Schmid A, Buhler B (2011) Cell physiology rather than enzyme kinetics can determine the efficiency of cytochrome P450-catalyzed C-H-oxyfunctionalization. J Ind Microbiol Biotechnol 38(9):1359–1370
23. Cuellar MC, Heijnen JJ, van der Wielen LAM (2013) Large-scale production of diesel-like biofuels—process design as an inherent part of microorganism development. Biotechnol J 8 (6):682–689
24. Daugulis AJ (1997) Partitioning bioreactors. Curr Opin Biotechnol 8(2):169–174
25. de Bont JAM (1998) Solvent-tolerant bacteria in biocatalysis. Trends Biotechnol 16 (12):493–499
26. de Carvalho CCCR, da Fonseca MMR (2002) Maintenance of cell viability in the biotransformation of (−)-carveol with whole cells of *Rhodococcus erythropolis*. J Mol Catal B Enzym 19–20:389–398
27. de Carvalho CCR, da Fonseca MMR (2002) Influence of reactor configuration on the production of carvone from carveol by whole cells of *Rhodococcus erythropolis* DCL14. J Mol Catal B: Enzym 19–20:377–387
28. Déziel E, Comeau Y, Villemur R (1999) Two-liquid-phase bioreactors for enhanced degradation of hydrophobic/toxic compounds. Biodegradation 10(3):219–233
29. Doig SD, Boam AT, Leak DI, Livingston AG, Stuckey DC (1998) A membrane bioreactor for biotransformations of hydrophobic molecules. Biotechnol Bioeng 58(6):587–594
30. Doig SD, Boam AT, Livingston AG, Stuckey DC (1999) Mass transfer of hydrophobic solutes in solvent swollen silicone rubber membranes. J Membr Sci 154(1):127–140
31. Dunlop M (2011) Engineering microbes for tolerance to next-generation biofuels. Biotechnol Biofuels 4(1):32
32. Dunlop MJ, Dossani ZY, Szmidt HL, Chu HC, Lee TS, Keasling JD, Hadi MZ, Mukhopadhyay A (2011) Engineering microbial biofuel tolerance and export using efflux pumps. Mol Sys Biol 7(1):n/a–n/a

33. Fichan I, Larroche C, Gros JB (1999) Water solubility, vapor pressure, and activity coefficients of terpenes and terpenoids. J Chem Eng Data 44(1):56–62
34. Fontanille P, Larroche C (2003) Optimization of isonovalal production from α-pinene oxide using permeabilized cells of Pseudomonas rhodesiae CIP 107491. Appl Microbiol Biotechnol 60(5):534–540
35. Freeman A, Woodley JM, Lilly MD (1993) In situ product removal as a tool for bioprocessing. Biotechnology (N Y) 11(9):1007–1012
36. Gershenzon J, Dudareva N (2007) The function of terpene natural products in the natural world. Nat Chem Biol 3(7):408–414
37. Girhard M, Machida K, Itoh M, Schmid RD, Arisawa A, Urlacher VB (2009) Regioselective biooxidation of (+)-valencene by recombinant E. coli expressing CYP109B1 from *Bacillus subtilis* in a two-liquid-phase system. Microb Cell Fact 8:36
38. Halan B, Buehler K, Schmid A (2012) Biofilms as living catalysts in continuous chemical syntheses. Trends Biotechnol 30(9):453–465
39. Heeres AS, Picone CSF, van der Wielen LAM, Cunha RL, Cuellar MC (2014) Microbial advanced biofuels production: overcoming emulsification challenges for large-scale operation. Trends Biotechnol 32(4):221–229
40. Heipieper H, Neumann G, Cornelissen S, Meinhardt F (2007) Solvent-tolerant bacteria for biotransformations in two-phase fermentation systems. Appl Microbiol Biotechnol 74 (5):961–973
41. Isken S, Bont de JAM (1998) Bacteria tolerant to organic solvents. Extremophiles 2:229–238
42. Jahn M, Seifert J, von Bergen M, Schmid A, Bühler B, Müller S (2013) Subpopulation-proteomics in prokaryotic populations. Curr Opin Biotechnol 24(1):79–87
43. Kampranis SC, Makris AM (2012) Developing a yeast cell factory for the production of terpenoids. Comput Struct Biotechnol J 3:e201210006
44. Kaspera R, Krings U, Pescheck M, Sell D, Schrader J, Berger RG (2005) Regio- and stereoselective fungal oxyfunctionalisation of limonenes. Z Naturforsch C 60(5–6):459–466
45. Krings U, Berger R (2008) In situ recovery of the aroma compound perillene from stirred-tank cultured *Pleurotus ostreatus* using gas stripping and adsorption on polystyrene. Biotechnol Lett 30(8):1347–1351
46. Krings U, Lehnert N, Fraatz MA, Hardebusch B, Zorn H, Berger RG (2009) Autoxidation versus Biotransformation of α-Pinene to Flavors with *Pleurotus sapidus*: Regioselective Hydroperoxidation of α-Pinene and Stereoselective Dehydrogenation of Verbenol. J Agric Food Chem 57(21):9944–9950
47. Laane C, Boeren S, Vos K, Veeger C (1987) Rules for optimization of biocatalysis in organic solvents. Biotechnol Bioeng 30(1):81–87
48. Linares D, Fontanille P, Larroche C (2009) Exploration of α-pinene degradation pathway of *Pseudomonas rhodesiae* CIP 107491. Application to novalic acid production in a bioreactor. Food Res Int 42(4):461–469
49. Lye GJ, Woodley JM (1999) Application of in situ product-removal techniques to biocatalytic processes. Trends Biotechnol 17(10):395–402
50. Lye GJ, Woodley JM (2001) Advances in the selection and design of two-liquid phase biocatalytic reactors. In: Mota M, Tramper J (eds) Cabral JMS. Taylor and Francis, London, pp 115–134
51. Malik S, Cusidó RM, Mirjalili MH, Moyano E, Palazón J, Bonfill M (2011) Production of the anticancer drug taxol in *Taxus baccata* suspension cultures: A review. Process Biochem 46(1):23–34
52. Malinowski JJ (2001) Two-phase partitioning bioreactors in fermentation technology. Biotechnol Adv 19(7):525–538
53. Mi J, Becher D, Lubuta P, Dany S, Tusch K, Schewe H, Buchhaupt M, Schrader J (2014) De novo production of the monoterpenoid geranic acid by metabolically engineered *Pseudomonas putida*. Microb Cell Fact 13(1):170
54. Mirata MA, Heerd D, Schrader J (2009) Integrated bioprocess for the oxidation of limonene to perillic acid with *Pseudomonas putida* DSM 12264. Process Biochem 44(7):764–771

55. Molina G, Pimentel M, Pastore G (2013) Pseudomonas: a promising biocatalyst for the bioconversion of terpenes. Appl Microbiol Biotechnol 97(5):1851–1864
56. Morrish JL, Brennan ET, Dry HC, Daugulis AJ (2008) Enhanced bioproduction of carvone in a two-liquid-phase partitioning bioreactor with a highly hydrophobic biocatalyst. Biotechnol Bioeng 101(4):768–775
57. Morrish JL, Daugulis AJ (2008) Improved reactor performance and operability in the biotransformation of carveol to carvone using a solid-liquid two-phase partitioning bioreactor. Biotechnol Bioeng 101(5):946–956
58. Müller S, Harms H, Bley T (2010) Origin and analysis of microbial population heterogeneity in bioprocesses. Curr Opin Biotechnol 21(1):100–113
59. Nacke C, Hüttmann S, Etschmann MW, Schrader J (2012) Enzymatic production and in situ separation of natural β-ionone from β-carotene. J Ind Microbiol Biotechnol 39(12):1771–1778
60. Newman JD, Marshall J, Chang M, Nowroozi F, Paradise E, Pitera D, Newman KL, Keasling JD (2006) High-level production of amorpha-4,11-diene in a two-phase partitioning bioreactor of metabolically engineered *Escherichia coli*. Biotechnol Bioeng 95(4):684–691
61. Oda S, Inada Y, Kato A, Matsudomi N, Ohta H (1995) Production of (S)-citronellic acid and (R)-citronellol with an interface bioreactor. J Ferment Bioeng 80(6):559–564
62. Oda S, Sugai T, Ohta H (1999) Optical resolution of racemic citronellol via a double coupling system in an interface bioreactor. J Biosci Bioeng 87(4):473–480
63. Oda S, Sugai T, Ohta H (2001) Interface bioreactor. In: Vulfson E, Halling P, Holland H (eds) Enzymes in nonaqueous solvents. Humana Press, New York, p 401–416
64. Onken J, Berger RG (1999) Biotransformation of citronellol by the basidiomycete *Cystoderma carcharias* in an aerated-membrane bioreactor. Appl Microbiol Biotechnol 51 (2):158–163
65. Paddon CJ, Westfall PJ, Pitera DJ, Benjamin K, Fisher K, McPhee D, Leavell MD, Tai A, Main A, Eng D et al (2013) High-level semi-synthetic production of the potent antimalarial artemisinin. Nature 496(7446):528–532
66. Peralta-Yahya PP, Keasling JD (2010) Advanced biofuel production in microbes. Biotechnol J 5(2):147–162
67. Peralta-Yahya PP, Ouellet M, Chan R, Mukhopadhyay A, Keasling JD, Lee TS (2011) Identification and microbial production of a terpene-based advanced biofuel. Nat Commun 2:483
68. Pollak FC, Berger RG (1996) Geosmin and related volatiles in bioreactor-cultured *Streptomyces citreus* CBS 109.60. Appl Environ Microbiol 62(4):1295–1299
69. Pursell MR, Mendes-Tatsis MA, Stuckey DC (2004) Effect of fermentation broth and biosurfactants on mass transfer during liquid–liquid extraction. Biotechnol Bioeng 85 (2):155–165
70. Ramos JL, Duque E, Gallegos M-T, Godoy P, Ramos-González MI, Rojas A, Terán W, Segura A (2002) Mechanisms of solvent tolerance in gram-negative bacteria. Ann Rev Microbiol 56(1):743–768
71. Ramos JL, Duque E, Rodriguez-Herva JJ, Godoy P, Haidour A, Reyes F, Fernandez-Barrero A (1997) Mechanisms for solvent tolerance in bacteria. The J biol chem 272(7):3887–3890
72. Rehmann L, Daugulis AJ (2007) Biodegradation of biphenyl in a solid–liquid two-phase partitioning bioreactor. Biochem Eng J 36(3):195–201
73. Rehmann L, Sun B, Daugulis AJ (2007) Polymer selection for biphenyl degradation in a solid-liquid two-phase partitioning bioreactor. Biotechnol Prog 23(4):814–819
74. Renninger NS, Newman J, Reiling KK, Regentin R, Paddon CJ, Amyris Biotechnologies, Inc., assignee (2010). Production of isoprenoids. U.S.A. patent US 7659097 B2
75. Sardessai Y, Bhosle S (2002) Tolerance of bacteria to organic solvents. Res Microbiol 153 (5):263–268
76. Savithiry N, Cheong T, Oriel P (1997) Production of α-Terpineol from escherichia coli cells expressing thermostable limonene hydratase. In: Davison B, Wyman C, Finkelstein M (eds) Biotechnology for fuels and chemicals. Humana Press, New York, p 213–220

77. Scalcinati G, Knuf C, Partow S, Chen Y, Maury J, Schalk M, Daviet L, Nielsen J, Siewers V (2012) Dynamic control of gene expression in *Saccharomyces cerevisiae* engineered for the production of plant sesquitepene α-santalene in a fed-batch mode. Metab Eng 14(2):91–103
78. Scalcinati G, Partow S, Siewers V, Schalk M, Daviet L, Nielsen J (2012) Combined metabolic engineering of precursor and co-factor supply to increase alpha-santalene production by *Saccharomyces cerevisiae*. Microb Cell Fact 11:117
79. Schalk M, Pastore L, Mirata MA, Khim S, Schouwey M, Deguerry F, Pineda V, Rocci L, Daviet L (2012) Toward a biosynthetic route to sclareol and amber odorants. J Am Chem Soc 134(46):18900–18903
80. Schewe H, Holtmann D, Schrader J (2009) P450(BM-3)-catalyzed whole-cell biotransformation of a-pinene with recombinant *Escherichia coli* in an aqueous-organic two-phase system. Appl Microbiol Biotechnol 83(5):849–857
81. Schewe H, Kaup BA, Schrader J (2008) Improvement of P450(BM-3) whole-cell biocatalysis by integrating heterologous cofactor regeneration combining glucose facilitator and dehydrogenase in *E. coli*. Appl microbiol biotechnol 78(1):55–65
82. Schmid A, Kollmer A, Witholt B (1998) Effects of biosurfactant and emulsification on two-liquid phase pseudomonas oleovorans cultures and cell-free emulsions containing n-Decane. Enzyme Microb Technol 22(6):487–493
83. Schrader J (2007) Microbial flavour production. In: Berger RG (ed) Flavours and fragrances. Springer, Berlin, pp 507–574
84. Schügerl K, Hubbuch J (2005) Integrated bioprocesses. Curr Opin Microbiol 8(3):294–300
85. See-Toh YH, Ferreira FC, Livingston AG (2007) The influence of membrane formation parameters on the functional performance of organic solvent nanofiltration membranes. J Membr Sci 299(1–2):236–250
86. Segura A, Molina L, Fillet S, Krell T, Bernal P, Muñoz-Rojas J, Ramos J-L (2012) Solvent tolerance in Gram-negative bacteria. Curr Opin Biotechnol 23(3):415–421
87. Sikkema J, de Bont JA, Poolman B (1994) Interactions of cyclic hydrocarbons with biological membranes. J Biol Chem 269(11):8022–8028
88. Sikkema J, de Bont JA, Poolman B (1995) Mechanisms of membrane toxicity of hydrocarbons. Microbiol Rev 59(2):201–222
89. Stark D, von Stockar U (2003) In situ product removal (ISPR) in whole cell biotechnology during the last twenty years. Adv Biochem Eng/Biotechnol 80:149–175
90. Summers DK, Sherratt DJ (1984) Multimerization of high copy number plasmids causes instability: cole 1 encodes a determinant essential for plasmid monomerization and stability. Cell 36(4):1097–1103
91. Takors R (2004) Ganzzell-ISPR-Prozessentwicklung: chancen und risiken. Chemie Ingenieur Technik 76(12):1857–1864
92. Tan Q, Day DF (1998) Bioconversion of limonene to α-terpineol by immobilized *Penicillium digitatum*. Appl Microbiol Biotechnol 49(1):96–101
93. Thomas CM, Nielsen KM (2005) Mechanisms of, and barriers to, horizontal gene transfer between bacteria. Nat Rev Micro 3(9):711–721
94. Tsuruta H, Paddon CJ, Eng D, Lenihan JR, Horning T, Anthony LC, Regentin R, Keasling JD, Renninger NS, Newman JD (2009) High-level production of amorpha-4,11-diene, a precursor of the antimalarial agent artemisinin, in *Escherichia coli*. PLoS One 4(2):e4489
95. Vaidya AM, Halling PJ, Bell G (1994) Surfactant-induced breakthrough effects during the operation of two-phase biocatalytic membrane reactors. Biotechnol Bioeng 44(6):765–771
96. Valadez-Blanco R, Ferreira FC, Jorge RF, Livingston AG (2008) A membrane bioreactor for biotransformations of hydrophobic molecules using organic solvent nanofiltration (OSN) membranes. J Membr Sci 317(1–2):50–64
97. van Beilen JB, Holtackers R, Lüscher D, Bauer U, Witholt B, Duetz WA (2005) Biocatalytic production of perillyl alcohol from limonene by using a novel *Mycobacterium* sp. Cytochrome P450 alkane hydroxylase expressed in *Pseudomonas putida*. Appl Environ Microbiol 71(4):1737–1744

98. Van Sonsbeek HM, Beeftink HH, Tramper J (1993) Two-liquid-phase bioreactors. Enzyme Microb Technol 15(9):722–729
99. Wang DIC, Ochoa A (1972) Measurements on the interfacial areas of hydrocarbon in yeast fermentations and relationships to specific growth rates. Biotechnol Bioeng 14(3):345–360
100. Wang Y, Lim L, DiGuistini S, Robertson G, Bohlmann J, Breuil C (2013) A specialized ABC efflux transporter GcABC-G1 confers monoterpene resistance to Grosmannia clavigera, a bark beetle-associated fungal pathogen of pine trees. New Phytol 197(3):886–898
101. Wery J, Hidayat B, Kieboom J, de Bont JAM (2001) An insertion sequence prepares *Pseudomonas putida* S12 for severe solvent stress. J Biol Chem 276(8):5700–5706
102. Westfall PJ, Gardner TS (2011) Industrial fermentation of renewable diesel fuels. Curr Opin Biotechnol 22(3):344–350
103. Westfall PJ, Pitera DJ, Lenihan JR, Eng D, Woolard FX, Regentin R, Horning T, Tsuruta H, Melis DJ, Owens A et al (2012) Production of amorphadiene in yeast, and its conversion to dihydroartemisinic acid, precursor to the antimalarial agent artemisinin. PNAS 109(3):E111–E118
104. Westgate S, Vaidya AM, Bell G, Halling PJ (1998) High specific activity of whole cells in an aqueous-organic two-phase membrane bioreactor. Enzyme Microb Technol 22(7):575–577
105. Whited GM, Feher FJ, Benko DA, Cervin MA, Chotani GK, McAuliffe JC, LaDuca RJ, Ben-Shoshan EA, Sanford KJ (2010) Development of a gas-phase bioprocess for isoprene-monomer production using metabolic pathway engineering. Ind Biotechnol 6(3):152–163
106. Willemsen JHA, Dijkink BH, Togtema A (2004) Organophilic pervaporation for aroma isolation—industrial and commercial prospects. Membr Technol 2004(2):5–10
107. Willrodt C, David C, Cornelissen S, Bühler B, Julsing MK, Schmid A (2014) Engineering the productivity of recombinant *Escherichia coli* for limonene formation from glycerol in minimal media. Biotechnol J. doi:10.1002/biot.201400023
108. Woodley JM, Bisschops M, Straathof AJJ, Ottens M (2008) Future directions for in situ product removal (ISPR). J Chem Technol Biotechnol 83(2):121–123
109. Wriessnegger T, Augustin P, Engleder M, Leitner E, Müller M, Kaluzna I, Schürmann M, Mink D, Zellnig G, Schwab H et al (2014) Production of the sesquiterpenoid (+)-nootkatone by metabolic engineering of *Pichia pastoris*. Metab Eng 24:18–29
110. Yang T, Stoopen G, Yalpani N, Vervoort J, de Vos R, Voster A, Verstappen FWA, Bouwmeester HJ, Jongsma MA (2011) Metabolic engineering of geranic acid in maize to achieve fungal resistance is compromised by novel glycosylation patterns. Metab Eng 13 (4):414–425
111. Zelder O, Hauer B (2000) Environmentally directed mutations and their impact on industrial biotransformation and fermentation processes. Curr Opin Microbiol 3(3):248–251