

Alleviating Monoterpene Toxicity Using a Two-Phase Extractive Fermentation for the Bioproduction of Jet Fuel Mixtures in *Saccharomyces cerevisiae*

Timothy C.R. Brennan, Christopher D. Turner, Jens O. Krömer, Lars K. Nielsen

Australian Institute for Bioengineering and Nanotechnology (AIBN),

University of Queensland, Brisbane Qld 4072, Australia; telephone: 61-7-3346-3222;

fax: 61-7-3346-3973; e-mail: j.kromer@uq.edu.au

Received 2 February 2012; revision received 20 March 2012; accepted 16 April 2012

Published online in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/bit.24536

ABSTRACT: Monoterpenes are a diverse class of compounds with applications as flavors and fragrances, pharmaceuticals and more recently, jet fuels. Engineering biosynthetic pathways for monoterpene production in microbial hosts has received increasing attention. However, monoterpenes are highly toxic to many microorganisms including *Saccharomyces cerevisiae*, a widely used industrial biocatalyst. In this work, the minimum inhibitory concentration (MIC) for *S. cerevisiae* was determined for five monoterpenes: β -pinene, limonene, myrcene, γ -terpinene, and terpinolene (1.52, 0.44, 2.12, 0.70, 0.53 mM, respectively). Given the low MIC for all compounds tested, a liquid two-phase solvent extraction system to alleviate toxicity during fermentation was evaluated. Ten solvents were tested for biocompatibility, monoterpene distribution, phase separation, and price. The solvents dioctyl phthalate, dibutyl phthalate, isopropyl myristate, and farnesene showed greater than 100-fold increase in the MIC compared to the monoterpenes in a solvent-free system. In particular, the MIC for limonene in dibutyl phthalate showed a 702-fold (308 mM, 42.1 g L⁻¹ of limonene) improvement while cell viability was maintained above 90%, demonstrating that extractive fermentation is a suitable tool for the reduction of monoterpene toxicity. Finally, we estimated that a limonane to farnesane ratio of 1:9 has physicochemical properties similar to traditional Jet-A aviation fuel. Since farnesene is currently produced in *S. cerevisiae*, its use as a co-product and extractant for microbial terpene-based jet fuel production in a two-phase system offers an attractive bioprocessing option.

Biotechnol. Bioeng. 2012;xxx: xxx–xxx.

© 2012 Wiley Periodicals, Inc.

KEYWORDS: monoterpene; extractive fermentation; jet fuel; *Saccharomyces cerevisiae*

Introduction

Isoprenoids are a large and diverse class of natural compounds derived from a common 5 carbon isoprene unit (Keasling, 2010). Fermentative production of isoprenoids using engineered microorganisms is a potential route to deliver petroleum-compatible fuels [e.g., isopentanol (Hull et al., 2006), farnesene (Renninger and McPhee, 2008), bisabolene (Peralta-Yahya et al., 2011)] from simple sugars supplied by renewable feedstocks such as lignocellulosic biomass and sugarcane (Fortman et al., 2008; Renouf et al., 2008). Monoterpenes are a subclass of isoprenoids built from two isoprene (C₅) units (Fig. 1). These C₁₀ olefins and their derivatives are major constituents of essential oils and find applications as flavors and fragrances (e.g., menthol and pinene) (van der Werf et al., 1997), antiseptics (e.g., thymol) (Lambert et al., 2001), and anticancer agents (e.g., limonene and perillyl alcohol) (Gould, 1997). Saturated paraffins generated from monoterpenes have properties similar to the light end of traditional kerosene aviation fuel (Jet-A) making them ideal components for “drop in” replacements (Fortman et al., 2008; Harvey et al., 2009; Renninger et al., 2008). For example, Amyris, Inc. plans to use a mixture of 50% limonane (C₁₀ cycloparaffin), 10% cymene (C₁₀ aromatic), and 40% farnesane (C₁₅ branched chain paraffin), termed AMJ-700, in test flights in 2012 (Amyris, 2009; Ryder, 2009).

Synthesis of monoterpenes in whole cell biocatalysts such as *Escherichia coli* (Carter et al., 2003; Dunlop et al., 2011;

Conflicts of interest: None.

Dr. Jens O. Krömer's present address is Centre for Microbial Electrosynthesis (CEMES), The University of Queensland, Queensland 4072, Australia.

Correspondence to: J. O. Krömer

Contract grant sponsor: Queensland government (National and International Research Alliances Program)

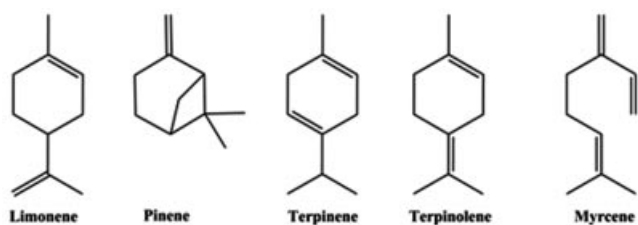


Figure 1. *d*-limonene, β -pinene, γ -terpinene, terpinolene and myrcene were the five monoterpenes analyzed in this work.

Reiling et al., 2004) and *S. cerevisiae* (Carrau et al., 2005; Fischer et al., 2011; Oswald et al., 2007) has been limited (e.g., $30 \mu\text{g L}^{-1}$ to 60 mg L^{-1} of monoterpene product) by insufficient supply of the monoterpene precursor geranyl pyrophosphate and monoterpene synthase activity (Fischer et al., 2011; Oswald et al., 2007; Reiling et al., 2004). In principle, engineering microbes to produce monoterpenes should be no more difficult than the already completed engineering of *S. cerevisiae* to produce farnesene, a C_{15} sesquiterpene replacement for diesel fuel (Renninger and McPhee, 2008), which is produced from the same precursors in the cell. However, unlike farnesene, monoterpenes are generally highly toxic to microorganisms. Therefore, the realization of microbial monoterpene production will rely extensively on solving this core issue.

Monoterpenes are lipophilic compounds and their toxicity is attributed to the interference with membrane properties (Andrews et al., 1980; Uribe and Pena, 1990; Uribe et al., 1985). For instance, β -pinene inhibits the respiration and essential ion (K^+ and H^+) transport in whole yeast cells, while loss of respiratory control, ATP synthesis inhibition and increased membrane fluidity is observed in isolated yeast mitochondria (Uribe et al., 1985). These observations indicate that β -pinene interferes with mitochondrial membrane integrity and ATP production, while release of cytoplasmic material in the presence of α -pinene (Andrews et al., 1980) indicates that monoterpenes can also cause severe damage to the plasma membrane. Limonene similarly causes severe interference with cell functions. Cell viability and ethanol production by *S. cerevisiae* using enzymatically digested citrus peel waste was significantly reduced in the presence of 0.02 – 0.10% v v $^{-1}$ orange peel oil, which contains 95 – 97% limonene (Pourbafrani et al., 2007; Wilkins et al., 2007). Although growth inhibition by orange peel oil, limonene, and other non-substituted monoterpenes in yeast and bacteria is well-established (Grohmann et al., 1994; Murdock and Allen, 1960; Subba et al., 1967; Uribe and Pena, 1990; Uribe et al., 1985; Wilkins et al., 2007), quantitative data on monoterpene toxicity are limited and the exact mechanism remains poorly understood.

A physical approach used to overcome toxicity limitations is to remove inhibitory products in situ during fermentation

using an extractive solvent. Numerous in situ product recovery (ISPR) techniques (e.g., pervaporation, perstraction, gas stripping) have been reported for the recovery of aromas, alcohols, and organic acids and an extensive review of ISPR projects and process designs have been covered by Stark and von Stockar (2003). Liquid–liquid extraction has shown the greatest potential because it is simple and scalable (Janusz, 2001) and provides the cells with an efficient product sink (Malinowski, 2001). Two-phase extractive fermentation has been reported to enhance the production and recovery of short-chain alcohols [e.g., ethanol (Daugulis et al., 1987), butanol (Roffler et al., 1988)], acetone (Roffler et al., 1988), organic acids (Bar and Gainer, 1987), and volatile sesquiterpene products such as amorpho-4,11-diene (Newman et al., 2006). However, this technique has not been applied to overcome monoterpene inhibition. Solvent selection is the first crucial step in the development of an effective two-phase bioprocess because solvents are generally both product and host specific (Bruce and Daugulis, 1991; León et al., 1998). The solvent needs to meet the following key criteria: Biocompatibility with the producing organism, high product distribution coefficients, favorable phase separation (e.g., low emulsion formation), and low cost (Bruce and Daugulis, 1991).

A proof-of-concept two-phase extractive system for monoterpene production with *S. cerevisiae* was defined in this work. First, we quantitatively determined the inhibitory concentrations for five common monoterpene products in a solvent-free system. In an attempt to evolve a monoterpene tolerant phenotype, we subjected the wild type strain to several hundred generations of limonene challenged chemostat cultivation without significant improvement. We then screened ten organic solvents for biocompatibility, monoterpene distribution, phase separation, and price to select suitable solvent candidates to be used for extractive fermentation. We demonstrated that using this biphasic approach, *S. cerevisiae* was able to withstand significantly higher monoterpene concentrations compared to the solvent-free system and the chemostat challenged strain. Finally, we explored the use of the yeast product farnesene as an extractant and a co-product for jet fuel production by estimating fuel properties of a farnesane-limonane blend.

Materials and Methods

Strains, Media, and Chemicals

The *S. cerevisiae* strain S288C (MAT α SUC2 gal2 mal mel flo1 flo8-1 hap1) was kindly provided by the Australian Wine Research Institute (AWRI, Adelaide, SA, Australia). Rich medium (YPD) was used to store cultures at -80°C in 40% glycerol. The YPD medium contained 10 g L^{-1} yeast extract, 10 g L^{-1} polypeptone, and 20 g L^{-1} dextrose (glucose). Cells were reactivated from frozen glycerol stocks by streaking out on chemically defined medium (CDM) agar plates (15 g L^{-1} agar) and incubated at 30°C . CDM

contained the following components per liter of solution: 5 g sucrose, 1 g $(\text{NH}_4)_2\text{HPO}_4$, 2 g $(\text{NH}_4)_2\text{SO}_4$, 0.99 g KCl, 0.15 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 7.8 g NaH_2PO_4 , 7.1 g Na_2HPO_4 , 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, trace metals: 4.5 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 15 mg EDTA, 0.84 mg $\text{MnCl}_2 \cdot 2\text{H}_2\text{O}$, 0.3 mg $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.3 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.4 mg $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 4.5 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 3 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1 mg H_3BO_3 , 0.1 mg KI, vitamins: 0.05 d-biotin, 1 mg Ca pantothenate, 1 mg nicotinic acid, 1 mg myoinositol, 25 mg thiamine hydrochloride, 1 mg pyridoxal hydrochloride, 0.2 mg *p*-amino benzoic acid. All experiments were carried out aerobically in CDM. Chemicals were obtained from Sigma Aldrich (Castle Hill, NSW, Australia) and were analytical grade ($\geq 99\%$) except for the following purities: *d*-limonene (93%), terpenolene ($\geq 85\%$), oleyl alcohol (85%), farnesene (mixture of isomers), isopropyl myristate (90%).

Growth Conditions

Single colonies from CDM solid media were used as inocula for the pre-cultures. Pre-cultures in 10 mL of CDM were incubated in 100 mL baffled shake flasks in a shaking incubator (Multitron, Infors, Bottmingen, Switzerland) at 200 rpm (Orbit 25 mm) and 30°C . Pre-cultures were grown overnight until mid-log phase and then appropriate aliquots of the pre-culture broth were used to inoculate 25 mL of fresh CDM medium to a target OD_{660} of 0.2, respectively. Cultures were grown at 30°C aerobically in 250 mL baffled screw top shake flasks sealed with TeflonTM lining to avoid any monoterpene evaporation. Monoterpene evaporation in sealed versus non-sealed shake flasks was determined hourly for 12 h and significant monoterpene losses occurred in non-sealed flasks. For sealed and non-sealed flasks we observed similar growth rates ($0.35 \pm 0.01 \text{ h}^{-1}$, respectively) and biomass yields after 12 h of growth ($0.22 \pm 0.02 \text{ g}_{\text{DCW}} \text{ g}_{\text{sucrose}}^{-1}$, respectively), showing that sufficient oxygen was available in the headspace of sealed flasks to ensure aerobic conditions. At 5 h after inoculation ($\sim \text{OD}_{660} = 1.0$) varying amounts of monoterpene were added aseptically into the culture medium using a 10 μL glass-syringe. Cell growth was determined by measuring the optical density at 660 nm against water in a spectrophotometer (Libra S4, Cambridge, England) and all growth experiments were performed in triplicate. Specific growth rates were determined from OD_{660} values during the exponential growth phase after the addition of monoterpene. For aqueous-organic two-phase growth conditions an equal volume (10.2 mL) of organic solvent-limonene mixture was added to exponentially growing cultures at 5 h and growth was monitored as described. The following amounts of limonene in the solvent mixture were tested: 1, 2.5, and 5% ($v_{\text{limonene}} v_{\text{solvent-limonene mix}}^{-1}$). Evaporation of limonene from the solvents butyl oleate, dioctyl phthalate, dibutyl phthalate, isopropyl myristate, and farnesene was tested by measuring limonene concentration before and after two-phase cultivation by GC/MS as described below.

Organic Solvent Biocompatibility

Ten organic solvents were tested for biocompatibility with *S. cerevisiae*. A 10% ($v_{\text{solvent}} v_{\text{culture}}^{-1}$) volume of solvent was added to exponentially growing cultures at 5 h and growth was monitored as described previously. The growth rate of cultures with solvent was compared to a control with no solvent and the ratio was reported as $\mu \mu_{\text{max}}^{-1}$.

GC/MS analysis

Monoterpenes in the organic and aqueous phases were quantified in GC/MS. For this the organic and aqueous phases were separated using centrifugation. The aqueous phase underwent a liquid-liquid extraction using hexane. Equal volumes of hexane and aqueous phase (0.5 mL) were mixed, vortexed for 5 min and allowed to phase-separate at 25°C . Aliquots of the hexane extract of the aqueous phase and of the organic phase were diluted in hexane prior to quantification in order for the signals to fall within the calibration curve. A linear standard curve was achieved for standard solutions of known monoterpene stocks in hexane (100, 250, 500, 750, 1,000 μM). Menthol was used as an internal standard and was added to the hexane samples and the standards with a 10 μL glass-syringe (Gerstel, Mulheim, Germany) to achieve a final concentration of 500 μM prior to injection. For quantification, 3 μL sample was injected into the GC program temperature vaporizer (PTV) inlet in splitless mode at 250°C using helium as a carrier gas with a constant flow rate of 1 mL min^{-1} . Compounds were separated using a Varian factorFOUR capillary column (VF-5 ms: 0.25 mm inner diameter, 0.25 μm film, 30 m length with a 10 m fused guard column) on an Agilent 7890A gas chromatograph attached to an Agilent 5975C MSD. Initially the oven temperature was set to 40°C and was held for 5 min followed by a temperature ramp of $7.5^\circ\text{C min}^{-1}$ to 100°C , then $35^\circ\text{C min}^{-1}$ to 350°C and held for 5 min. Detection was achieved in scan mode at 9.26 scans sec^{-1} from 30 to 300 amu.

Monoterpene Partitioning Coefficients

The partitioning coefficient K_d is defined as the ratio of monoterpene concentration in the organic phase to aqueous phase. In order to determine K_d , equal volumes (0.5 mL) of aqueous CDM media and each organic solvent were vortexed vigorously overnight at 30°C . Known amounts of monoterpene were added directly into the organic solvent prior to mixing and each monoterpene K_d was determined in triplicate for each solvent. Samples were allowed to phase separate for 5 min after mixing at 30°C . Due to emulsion formation, samples with corn oil, oleyl alcohol, and dodecanol were centrifuged at 5,900 rcf for 5 min at 25°C (Eppendorf centrifuge 5415R, rotor: FA-45-24-11) to separate the two phases. After phase separation, monoterpene quantification in the aqueous and organic phases was determined by GC/MS as described above.

Viability Assessment

Propidium iodide (PI) was used to measure cell viability and membrane integrity. During limonene treated growth experiments, aliquots from the culture broth were analyzed at 2, 4, and 6 h after the addition of limonene or limonene-solvent mixtures. The final PI concentration in the sample was $2.5 \mu\text{g mL}^{-1}$ and samples were shaken as set by Quanta Cell Lab software well-prep mode in 96-well plates for 0.5 h at 25°C before analysis. As positive controls non-limonene treated cells were incubated in 70% ethanol at 30°C for 0.5 h, washed and resuspended in PBS. This caused 99.5% of the cells to be stained with PI. Cell suspensions were analyzed using a Beckman Coulter Cell Lab SC MPL flow-cytometer with an argon-ion laser emitting 488 nm beam at 22 mW. The intensity of fluorescence at FL3 (red fluorescence, 620 nm) was recorded using a logarithmic scale and the sample flow rate was $30 \mu\text{L min}^{-1}$.

Jet fuel Physicochemical Property Estimation

Physicochemical properties of limonane and limonane-farnesane fuel blends were estimated using suitable modeling software and compared to previously reported (Renninger et al., 2008) fuel specifications. Modeling in AspenPlus[®] (AspenTech, 2000) was used for transport and vapor liquid equilibrium properties (e.g., density, flash point, enthalpy of combustion, and viscosity) while the Dortmund Data Bank software (Onken et al., 1989) was used for freezing point calculations. The UNIFAC group contribution method (Fredenslund et al., 1977) was used for thermodynamic properties.

Chemostat Adaptation

A sucrose limited continuous fermentation was carried out in a 1.4 L Multifors benchtop bioreactor (InforsHT, Bottmingen, Switzerland) attached to a HPR-20 QIC gas analysis system (Hiden, Warrington, England). Synthetic media (CBS) containing 2 g L^{-1} sucrose, 5 g L^{-1} $(\text{NH}_4)_2 \text{SO}_4$, 3 g L^{-1} KH_2PO_4 , 0.5 g L^{-1} $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and identical concentrations of vitamins and trace metals of CDM, were used for chemostat experiments. An aerobic culture of 350 mL was run at 30°C , 500 rpm and was sparged with air at 0.35 L h^{-1} . The pH was maintained at 5 by automatic addition of 2 M NaOH. A dilution rate of 0.2 h^{-1} was used with an inlet feed of 72 mL h^{-1} . Limonene was continuously fed at $125 \mu\text{L h}^{-1}$ directly into the culture using an electronic syringe pump (New Era Pump System Inc., Farmingdale, NY) controlled by the Syringe Pump Pro software program (Version 1.54, Gawler, South Australia). The limonene concentration in the chemostat was determined directly. 1 mL of culture was removed and harvested by centrifugation at 5,900 rcf for 1 min at 25°C . The supernatant was separated from the cell pellet and immediately overlaid with $200 \mu\text{L}$ of hexane and then

vortexed for 30 s. The limonene concentration in the hexane layer was analyzed by GC/MS. The steady state limonene concentration was measured to be $0.53 \pm 0.04 \text{ mM}$ ($n = 3$). Culture purity was routinely monitored by light microscopy. Culture from the reactor was used to inoculate shake flask cultures as described in the growth conditions section to determine the MIC for limonene.

Results

Growth Inhibition by Monoterpenes

The toxicity of pure monoterpenes was determined by adding different amounts of the monoterpene to yeast measuring the growth rate over the ensuing 5 hr (Fig. 2). The monoterpene was added to yeast cells already in mid-exponential growth, in order to avoid confounding by lag phase phenomena. The minimum inhibitory concentration (MIC) was defined as the amount of monoterpene required to reduce the specific growth rate by 50% compared to control cultures with no monoterpene present. As shown in Figure 3, very low concentrations (MIC $0.4\text{--}2 \text{ mM}$, $0.0075\text{--}0.033\% \text{ v v}^{-1}$) for all five monoterpenes caused growth inhibition. Limonene was the most toxic (MIC = 0.44 mM) while the acyclic terpene myrcene was the least toxic (MIC = 2.12 mM). Even when growth is completely inhibited by limonene (0.6 mM), viability remains high 6 h after addition; it requires another magnitude increase in concentration to see substantial loss of viability, e.g., 73% reduction in viability at 6 mM (Fig. 2B). In contrast, if limonene is added to cultures immediately after inoculation, significant reduction in viability is seen even at MIC (data not shown).

Though low, the MIC of limonene is approximately an order of magnitude higher than its aqueous saturation point 0.045 mM (Schmid et al., 1992) (S_{Limonene} in Fig. 2 B). Cultures with limonene present in amounts beyond its solubility point (e.g., in Fig. 2 B, the 2nd and 3rd data points are 0.14 mM and 0.34 mM , respectively) were able to grow within 97% of the maximum growth rate with no limonene present. In fact, all five monoterpenes had MICs well above their solubility points (Table I).

An unsuccessful attempt was made to alleviate monoterpene toxicity via cell adaptation. After 885 h (225 generations) of continuous cultivation in the presence of 0.54 mM limonene, no significant improvement in the MIC (0.45 mM) was observed. Shown in Figure 2 B, identical limonene concentrations for the mutant and wild type had very similar impact on growth.

Solvent Selection for a Two-Phase Bioprocess to Reduce Monoterpene Toxicity In situ

In order to alleviate the observed monoterpene toxicity, 10 organic solvents were chosen from the literature based on

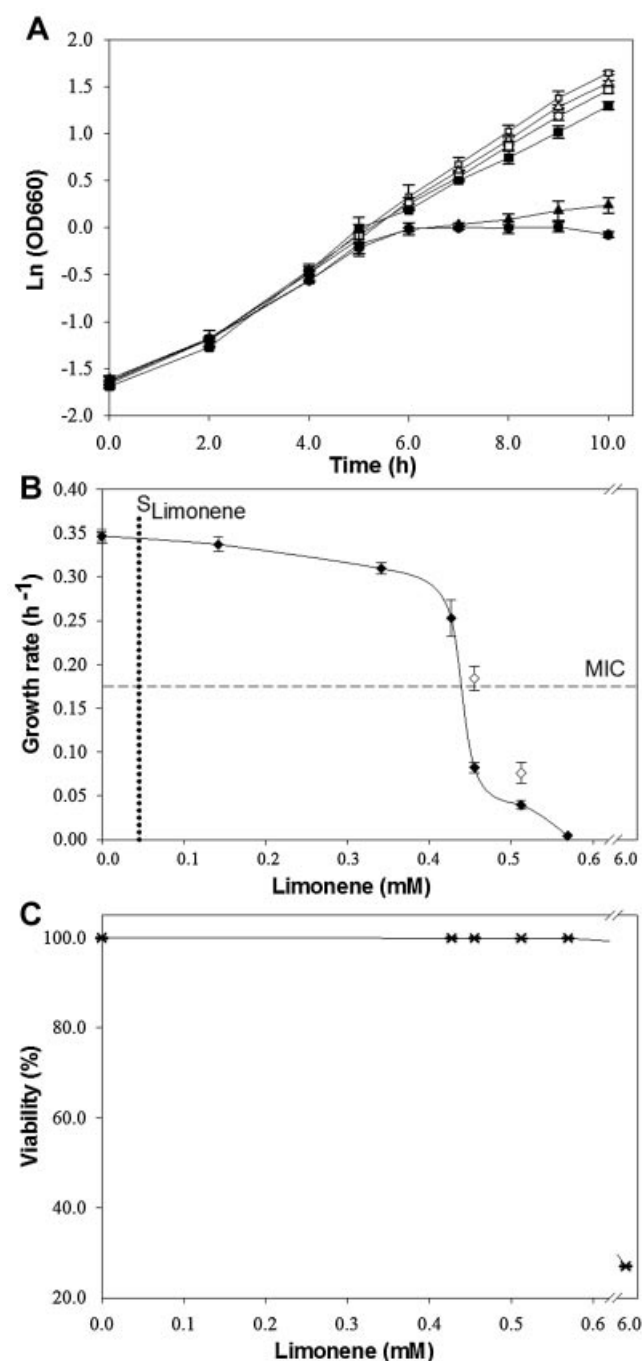


Figure 2. Growth and viability of limonene dosed cultures. **A:** Cultures were grown exponentially and then various amounts of limonene (□ control, △ 0.14 mM, ○ 0.34 mM, ■ 0.43 mM, ▲ 0.46 mM, ● 0.51 mM) were added exogenously at $t = 5$ h. **B:** Growth rate versus increasing limonene concentration (◆ wild-type, ◇ mutant); the aqueous solubility of limonene (S_{Limonene}) is 0.045 mM (Schmid et al., 1992). A 50% reduction in growth rate compared to the control without monoterpene is approximately 0.175 h^{-1} , this concentration is defined as the MIC. The mutant represents the limonene challenged chemostat strain after 885 h of continuous cultivation. **C:** Cell viability × was determined for the wild type after 6 h of limonene exposure. Error bars for growth rate and viability represent one standard deviation of biological triplicates (viability deviations were less than 0.02 and therefore are not visible).

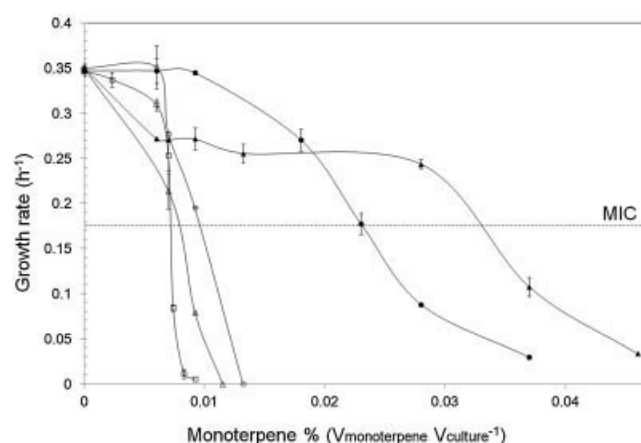


Figure 3. Growth rates of monoterpene dosed cultures (□-Limonene, △-Terpinolene, ○-γ-Terpinene, ●-β-pinene, ▲-Myrcene) versus increasing monoterpene concentration. Growth was monitored as described for limonene in the Figure 2. Error bars represent one standard deviation for biological triplicates.

their $\log P_{ow}$ coefficients, which is defined as the logarithm of the ratio of a compound's equilibrium concentration in the octanol phase to the aqueous phase (Laane et al., 1987; Vermue and Tramper, 1995). Although it is highly dependent on the microorganism, it is generally supported that an organic compound with $\log P_{ow} > 5$ should be biocompatible with *S. cerevisiae* (Bruce and Daugulis, 1991). Using a 10% ($v v^{-1}$) solvent phase, as described previously for similar experiments (Schewe et al., 2009), we confirmed that all 10 solvents met the criterion for biocompatibility with *S. cerevisiae* ($\mu \mu_{max}^{-1}$ values 0.65–1.0, see Table II). All five monoterpenes partitioned preferentially into the organic phase over the aqueous media ($\log K_d$ 1.8–2.7, see Table III). However, corn oil, oleyl alcohol, and dodecanol, caused emulsions that required separation by centrifugation (Table II). These solvents were excluded from the remaining experiments, since interference with biomass quantification could not be ruled out. Considering these characteristics, along with the solvent price, butyl oleate, dioctyl phthalate,

Table I. Monoterpene minimum inhibitory concentrations.

Monoterpene	MIC ^a (mM)	Aqueous solubility ^b (mM, 25°C)	Log P_{o-w} ^b
Limonene	0.44	0.045	4.4
Terpinolene	0.53	0.068	4.47
γ-Terpinene	0.70	0.064	4.5
β-Pinene	1.45	0.052	4.42
Myrcene	2.12	0.043	4.5

^aThe MIC is defined as the amount of terpene required to cause a 50% reduction in growth rate compared to the control with no monoterpene present. Although the aqueous solubility is exceeded, concentration units (mM) are used for explanatory reasons.

^bAqueous solubilities and $\log P_{ow}$ are taken from literature (Schmid et al., 1992).

Table II. Solvent selection criteria.

Solvent	$\mu \mu_{\max}^{-1} \pm \text{SD}$	Log P_{ow}	Log K_d limonene $\pm \text{SD}$	Phase separation	Price USD L ⁻¹
Corn oil	1.0 $\pm$ 0.09	7.4	2.15 $\pm$ 0.23	—	7.4
Dodecane	0.65 $\pm$ 0.09	6.6	2.20 $\pm$ 0.22	+	50.2
Hexadecane	0.90 $\pm$ 0.08	8.8	2.57 $\pm$ 0.06	+	67.1
Oleyl alcohol	0.90 $\pm$ 0.01	5.6	2.50 $\pm$ 0.16	—	17.2
Butyl oleate	0.95 $\pm$ 0.05	9.8 ^a	2.29 $\pm$ 0.15	+	6.1
Dibutyl phthalate	0.71 $\pm$ 0.12	4.63 ^b	1.60 $\pm$ 0.01	+	12.0
Dodecanol	0.65 $\pm$ 0.09	5.32	2.47 $\pm$ 0.15	—	8.5
Diocetyl phthalate	0.73 $\pm$ 0.06	7.54 ^a	2.53 $\pm$ 0.11	+	7.1
Farnesene	0.81 $\pm$ 0.08	7.1 ^c	ND	+	—
Isopropyl myristate	0.95 $\pm$ 0.04	7.4	ND	+	12.8

$\mu \mu_{\max}^{-1}$ was determined for biocompatibility by comparing the growth rate in the presence of 10% (v/v) solvent addition to cultures with no solvent ($\mu_{\max}$); log P_{ow} is the standard partitioning coefficient in an octanol-water system and values are from the previous report (Bruce and Daugulis, 1991); (—) indicates poor phase separation and centrifugation was required, (+) no centrifugation needed; bulk solvent price values were taken from (Spectrum, 2011). Standard deviations ($\pm \text{SD}$) are given ($n = 3$).

^aMattiasson and Holst (1991).

^bStales et al. (1997).

^cKishimoto et al. (2005).

dibutyl phthalate, isopropyl myristate, and farnesene were selected for further analysis.

The five selected solvents were then investigated in a limonene-solvent two-phase system. Limonene was the most inhibitory monoterpene (Fig. 3) and thus was chosen to screen the selected solvents for their ability to reduce toxicity. Based on the relative MICs for all five monoterpenes, we assumed that solvent candidates that were successful with limonene could be applied as extractants for the other four monoterpene products. The maximum amount of limonene that could be loaded into the solvent mixture before growth was severely inhibited was determined. In Figure 4, at 5% ($v v^{-1}$) limonene in dibutyl phthalate, growth was reduced by 50% (MIC 308.7 mM) with the amount of limonene present (42.1 g L⁻¹ aqueous culture) being 702-fold higher than the MIC for limonene in solvent-free cultures (Table IV). Butyl oleate was the least effective solvent with the growth rate being reduced by 80% in a 1% limonene load (Fig. 4). However, solvents dioctyl phthalate, isopropyl myristate, and farnesene also significantly reduced limonene toxicity with MICs over two-orders

of magnitude higher than the MIC for limonene with no solvent present (Table IV). Cell viability at these critical concentrations for each limonene-solvent system was measured using the PI staining method described above. Viability at each of the reported MICs was over 90% (Table IV).

Modeling Mono and Sesquiterpene Jet Fuel Blends

The fermentation products, limonene and farnesene, require an additional processing step to be used as jet fuels. For example, chemical hydrogenation is used to saturate C–C double bonds that exist in terpene compounds to produce alkane fuel targets such as limonane and farnesane (Ryder, 2009). In order demonstrate that our thermodynamic model accurately predicted fuel properties, we used our model to predict the previously published properties of a 97.1% limonane fuel composition (AMJ-300 in Table V) that was tested using industry standard (ASTM) methods (Renninger et al., 2008). Our predicted properties of AMJ-300 (UNIFAC

Table III. Monoterpene-solvent log K_d values.

Solvent	γ -Terpinene	Terpinolene	Myrcene	β -Pinene	<i>d</i> -Limonene
Corn oil	2.63 $\pm$ 0.13	ND	2.18 $\pm$ 0.24	1.21 $\pm$ 0.18	2.15 $\pm$ 0.23
Dodecane	2.64 $\pm$ 0.12	ND	2.16 $\pm$ 0.20	2.12 $\pm$ 0.09	2.20 $\pm$ 0.22
Hexadecane	2.73 $\pm$ 0.10	ND	2.45 $\pm$ 0.03	1.89 $\pm$ 0.08	2.57 $\pm$ 0.06
Oleyl alcohol	2.71 $\pm$ 0.05	ND	1.92 $\pm$ 0.30	2.08 $\pm$ 0.12	2.50 $\pm$ 0.16
Butyl oleate	2.5 $\pm$ 0.24	ND	1.84 $\pm$ 0.54	2.01 $\pm$ 0.18	2.29 $\pm$ 0.15
Dibutyl phthalate	2.46 $\pm$ 0.18	2.18 $\pm$ 0.46	2.06 $\pm$ 0.39	1.55 $\pm$ 0.14	1.60 $\pm$ 0.01
Dodecanol	2.72 $\pm$ 0.01	2.14 $\pm$ 0.40	2.30 $\pm$ 0.14	2.16 $\pm$ 0.06	2.47 $\pm$ 0.15
Diocetyl phthalate	2.64 $\pm$ 0.03	ND	2.33 $\pm$ 0.17	1.96 $\pm$ 0.26	2.53 $\pm$ 0.11
Farnesene	ND	2.85 $\pm$ 0.21	2.60 $\pm$ 0.26	ND	ND
Isopropyl myristate	ND	ND	ND	ND	ND

K_d is the ratio of monoterpene in the organic solvent phase to the aqueous media phase. Standard deviations are given ($n = 3$). ND, no monoterpene was detected in the aqueous phase.

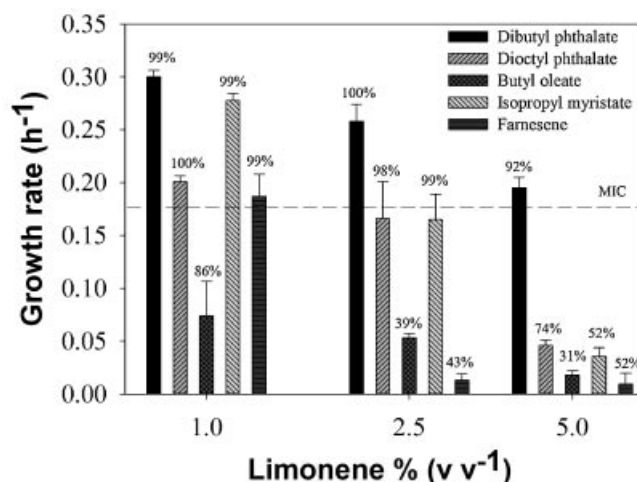


Figure 4. Growth rate versus limonene loading in butyl oleate, dibutyl phthalate, dioctyl phthalate, isopropyl myristate and farnesene. Limonene was loaded at 1, 2.5, 5% ($\text{v}_{\text{limonene}} \text{v}_{\text{limonene} + \text{solventmix}}^{-1}$) in a limonene-solvent mixture. The % of viable cells is given above each growth rate value. Growth was monitored as described in the Figure 2. Error bars are one standard deviation above the mean for biological triplicates.

AMJ-300 in Table V) were comparable with those previously reported ($<10\%$ error). Due to limonene's toxicity, we then used this model to find the minimum limonane load in farnesane that met Jet-A properties. The physicochemical properties of a 10% (v v^{-1}) blend of limonane in farnesane are very similar to Jet-A specifications.

Discussion

Monoterpenes are known fungicidal agents, but the exact mechanisms of action are not well understood. For solvent-caused toxicity in microbes, two types of toxicity have been distinguished: Molecular and phase (Bar, 1987). Molecular toxicity has been described as compounds that are soluble in the aqueous phase and due to their hydrophobicity, intercalate within the lipid bilayer and deteriorate membrane function (Sikkema et al., 1995). Increases in membrane fluidity, membrane damage, denaturation of membrane-bound proteins, and loss of energy transduction are some of the consequences of a solvent's impact at the

molecular level (Inoue and Horikoshi, 1991; León et al., 1998; Sikkema et al., 1994b; Vermue et al., 1993). Alternatively, phase toxicity occurs when the amount of solvent present in the culture medium exceeds the compounds solubility point and a distinct second phase exists. The presence of a second phase means that thermodynamically, the aqueous phase is fully saturated as well as the different compartments of the biomass (e.g., cytoplasmic membrane) (León et al., 1998; Osborne et al., 1990; Sikkema et al., 1994b). Phase toxicity could be caused by extraction of outer-cellular components during cell-solvent contact, extraction of nutrients from the media, or cell-coating (Bar, 1987). We observe severe toxicity for all five monoterpenes only well beyond their aqueous solubilities (Table I) and in addition, the most toxic monoterpene found in this work (limonene) caused no growth inhibition when the aqueous media was saturated with limonene (Fig. 2B). It was previously suggested that monoterpene toxicity in microbes occurs at the molecular level causing structural damage to the plasma membrane and interfering with its normal function (Sikkema et al., 1994a; Uribe et al., 1985). In contrast, we observed no loss of viability at the MIC of limonene (Fig. 2B). Only at very high limonene loadings (6.2 mM) was membrane damage observed, resulting in 73% of the total population being no longer viable (Fig. 2B). We did observe significant loss of viability at the MIC of limonene, when added to the culture immediately after inoculation, and this may explain why previous studies (Andrews et al., 1980) have observed molecular toxicity. We conclude that toxicity observed when limonene is added (or slowly accumulating) during *S. cerevisiae* culture must be due to phase effects.

The observation of phase toxicity has implications for strain optimization. We have shown that although growth stops, the cells are still viable. Controlling strains to begin monoterpene production after the initial growth phase is a potential route to address toxicity constraints. For example, the highest production of lactic acid (100 to 154 g L^{-1}), which is also considered a toxic end product, in *Lactobacillus* occurs during the stationary phase of cell growth (Wee et al., 2005). Another important implication of phase toxicity is with respect to engineering tolerance in the biocatalyst. For instance, engineering efflux systems to export compounds from the membrane, as described recently for limonene and the other biofuel targets *n*-butanol and α -pinene in *E. coli* (Dunlop et al., 2011), should not alleviate monoterpene toxicity in *S. cerevisiae* because, as demonstrated here, the

Table IV. Solvent-limonene mixture MICs and cell viability.

Solvent	Limonene MIC (mM)	Limonene (g L^{-1})	Fold increase vs. solvent free system	Cell viability (%)
No solvent	0.44	0.06	—	99
Dibutyl phthalate	308.7	42.1	702	92
Dioctyl phthalate	154.3	21.0	351	98
Isopropyl myristate	154.3	21.0	351	99
Farnesene	61	8.4	140	99

Table V. Predicted jet fuel thermodynamic and physicochemical properties.

Property	Units	Jet A (ASTM)	AMJ-300 (ASTM)	AMJ-300 (UNIFAC)	UQJ-1 (UNIFAC)
Flash point ^a	°C	Min. 38	43	44.37	56.45
Density (at 15°C/288.15 K)	Kg m ⁻³	775–840	800.9	768.27	778.75
Freezing point ^b	°C	Max -40	<-70	-97.65	-40.36
Viscosity (at -20°C/253.15 K)	mm ² s ⁻¹	Max 8.0	2.849	3.818	7.714
dH° combustion (NET)	MJ kg ⁻¹	Min 42.8	43.4125	43.3341	43.9351

AMJ-300 values are from the previous report (Renninger et al., 2008) and contain limonane 97.1 wt% and *p*-cymene 1.6 wt%; UQJ-1 contains 10% v v⁻¹ limonane and 90% v v⁻¹ farnesane.

^aRiazi (1986).

^bConstantinou and Gani (1994).

monoterpene aqueous concentration and consequently, the membrane concentration, is saturated well before we observe any growth inhibition (Table I). Furthermore, we showed that simply evolving the cells over time, which is one of the most straightforward methods for generating improved phenotypes (Kwon et al., 2011), showed negligible improvement in limonene tolerance, MIC increased from 0.44 to 0.45 (see mutant in Fig. 2B).

In contrast to the limited success of cellular engineering, manipulating phase properties by introducing an inert solvent phase shows substantial potential (e.g., MIC for limonene with the solvent dibutyl phthalate is 702-fold higher than the MIC with no solvent). The monoterpene partitioning coefficients (K_d) (Bruce and Daugulis, 1991) for the 10 biocompatible solvents studied are several orders of magnitude higher ($\log K_d = 2$ –2.85, see Table III) than those ($\log K_d = 0.78$) reported for an economically feasible extractive ethanol fermentation plant (Maiorella et al., 1984), indicating the commercial potential of such a process. Furthermore, high limonene loads can be achieved in the solvent phase without compromising cell viability. This means that the solvent can act as an effective product sink in situ without negatively impacting the producing organism. As shown in Table IV, cultures with 42.1 g L⁻¹ of limonene in dibutyl phthalate can maintain 92% cell viability. Dibutyl phthalate showed the highest limonene load and this may be explained by its similar hydrophobicity to limonene ($\log P_{ow}$ is 4.63 in Table II and 4.5 for limonene in Table I) compared to the other solvents tested. To our knowledge this is the highest monoterpene concentration reported during fermentation with baker's yeast.

The use of a solvent that does not require separation from the monoterpene products would simplify this technique. Although Figure 4 suggests that farnesene is not the best solvent option to reduce limonene toxicity, farnesene is of particular interest as an extractive solvent since it can be synthesized by yeast during fermentation (Renninger and McPhee, 2008) and, as shown above, a blend of 10% (v v⁻¹) limonane in farnesane exhibits physicochemical properties similar to Jet-A (Table V). Using farnesene as an extractant and a co-product will ultimately require careful process engineering that balances both monoterpene toxicity and desired fuel properties. An organism with an ability to produce both the extractant and monoterpene product in

situ will positively impact raw-material and downstream processing costs. This is likely to outweigh toxicity advantages of the other solvents tested. By holding the 10% (v v⁻¹) terpene fuel blend constant, we found that by reducing the ratio of organic to culture volume from 0.05, where we observed no growth and 88% loss in cell viability, to 0.005, both fast cell growth (80% of μ_{max}) and high viability (99%) can be achieved (data not shown). The amount of limonene in the latter system (2.8 mM, 380 mg L⁻¹), is 6.3-fold higher than the MIC without any solvent present.

Conclusion

End product inhibition is a critical problem for the microbial synthesis of monoterpene-derived fuels and chemicals. We have provided quantitative data for the inhibitory limits of five common monoterpene products and shown that in *S. cerevisiae*, the mechanism of toxicity is likely to be a phase effect. As an alternative to cellular engineering, we have demonstrated that a biphasic extractive system can significantly enhance fermentation by alleviating monoterpene toxicity in situ. To make this process even more efficient, we have shown that by using farnesene as the extractant, one could obtain a terpene mix that could be directly used for hydrogenation into jet fuel. Depending on the other components of the organic phase this could at the same time reduce the downstream processing cost (e.g., distillation).

We thank Chris Paddon (Amyris Inc, Emeryville, CA) and Andreas Schmid (Technical University Dortmund, Germany) for useful discussions and the Queensland government (National and International Research Alliances Program) for financial support. We also thank the Australian Wine Research Institute (AWRI, Adelaide, SA, Australia) for providing us with the yeast used in this study.

References

- Amyris. 2009. (Press release) Embraer, General Electric, Azul, and Amyris announce renewable jet fuel evaluation project. (<http://www.amyris.com/en/newsroom/83-renewable-jet-fuel-evaluation>).

- Andrews RE, Parks LW, Spence KD. 1980. Some effects of douglas-fir terpenes on certain microorganisms. *Appl Environ Microbiol* 40(2):301–304.
- AspenTech. 2000. ASPEN plus user's manual. Cambridge, USA: Aspen Technology Inc.
- Bar R. 1987. Phase toxicity in a water-solvent two-liquid phase microbial system. In: Laane C, Tramper J, Lilly MD, editors. *Studies in organic chemistry: 29. Biocatalysis in Organic Media*; International Symposium Wageningen, Netherlands December 7–10, 1986. Amsterdam: Elsevier. p 147–154.
- Bar R, Gainer JL. 1987. Acid Fermentation in water-organic solvent two-liquid phase systems. *Biotechnol Prog* 3(2):109–114.
- Bruce LJ, Daugulis AJ. 1991. Solvent selection strategies for extractive biocatalysis. *Biotechnol Prog* 7(2):116–124.
- Carrau FM, Medina K, Boido E, Farina L, Gaggero C, Dellacassa E, Versini G, Henschke PA. 2005. *De novo* synthesis of monoterpenes by *Saccharomyces cerevisiae* wine yeasts. *FEMS Microbiol Lett* 243(1):107–115.
- Carter OA, Peters RJ, Croteau R. 2003. Monoterpene biosynthesis pathway construction in *Escherichia coli*. *Phytochemistry* 64(2):425–433.
- Constantinou L, Gani R. 1994. New group contribution method for estimating properties of pure compounds. *AIChE J* 40(10):1697–1710.
- Daugulis AJ, Swaine DE, Kollerup F, Groom CA. 1987. Extractive fermentation-integrated reaction and product recovery. *Biotechnol Lett* 9(6):425–430.
- Dunlop MJ, Dossani ZY, Szmidi HL, Chu HC, Lee TS, Keasling JD, Hadi MZ, Mukhopadhyay A. 2011. Engineering microbial biofuel tolerance and export using efflux pumps. *Mol Syst Biol* 7:487.
- Fischer MJC, Meyer S, Claudel P, Bergdoll M, Karst F. 2011. Metabolic engineering of monoterpene synthesis in yeast. *Biotechnol and Bioeng* 108(8):1883–1892.
- Fortman JL, Chhabra S, Mukhopadhyay A, Chou H, Lee TS, Steen E, Keasling JD. 2008. Biofuel alternatives to ethanol: Pumping the microbial well. *Trends Biotechnol* 26(7):375–381.
- Fredenslund A, Gmehling J, Rasmussen P. 1977. Vapor-liquid equilibria using UNIFAC. Amsterdam: Elsevier.
- Gould MN. 1997. Cancer chemoprevention and therapy by monoterpenes. *Environ Health Perspect* 105:977–979.
- Grohmann K, Baldwin EA, Buslig BS. 1994. Production of ethanol from enzymatically hydrolyzed orange peel by the yeast *Saccharomyces cerevisiae*. *Appl Biochem Biotechnol* 45/46:315–327.
- Harvey BG, Wright ME, Quintana RL. 2009. High-density renewable fuels based on the selective dimerization of pinenes. *Energy Fuels* 24(1):267–273.
- Hull A, Golubkov I, Kronberg B, Marandzheva T, Stam J. 2006. An alternative fuel for spark ignition engines. *Int J Engine Res* 7(3): 203–214.
- Inoue A, Horikoshi K. 1991. Estimation of solvent-tolerance of bacteria by the solvent parameter log P. *J Ferment Bioeng* 71(3):194–196.
- Janusz JM. 2001. Two-phase partitioning bioreactors in fermentation technology. *Biotechnol Adv* 19(7):525–538.
- Keasling JD. 2010. Microbial production of isoprenoids. In: Timmis KN, editor. *Handbook of hydrocarbon and lipid microbiology*. Berlin: Springer. p. 2951–2966.
- Kishimoto T, Wanikawa A, Kagami N, Kawatsura K. 2005. Analysis of hop-derived terpenoids in beer and evaluation of their behavior using the stir bar—sorbent Extraction Method with GC-MS. *J Agric Food Chem* 53(12):4701–4707.
- Kwon Y-D, Kim S, Lee SY, Kim P. 2011. Long-term continuous adaptation of *Escherichia coli* to high succinate stress and transcriptome analysis of the tolerant strain. *J Biosci Bioeng* 111(1):26–30.
- Laane C, Boeren S, Vos K, Veeger C. 1987. Rules for optimization of biocatalysis in organic solvents. *Biotechnol Bioeng* 30(1):81–87.
- Lambert RJW, Skandamis PN, Coote PJ, Nychas GJE. 2001. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. *J Appl Microbiol* 91(3): 453–462.
- León R, Fernandes P, Pinheiro HM, Cabral JMS. 1998. Whole-cell biocatalysis in organic media. *Enzyme Microb Technol* 23(7–8):483–500.
- Maioresella BL, Blanch HW, Wilke CR. 1984. Economic evaluation of alternative ethanol fermentation processes. *Biotechnol Bioeng* 26(9): 1003–1025.
- Malinowski JJ. 2001. Two-phase partitioning bioreactors in fermentation technology. *Biotechnol Adv* 19(7):525–538.
- Mattiasson B, Holst O. 1991. *Extractive bioconversions*. New York: Marcel Dekker. p 211.
- Murdock DI, Allen WE. 1960. Germicidal effect of orange peel oil and *d*-limonene in water and orange juice. *Food Technol* 14(9):441–445.
- Newman J, Marshall J, Chang M, Nowroozi F, Paradise E, Pitera D, Newman K, Keasling J. 2006. High-level production of amorpho-4,11-diene in a two-phase partitioning bioreactor of metabolically engineered *Escherichia coli*. *Biotechnol Bioeng* 95:684–691.
- Onken U, Rarey-Nies J, Gmehling J. 1989. The Dortmund Data Bank: A computerized system for retrieval, correlation, and prediction of thermodynamic properties of mixtures. *Int J Thermophys* 10(3):739–747.
- Osborne SJ, Leaver J, Turner MK, Dunnill P. 1990. Correlation of biocatalytic activity in an organic aqueous 2-liquid phase system with solvent concentration in the cell-membrane. *Enzyme Microb Technol* 12(4): 281–291.
- Oswald M, Fischer M, Dirniger N, Karst F. 2007. Monoterpenoid biosynthesis in *Saccharomyces cerevisiae*. *FEMS Yeast Res* 7(3):413–421.
- 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.
- Pourbafrani M, Talebnia F, Niklasson C, Taherzadeh M. 2007. Protective effect of encapsulation in fermentation of limonene-contained media and orange peel hydrolyzate. *Int J Mol Sci* 8(8):777–787.
- Reiling KK, Yoshikuni Y, Martin VJJ, Newman J, Bohlmann J, Keasling JD. 2004. Mono and diterpene production in *Escherichia coli*. *Biotechnol Bioeng* 87(2):200–212.
- Renninger N, McPhee D. 2008. Fuel compositions including farnesane and farnesene derivatives and methods of making and using same. US Patent: WO2008045555.
- Renninger N, Ryder J, Fisher K. 2008. Jet fuel compositions and methods of making and using same. US Patent: WO2008133658.
- Renouf MA, Wegener MK, Nielsen LK. 2008. An environmental life cycle assessment comparing Australian sugarcane with US corn and UK sugar beet as producers of sugars for fermentation. *Biomass Bioenergy* 32(12):1144–1155.
- Riazi M. 1986. API databook, 5th edition. procedure 2B7.1.
- Roffler SR, Blanch HW, Wilke CR. 1988. In situ extractive fermentation of acetone and butanol. *Biotechnol Bioeng* 31(2):135–143.
- Ryder JA, Amyris Biotechnologies Inc (AMYR-Non-standard), assignee. 2009. Fuel composition, useful to power any equipment such as an emergency generator or internal combustion engine, which requires a fuel such as jet fuels or missile fuels, comprises limonene and farnesane. US Patent: 7589243.
- Schewe H, Holtmann D, Schrader J. 2009. P450BM-3-catalyzed whole-cell biotransformation of α -pinene with recombinant *Escherichia coli* in an aqueous-organic two-phase system. *Appl Microbiol Biotechnol* 83(5): 849–857.
- Schmid C, Steinbrecher R, Ziegler H. 1992. Partition-coefficients of plant cuticles for monoterpenes. *Trees* 6(1):32–36.
- Sikkema J, de Bont J, Poolman B. 1995. Mechanisms of membrane toxicity of hydrocarbons. *Microbiol Rev* 59(2):201–222.
- Sikkema J, de Bont JA, Poolman B. 1994a. Interactions of cyclic hydrocarbons with biological membranes. *J Biol Chem* 269(11):8022–8028.
- Sikkema J, Weber FJ, Heipieper HJ, Bont JAMD. 1994b. Cellular toxicity of lipophilic compounds: mechanisms, implications, and adaptations. *Biocatalysis* 10:113–122.
- Spectrum. 2011. Spectrum chemicals and laboratory products. https://www.spectrumchemical.com/OA_HTML/ibeCCtpSctDspRte.jsp?minisite=10020&site=&respid=50577.
- Stales CA, Peterson DR, Parkerton TF, Adams WJ. 1997. The environmental fate of phthalate esters: A literature review. *Chemosphere* 35(4):667–749.

- Stark D, von Stockar U. 2003. In situ product removal (ISPR) in whole cell biotechnology during the last twenty years. Process Integration in Biochemical Engineering. In: von Stockar U, van der Wielen L, Bruggink A, Cabral J, Enfors SO, Fernandes P, Jenne M, Mauch K, Prazeres D, Reuss M, editors. Advances in biochemical engineering/biotechnology. Berlin: Springer. p. 149–175.
- Subba MS, Soumithr TC, Rao RS. 1967. Antimicrobial action of citrus oils. J Food Sci 32(2):225–229.
- Uribe S, Pena A. 1990. Toxicity of allelopathic monoterpene suspensions on yeast dependence on droplet size. J Chem Ecol 16(4):1399–1408.
- Uribe S, Ramirez J, Pena A. 1985. Effects of β -pinene on yeast membrane functions. J Bacteriol 161(3):1195–1200.
- van der Werf M, de Bont J, Leak D. 1997. Opportunities in microbial biotransformation of monoterpenes. In: Berger R, Babel W, Blanch H, Cooney C, Enfors S, Eriksson K, Fiechter A, Klibanov A, Mattiasson B, Primrose S, editors. Biotechnology of aroma compounds. Berlin: Springer. p 147–177.
- Vermue M, Sikkema J, Verheul A, Bakker R, Tramper J. 1993. Toxicity of homologous series of organic solvents for the gram-positive bacteria *Arthrobacter* and *Nocardia* Sp. and the gram-negative bacteria *Acinetobacter* and *Pseudomonas* Sp. Biotechnol Bioeng 42(6):747–758.
- Vermue MH, Tramper J. 1995. Interrelations of chemistry and biotechnology. Pure Appl Chem 67:345–373.
- Wee Y-J, Kim J-N, Yun J-S, Ryu H-W. 2005. Optimum conditions for the biological production of lactic acid by a newly isolated lactic acid bacterium *Lactobacillus* sp. RKY2. Biotechnol Bioprocess Eng 10(1):23–228.
- Wilkins M, Suryawati L, Maness N, Chrz D. 2007. Ethanol production by *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* in the presence of orange-peel oil. World J Microbiol Biotechnol 23(8):1161–1168.