ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Production of Farnesene and Santalene by *Saccharomyces cerevisiae* Using Fed-Batch Cultivations With *RQ*-Controlled Feed

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ABSTRACT: Terpenes have various applications as fragrances, cosmetics and fuels. One of the most prominent examples is the sesquiterpene farnesene, which can be used as diesel substitute in its hydrogenated form farnesane. Recent metabolic engineering efforts have enabled efficient production of several terpenes in *Saccharomyces cerevisiae* and *Escherichia coli*. Plant terpene synthases take on an essential function for sesquiterpene production as they catalyze the specific conversion of the universal precursor farnesyl diphosphate (FPP) to the sesquiterpene of interest and thereby impose limitations on the overall productivity. Using farnesene as a case study, we chose three terpene synthases with distinct plant origins and compared their applicability for farnesene production in the yeast *S. cerevisiae*. Differences regarding the efficiency of these enzymes were observed in shake flask cultivation with maximal final titers of 4 mg/L using α-farnesene synthase from *Malus domestica*. By employing two existing platform strains optimized for sesquiterpene production, final titers could be raised up 170 mg/L in fed-batch fermentations with *RQ*-controlled exponential feeding. Based on these experiments, the difference between the selected synthases was not significant. Lastly, the same fermentation setup was used to compare these results to production of the fragrance sesquiterpene santalene, and almost equivalent titers were obtained with 163 mg/L, using the highest producing strain expressing a santalene synthase from *Clausena lansium*. However, a reduction of the product yield on biomass by 50% could indicate a higher catalytic efficiency of the farnesene synthase.

Biotechnol. Bioeng. 2016;113: 72–81.

KEYWORDS: isoprenoids; terpene synthases; biofuels; yeast; metabolic engineering; fed-batch

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Conflicts of interest: ST, GS, VN, and JN declare to have no competing interests.

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Received 19 March 2015; Accepted 15 June 2015
Accepted manuscript online 24 June 2015;
Article first published online 2 September 2015 in Wiley Online Library (http://onlinelibrary.wiley.com/doi/10.1002/bit.25683/abstract).
DOI 10.1002/bit.25683

Introduction

Isoprenoids have attracted great interest in the recent past based on their applications in the food and pharmaceutical industry (Tippmann et al., 2013). In addition, particularly mono- and sesquiterpenes have been identified as suitable diesel and jet fuel substitutes (Meylemans et al., 2012; Millo et al., 2014). Production of these compounds by plant extraction mostly fails to meet industrial scale and is often not sustainable or dependent on seasonal and geographical changes. The design of microbial cell factories by metabolic engineering, however, offers an alternative production route by redirecting cellular metabolism towards a desired product, which has been successfully demonstrated for production of several sesquiterpenes in the yeast *Saccharomyces cerevisiae* (Asadollahi et al., 2008; Paddon et al., 2013; Peralta-Yahya et al., 2011; Scalcinati et al., 2012a). All isoprenoids are produced from the activated isoprene units isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), which are both derived from the mevalonate pathway in *S. cerevisiae* (Fig. 1). The metabolic route to farnesene comprises nine enzymatic reactions from acetyl-CoA and is both highly energy and co-factor dependent, as the production of one mole of farnesene from three moles of acetyl-CoA requires nine moles of ATP and six moles of NADPH. Furthermore, the essential role of farnesyl diphosphate (FPP) for sterol synthesis limits the FPP availability for production of desired sesquiterpenes. Using a combination of different metabolic engineering strategies including engineering of the FPP branch point, increasing flux through the mevalonate pathway and improving co-factor balance, the production of the fragrance precursor α-santalene was recently enabled in *S. cerevisiae* (Scalcinati et al., 2012b).

The terminal conversion of FPP to farnesene is catalyzed by farnesene synthase, which is not expressed by *S. cerevisiae* endogenously, but found in various plants (Chen et al., 2011). By removal of the diphosphate group, which results in a rearrangement of the double bonds in the molecule, two isomers are formed, α- and β-farnesene, with six stereoisomers overall.

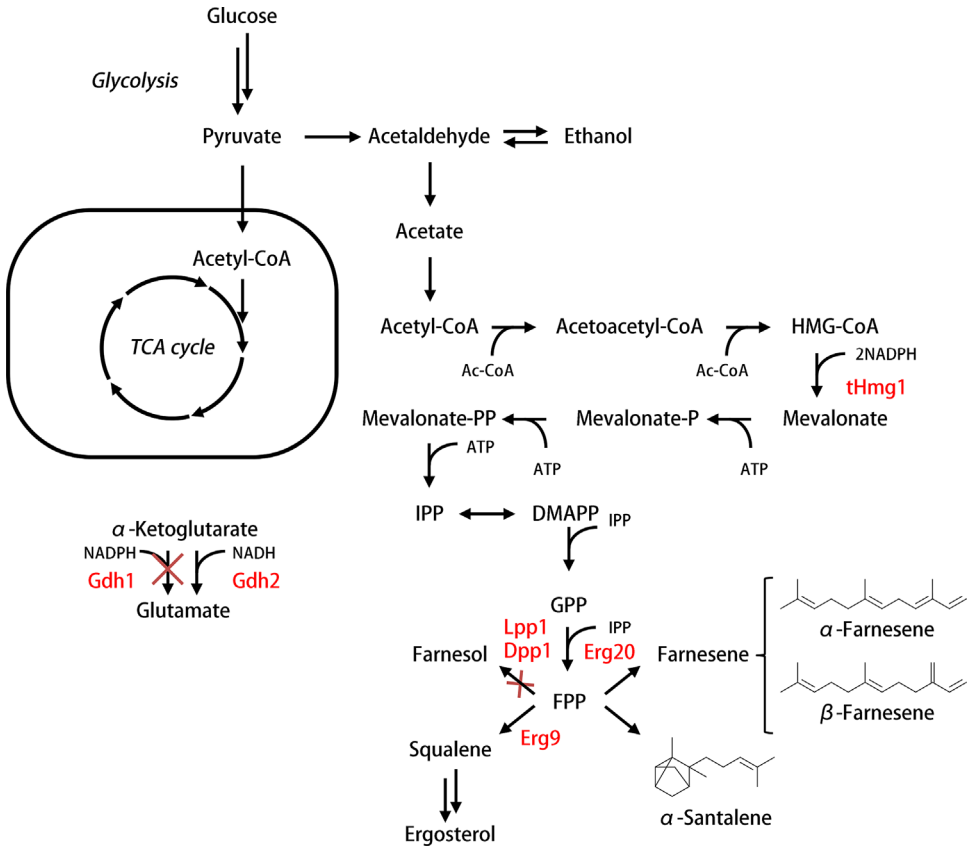

Figure 1. Production of farnesene and santalene from glucose in *S. cerevisiae*. Isoprenoids are synthesized via the mevalonate pathway from cytosolic acetyl-CoA, which is derived from pyruvate via the PDH bypass. The mevalonate pathway comprises seven steps and yields the isomers IPP and DMAPP. Condensations of the two isomers form the precursors for mono-, sesqui- or longer chain terpenoids. In the last step, FPP is converted by farnesene synthase to yield farnesene. Similarly, santalene is produced from FPP by santalene synthase. ATP and co-factor requirements are shown only for the mevalonate pathway. Enzymes in red denote engineering targets used in this study with tHmg1: truncated HMG-CoA reductase, Erg9: squalene synthase, Erg20: FPP synthase, Lpp1: lipid phosphate phosphatase, Dpp1: diacylglycerol diphosphate phosphatase, Gdh1/2: glutamate dehydrogenase. IPP: isopentenyl diphosphate, DMAPP: dimethylallyl diphosphate, GPP: geranyl diphosphate, FPP: farnesyl diphosphate, Ac-CoA: acetyl-coA.

Poor expression of these terpene synthases has been identified as a major constraint on production of sesquiterpenes (Martin et al., 2001). Moreover, substrate affinity and turnover of these enzymes are key parameters for an efficient production of farnesene from the FPP branch point, and have not been compared *in vivo* hitherto.

Farnesene synthases are widely spread in the plant kingdom and coding sequences are available for several organisms including *Zea mays*, *Cucumis melo*, *Pyrus communis* and *Mentha arvensis* (Nucleotide Database, National Center for Biotechnology Information). The objective of this study was to express three farnesene synthase genes from plants in *S. cerevisiae*, i.e., α-farnesene synthase from *Malus domestica* and ß-farnesene synthase from *Citrus junos* and *Artemisia annua*, respectively, which have previously been characterized regarding their reaction conditions, co-factor requirements and product specificities (Crock et al., 1997; Green et al., 2007; Maruyama et al., 2001; Pechous and Whitaker, 2004; Picaud et al., 2005; Rupasinghe et al., 2000) and compare production of farnesene with production of another sesquiterpene. For this purpose, an existing platform for sesquiterpene production was used to investigate production capacity in comparison to α-santalene. Down-regulation of sterol synthesis in this platform strain at low extracellular glucose concentrations (Scalcinati et al., 2012a) prompted us to investigate production of farnesene in glucose limited conditions. Fed-batch processes are widely used in industrial applications to attain high cell densities avoiding substrate inhibition and overflow metabolism, which is reported to occur in *S. cerevisiae* at glucose concentrations above 0.04 g/L and is known to reduce biomass yields (Geurts et al., 1980; Pham et al., 1998; Verduyn, 1991). We therefore chose an exponential feeding strategy to account for exponential growth of the host organism. Controlling the feed rate by the respiratory quotient (*RQ*) is an additional strategy to avoid overfeeding and carbon loss due to the Crabtree effect. Setting the *RQ* to a value of 1, which translates to the threshold for ethanol formation, aims at maintaining metabolism in a state optimized for growth.

Materials and Methods

Plasmid and Strain Construction

Synthetic, codon optimized genes for farnesene synthases from *M. domestica*, *C. junos*, and *A. annua* were purchased from GenScript

(GenScript, Piscataway, NJ) and cloned into plasmids pSP-G1 (Partow et al., 2010) and pICK01 (Scalcinati et al., 2012a) using restriction enzymes *Not*I and *Pac*I (Thermo Fisher Scientific, Waltham, MA, USA) (Table I). Strains with auxotrophy for uracil were grown on YPD plates containing 20 g/L glucose, 10 g/L yeast extract, 20 g/L peptone from casein, and 20 g/L agar. Plasmid containing strains were grown on selective growth medium containing 6.9 g/L yeast nitrogen base w/o amino acids (Formedium, Hunstanton, UK), 0.77 g/L complete supplement mixture w/o uracil (Formedium), 20 g/L glucose, and 20 g/L agar.

Growth Media

All batch cultivations in shake flasks and bioreactors were performed using minimal media as described by Verduyn et al. (1992) containing 20 g/L glucose, 5 g/L $(NH_4)_2SO_4$, 3 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 1 mL/L trace element solution and 50 μL/L antifoam. The medium was adjusted to pH 5 using 2 M KOH and 1 mL/L vitamin solution was added after sterilization. The feed medium for fed-batch cultivations was ten times concentrated and contained 200 g/L glucose.

Shake Flask Cultivations

Strains were grown on plates containing selective growth medium at 30°C. A single colony was used to inoculate 5 mL of minimal medium, which was incubated overnight at 30°C and 200 rpm. Subsequently, 100 mL shake flasks without baffles containing 18 mL of liquid medium were inoculated at an OD600 of 0.1 and an extractive dodecane overlay (≥99%, Sigma–Aldrich, St. Louis, MO) was added at 10% v/v to sequester farnesene. Shake flasks were incubated at 30°C and 160 rpm.

Bioreactor Cultivations

Similar to the shake flask cultivations described above, a single colony from a fresh plate containing selective medium was used to inoculate 5 mL minimal medium and incubated overnight at 30°C and 200 rpm. Cells were then transferred to 50 mL of liquid medium in 500 mL shake flasks and cultivated overnight at 30°C and 160 rpm. Subsequently, the preculture was harvested by centrifugation at 4000 rpm for 5 min and resuspended in 10 mL of fresh medium. The OD600 was measured using a Genesis20 spectrophotometer (Thermo Fisher Scientific) to inoculate the bioreactors at an OD600 of 0.1. All cultivations were performed in 2.5 L Applikon vessels (Applikon, Delft, The Netherlands) using the DasGip Microbiology PD system (DasGip, Jülich, Germany). Homogenous mixing was enabled using two six-blade Rushton turbines at 600 rpm. The temperature was controlled at 30°C and the pH was adjusted to 5 using 2 M KOH and 2 M HCl. The vessels were sparged with air at an initial rate of 60 L/h. To assure constant aeration at 1 vvm, the aeration rate was increased during the fed-batch phase according to the culture volume. The batch phase was started with an initial volume of 1 L. The feed was initiated after glucose and ethanol depletion, which was monitored by on-line CO_2 measurements obtained from the exhaust gas analysis. Prior to feed start, 200 mL of dodecane were added to the vessels under aseptic conditions.

RQ-Control and Feed Profile Development

Cultivations in bioreactors were conducted in fed-batch mode with *RQ*-controlled exponential feeding. The aerobic fed-batch process was performed in 2.5 L Applikon vessels (Applikon, Schiedam, The Netherlands) with an initial working volume of 1.0 L. Agitation at 800 rpm was maintained using an integrated stirrer (DasGip) and the temperature was monitored using a platinum RTD temperature sensor and kept at 30°C. The rate of aeration was set to 1 vvm by a mass flow controller. The pH of the medium was maintained at 5.0 by automatic addition of 2 M KOH and 2 M HCl. The temperature, agitation, gassing, pH and composition of the off-gas were monitored and controlled using the integrated DasGip monitoring and control system. Dissolved oxygen concentration was monitored with an autoclavable polarographic oxygen electrode (Mettler Toledo, Columbus, OH) and kept above 30% via stirrer speed and gas flow rate using the DasGip control system. The effluent gas from the fermentation was cooled, dried and analyzed for real-time determination of oxygen and CO_2 concentration by DasGip fedbatch pro® gas analysis systems with the off gas analyzer GA4 based on zirconium dioxide and two-beam infrared sensor. The integrated mass flow sensor allowed for on-line monitoring and calculation of oxygen transfer rate (*OTR*) (mmol/h), carbon dioxide transfer rate (*CTR*) (mmol/h) and respiratory quotient (*RQ*) by dividing *CTR* by *OTR*. The seed cultures for the cultivations were grown at 30°C in 500 mL shake flasks containing 100 mL of culture with agitation in an orbital shaker at 100 rpm. Pre-cultures were used to inoculate the fermenters at a final dry weight of 1 mg/L. All cultivations were performed in duplicate or triplicate. The fed-batch cultures were

Table I. Overview of plasmids used in this study.

Plasmid	Gene(s)	Reference
pSP-G1	*URA3* based expression vector containing bidirectional P_{TEF1}-P_{PGK1} promoter	(Partow et al., 2010)
pICK01	pSP-G1, P_{PGK1}-*tHMG1*, P_{TEF1}-*SanSyn*	(Scalcinati et al., 2012a)
pIST01	P_{TEF1}-*FarnSyn_Md*	This study
pIST02	P_{TEF1}-*FarnSyn_Cj*	This study
pIST03	P_{TEF1}-*FarnSyn_Aa*	This study
pIST04	P_{TEF1}-*FarnSyn_Md,* P_{PGK1}-*tHMG1*	This study
pIST05	P_{TEF1}-*FarnSyn_Cj,* P_{PGK1}-*tHMG1*	This study
pIST06	P_{TEF1}-*FarnSyn_Aa,* P_{PGK1}-*tHMG1*	This study

Md: *M. domestica*, *Cj*: *C. junos* and *Aa*: *A. annua*.

initiated as batch cultures using 20 g/L glucose. Feeding with fresh medium commenced only after residual ethanol produced from the glucose consumption phase was completely depleted. An exponential feed rate ν (t) (L/h) was calculated as previously described by Scalcinati et al. (2012a). Assuming substrate concentration is at steady state ($dS/dt = 0$), the exponential feed rate was calculated as

$$\nu(t) = \frac{\mu X_0 V_0 \exp(\mu t)}{Y_{SX}(S^{in} - S)} = \nu_0 \exp(\mu t) \quad (1)$$

with t as the feed time, μ as the specific growth rate, X_0 and V_0 as the initial biomass concentration and reaction volume, respectively, Y_{SX} as the specific biomass yield and S as the substrate concentration in the feed medium (superscript in) and in the cultivation medium (Nielsen et al., 2002; pp. 411–416). A specific growth rate of 0.1 1/h was used, since overflow metabolism is reported to start at $\mu > 0.25$ 1/h (Nielsen et al., 2002). Glucose was assumed to be the rate limiting substrate to which *S. cerevisiae* responds following the Monod kinetics, where $S \approx 0$ since $S^{in} >> S$. For calculation of the initial feed rate ν_0, available chemostat data for strain SCIGS28, which differs from strains SCIST04-6 only by the terpene synthase on the plasmid, at a dilution rate of $D = 0.1$ 1/h were used.

To facilitate strain comparison, the same feeding strategy was applied in all experiments with $\nu_0 = 0.003$ L/h, in order to keep the glucose concentration below the substrate consumption rate specified for these conditions (Scalcinati et al., 2012b). A correct feed rate was obtained using a modified single loop, set-point control method programming the fermenter fb-pro software (DasGip) and controlled using the DasGip control system. Additionally, the feed rate was controlled based on the respiratory culture response monitoring *RQ* values as indirect measure of the cell metabolic state. Online precise measurement of the exit gas composition allowed to adjust the feed rate through a proportional integral (PI) controller maintaining the *RQ* at the desired set point of 1.0 during feed phase, whereas pumps were started at $RQ < 1$ and turned off at $RQ > 1$ to avoid ethanol formation.

Measurement of Cell Growth

Cell growth was determined by measuring optical density at 600 nm using a Genesis 20 spectrophotometer (Thermo Fisher Scientific). For cell dry weight measurement, a 5 mL sample was taken from the bioreactor and pipetted onto a pre-weighted filter with a pore size of 0.45 μm (Sartorius, Göttingen, Germany). Subsequently, the filter was washed with 5 mL of water, dried in a microwave at 150 W for 15 min and stored in a desiccator until it was weighed again.

Quantification of Extracellular Metabolites

In order to measure glucose, ethanol, glycerol, acetate and succinate during the fermentation, a sample was taken from the two-phase cultivation broth and centrifuged at 4000 rpm for 3 min. The organic overlay was removed and the cell suspension was filtered using a 0.45 μm nylon filter (VWR International, Radnor, PA). Subsequently, the sample was analyzed by HPLC using a Dionex Ultimate 3000 (Dionex, Sunnyvale, CA) equipped with an Aminex HPX-87H column (300 mm x 7.8 mm, Bio-Rad Laboratories, Hercules, CA) in connection with a variable wavelength detector (210 nm) and a refractive index detector (512 μRIU). The system was run at 45°C and a flow rate of 0.6 mL/min with 5 mM H_2SO_4 as solvent. The analytes were detected by comparison with a known standard and quantified by external calibration.

Quantification of Farnesene and Santalene

For quantification of farnesene, a sample was removed from the cultivation broth and separation of the two phases (aqueous and dodecane) was performed by centrifugation at 5000 rpm for 5 min. An aliquot was taken from the dodecane phase and either injected directly or diluted ten times in hexane. Farnesene isomers were detected on a Focus GC ISQ single quadrupole GC-MS and quantified on a Focus GC-FID (Thermo Fisher Scientific, USA) with identical analytical conditions as described by Tippmann et al. (2015). Since the signal intensity of the FID primarily depends on the number of carbon atoms (Holm, 1999), quantification of both isomers, α- and β-farnesene, was performed by external calibration using trans-β-farnesene analytical standards (≥90%, Sigma–Aldrich) as presented by Wang et al. (2011). Santalene was quantified as described previously by Scalcinati et al. (2012b) with minor modifications. The organic overlay was collected by centrifugation at 5000 rpm for 5 min, diluted with an equal volume of 2-ethyl acetate (Sigma, St Luis, MO) containing α-humulene (≥96%, Sigma Aldrich) as internal standard and subsequently analyzed by GC-MS. Identification of santalene was performed comparing mass spectra and retention time with an authentic standard, while santalene concentrations were calculated using external calibration and corrected by recovery of the internal standard α-humulene.

Results

Farnesene Production in Shake Flask Cultivations

In the first part of our study, three farnesene synthases were compared for farnesene production. For this purpose, *S. cerevisiae* CEN.PK113-5D strains harboring plasmids pIST01-03 were cultivated for 48 h in minimal medium overlayed with 10% v/v of dodecane to harvest the product. Farnesene titers are reported in Figure 2A. Highest farnesene titers were observed for α-farnesene synthase from *M. domestica* with approximately 4 mg/L, whereas β-farnesene titers obtained with the synthases from *C. junos* and *A. annua* were slightly lower, i.e. approximately 3.5 and 3.0 mg/L, respectively. Furthermore, sesquiphellandrene, which is like farnesene directly derived from FPP, was detected as byproduct for farnesene synthase from *C. junos*, whereas the other two synthases showed high product specificity for farnesene in our experiments. In order to enhance farnesene production, the farnesene synthase genes were expressed from 2 μm plasmids together with a truncated version of the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase gene, *tHMG1*, which encodes a flux controlling enzyme of the mevalonate pathway

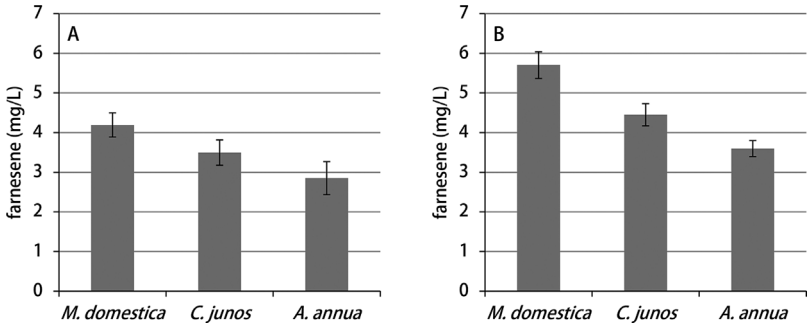

Figure 2. Comparison of yeast strains carrying three different farnesene synthase genes in shake flask cultivation. *S. cerevisae* strain CEN.PK113-5D was transformed with plasmids pIST01-03 (Panel A) and pIST04-06 (Panel B) to compare farnesene synthase genes from different plant sources, i.e. *M. domestica, C. junos,* and *A. annua* and to study the effect of truncated HMG-CoA reductase tHmg1. Cells were cultivated for 48 h and farnesene was extracted using a dodecane overlay. Values represent average concentrations normalized to total culture volume of three biological replicates with standard deviation.

(Ohto et al., 2009). Opposed to native Hmg1, which is located in the ER membrane, tHmg1 represents the free cytosolic form of the enzyme as it misses the regulatory membrane binding domain (Polakowski et al., 1998). Evaluation of this plasmid set (pIST04-06) in shake flasks revealed an increase in titers by at least 25% after 48 h of cultivation time with farnesene synthase from *M. domestica* producing approximately 6 mg/L of α-farnesene and farnesene synthases from *C. junos* and *A. annua* producing approximately 4.5 and 3.5 mg/L, respectively (Fig. 2B).

Production of Farnesene in Fed-Batch Cultivations With *RQ*-Controlled Exponential Feed

The comparison of three farnesene synthases in shake flasks revealed clear differences regarding their efficiency when tested in *S. cerevisiae* CEN.PK113-5D. In accordance with the literature, co-expression of *tHMG1* led to a significant improvement in sesquiterpene titers. In order to further increase farnesene production, strains SCICK16 and SCIGS22, which were constructed for improved production of sesquiterpenes by engineering of the FPP branch point and increasing the flux through the mevalonate pathway (Scalcinati et al., 2012a; Scalcinati et al., 2012b), were transformed with plasmids pIST04-06 resulting in strains SCIST07-12 (Table II). Fed-batch cultivations were used for strain comparison as glucose limited conditions were necessary to benefit from down-regulation of *ERG9* encoding squalene synthase by the *HXT1* promoter (Ozcan and Johnston, 1995; Reifenberger et al., 1995). Additionally, the *RQ* was utilized in a feedback loop to indirectly control the glucose concentration and avoid ethanol formation. Typical fermentation profiles for volume, *RQ* and feed rate during the fed-batch phase are shown in Figure 3. As described earlier, feeding was initiated subsequent to glucose and ethanol depletion, where $RQ < 1$. With rising availability of glucose in the medium, the *RQ* approached the threshold of 1, where pumps were

Table II. Overview of *S. cerevisiae* strains used in this study.

Strain	Genotype	Plasmid	Reference
CEN.PK113-5D	*MAT***a** *MAL2-8^c SUC2 ura3-52*	None	P. Kötter, University of Frankfurt, Germany
SCICK16	*MAT***a** *MAL2-8^c SUC2 ura3-52 lpp1*Δ::*loxP dpp1*Δ::*loxP* P_{ERG9}Δ::*loxP*-P_{HXT1}	None	(Scalcinati et al., 2012a)
SCIGS22	*MAT***a** *MAL2-8^c SUC2 ura3-52 lpp1*Δ::*loxP dpp1*Δ::*loxP* P_{ERG9}Δ::*loxP*-P_{HXT1} gdh1Δ::*loxP* P_{TEF1}-ERG20 P_{PGK1}-GDH2	None	(Scalcinati et al., 2012b)
SCIGS24	*MAT***a** *MAL2-8^c SUC2 ura3-52 lpp1*Δ::*loxP dpp1*Δ::*loxP* P_{ERG9}Δ::*loxP*-P_{HXT1} gdh1Δ::*loxP* P_{TEF1}-ERG20 P_{PGK1}-GDH2	pISP15	(Scalcinati et al., 2012b)
SCIST01	CEN.PK113-5D	pIST01	This study
SCIST02	CEN.PK113-5D	pIST02	This study
SCIST03	CEN.PK113-5D	pIST03	This study
SCIST04	CEN.PK113-5D	pIST04	This study
SCIST05	CEN.PK113-5D	pIST05	This study
SCIST06	CEN.PK113-5D	pIST06	This study
SCIST07	SCICK16	pIST04	This study
SCIST08	SCICK16	pIST05	This study
SCIST09	SCICK16	pIST06	This study
SCIST10	SCIGS22	pIST04	This study
SCIST11	SCIGS22	pIST05	This study
SCIST12	SCIGS22	pIST06	This study

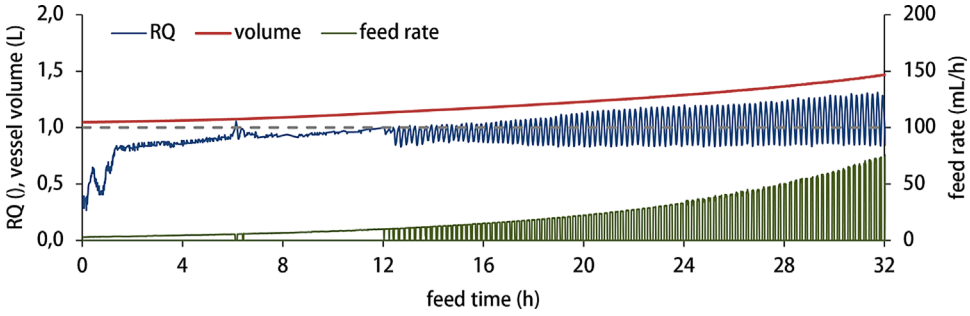

Figure 3. Time course of respiratory quotient (*RQ*), feed rate and volume during fed-batch phase with *RQ*-controlled exponential feeding. Data represent on-line measurements from cultivation of strain SCIST05.

turned off. The on/off switch of the feed at this value resulted in an oscillation of the *RQ* with slightly increasing amplitude over time. However, the *RQ* remained below 1.4 in all experiments.

Figure 4 illustrates the development of biomass, glucose, ethanol and farnesene concentration for all nine strains during the fed-batch phase, while Table III depicts physiological parameters obtained from the data set. As it can be seen, *RQ* values were kept close to 1 on average throughout the fermentations, indicating successful functioning of the feedback control. This observation is also reflected by the ethanol concentration, which remained close to zero in all experiments. However, in some cases ethanol accumulation was observed at the

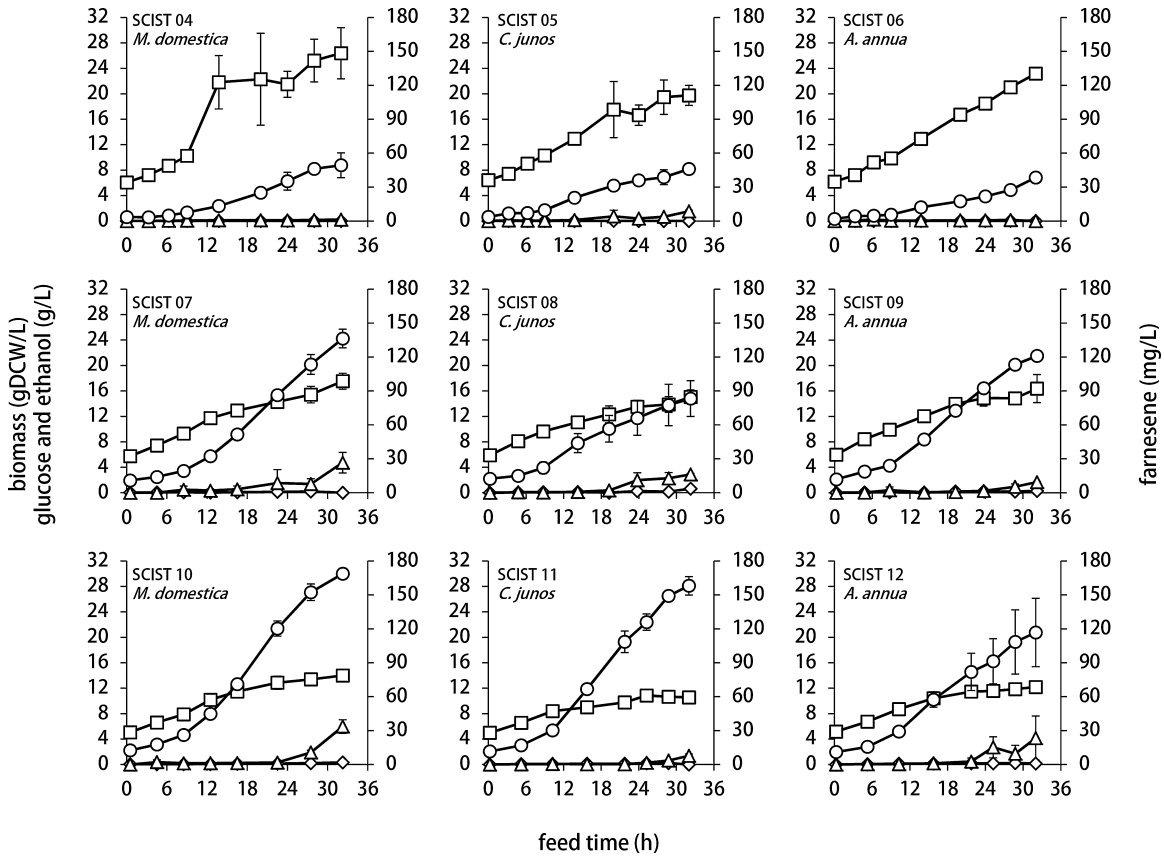

Figure 4. Production of farnesene in fed-batch cultivations with *RQ*-controlled exponential feeding. Background *S. cerevisiae* strains CEN.PK113-5D, SCICK16 and SCIGS22 were transformed with plasmids pIST04-06 carrying a farnesene synthase gene from *M. domestica*, *C. junos* or *A. annua* and HMG-CoA reductase gene tHMG1, resulting in strains SCIST04-12. The background strains carry the following modifications: P*HXT1-ERG9*, *dpp1*Δ, *lpp1*Δ (SCIST07-09) and P*HXT1-ERG9*, *dpp1*Δ, *lpp1*Δ, *ERG20*↑, *GDH2*↑, *gdh1*Δ (SCIST10–12). Data were obtained during the feed phase and represent average values of two or three independent biological replicates. Squares: biomass, diamonds: glucose, triangles: ethanol, circles: farnesene.

Table III. Physiological parameters of farnesene producing strains SCIST04-12 during fed-batch cultivations with *RQ*-controlled exponential feed. μ: specific growth rate, r_S: specific substrate uptake rate, Y_{SX}: biomass yield, Y_{XFar}: farnesene yield and *RQ*: respiratory quotient.

Background strain	Farnesene synthase	μ h^{-1}	r_S g/(gDCW · h)	Y_{SX} gDCW/g	Y_{XFar} g/gDCW	Final farnesene mg/$L_{aqueous}$	*RQ* -
SCIST04	*M. domestica*	0.062 ± 0.004	0.149 ± 0.003	0.418 ± 0.017	0.002 ± 0.001	49.32 ± 10.95	0.96 ± 0.02
SCIST05	*C. junos*	0.048 ± 0.007	0.163 ± 0.016	0.297 ± 0.073	0.003 ± 0.000	46.03 ± 4.71	1.00 ± 0.02
SCIST06	*A. annua*	0.053 ± 0.002	0.170 ± 0.010	0.311 ± 0.007	0.002 ± 0.000	38.46 ± 0.28	1.00 ± 0.01
SCIST07	*M. domestica*	0.046 ± 0.004	0.172 ± 0.017	0.268 ± 0.002	0.009 ± 0.000	136.37 ± 8.23	0.97 ± 0.03
SCIST08	*C. junos*	0.035 ± 0.003	0.122 ± 0.007	0.288 ± 0.038	0.007 ± 0.001	83.30 ± 15.76	1.04 ± 0.02
SCIST09	*A. annua*	0.038 ± 0.005	0.146 ± 0.033	0.263 ± 0.025	0.010 ± 0.002	121.00 ± 1.91	1.02 ± 0.02
SCIST10	*M. domestica*	0.043 ± 0.001	0.193 ± 0.001	0.222 ± 0.007	0.015 ± 0.001	168.75 ± 4.68	0.98 ± 0.02
SCIST11	*C. junos*	0.032 ± 0.001	0.221 ± 0.003	0.146 ± 0.007	0.022 ± 0.002	158.09 ± 8.13	1.00 ± 0.01
SCIST12	*A. annua*	0.034 ± 0.000	0.199 ± 0.021	0.172 ± 0.017	0.013 ± 0.003	116.74 ± 30.26	1.01 ± 0.02

Values represent mean ± standard deviation of two or three biological replicates.

end of the fermentation for the heavily engineered strains SCIST07-12. Likewise, glucose concentration remained close to zero, which was the target condition to ensure down-regulation of *ERG9*. In consequence, production of ergosterol was limited, which led to reduced biomass formation as shown by the biomass yields in Table III. For strains SCIST07-12, final biomass concentrations did not exceed 15 gDCW/L, whereas up to 26 gDCW/L were obtained for strains SCIST04-06. However, while growth was impaired, farnesene production was increased substantially and reached final titers of up to ~170 mg/L. Similar to the results in shake flasks, highest titers were attained using α-farnesene synthase from *M. domestica*, whereas expression of β-farnesene synthase from *C. junos* and *A. annua* resulted in final farnesene titers of approximately 160 and 120 mg/L, respectively. Surprisingly, while production could be significantly improved from background strain SCICK16 to SCIGS22 using farnesene synthases from *M. domestica* and *C. junos*, a similar increase was not observed with the farnesene synthase from *A. annua*. Ion chromatograms and mass spectra from GC-MS analysis of a dodecane sample from cultivation of strains SCIST04, SCIST06 and an authentic standard for β-farnesene are shown in Figure 5. While the retention time of β-farnesene from yeast was slightly shifted, most likely caused by dodecane, its mass spectrum revealed high resemblance to the analytical standard. In

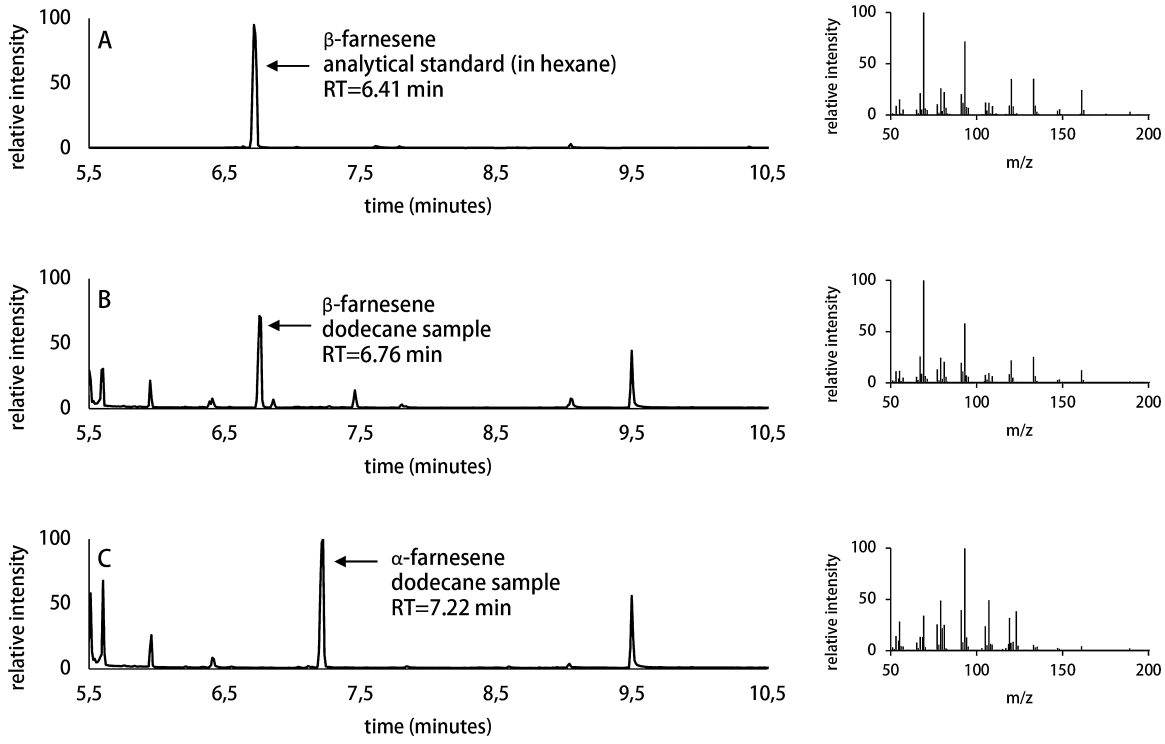

Figure 5. Total ion chromatograms (Left Panel) and mass spectra (Right Panel) obtained from GC-MS analysis of an authentic standard for β-farnesene (**A**) and dodecane samples from cultivation of strains SCIST06 (**B**) andSCIST04 (**C**) expressing the β-farnesene synthase gene from *A. annua* and the α-farnesene synthase gene from *M. domestica*, respectively.

comparison, α-farnesene had a longer retention time, but was identified by its mass spectrum.

Production of Santalene in Fed-Batch Cultivations With *RQ*-Controlled Exponential Feed

Santalene is another commercially interesting sesquiterpene, which is directly produced from FPP by a specific plant terpene synthase. In order to evaluate the efficiency of the farnesene synthases in comparison to another sesquiterpene synthase, a similar *RQ*-controlled fed-batch fermentation with strain SCIGS24 was performed (Scalcinati et al., 2012b). This strain is identical to SCIST10-12 regarding its genetic background and only differs by carrying plasmid pISP15, which contains a codon optimized santalene synthase from *Clausena lansium* (see Table II). Similar to the fed-batch cultivations performed to investigate farnesene production, the feed was controlled using the *RQ*, which was maintained at 0.98 on average throughout the experiment. However, as the batch phase medium contained 30 g/L glucose, higher biomass concentrations were attained overall reaching up to 25 gDCW/L (Fig. 6). This is also indicated by the biomass yield of 0.281 gDCW/g, which was significantly higher. However, the product yield of 0.006 g/gDCW amounts only to half compared to the one observed for the farnesene synthases. Considering the production of santalene, with 163 mg/L of α-santalene after approximately 30 h of feeding time, final titers were almost equivalent to cultivations using strains SCIST10-12.

Discussion

The sesquiterpene farnesene fulfills various functions in plants and represents a biofuel precursor that can be obtained efficiently from yeast fermentation. Terpene production is strongly dependent on the availability of the precursors GPP, FPP and GGPP. Furthermore, limitations to the overall productivity are added by terpene synthases, which catalyze the conversion of these precursors, but mostly originate from plant sources. The impact of these aspects was recently illustrated for mono-, sesqui- and diterpene production. Ding et al. (2014) selected suitable GGPP synthases by predicting their catalytic efficiency *in silico* and thereby raised production of the diterpene taxadiene up to 73 mg/L. Likewise, screening of different combinations of GPP and pinene synthases revealed to significantly improve production of pinene in *E. coli* (Sarria et al., 2014). Furthermore, Xie et al. (2012) isolated novel terpene synthases from *Ricinus communis*, which were tested for production of sesquiterpenes in *E. coli* and *S. cerevisiae*.

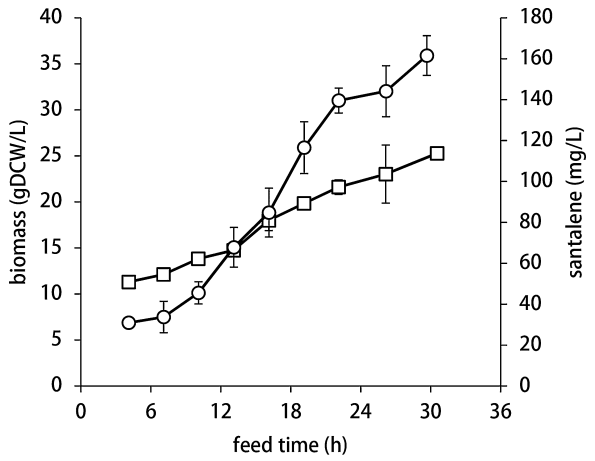

Figure 6. Production of santalene in fed-batch cultivations with *RQ*-controlled exponential feeding, using strain SCISG24 expressing HMG-CoA reductase gene *tHMG1* and the α-santalene synthase gene from *C. lansium* from plasmid pISP15. Data shown were obtained during the fed-batch phase and represent average values ofthree independent biological replicates. Squares: biomass, circles: santalene.

Recent studies have described production of farnesene in *E. coli* and characterization of several farnesene synthases *in vitro*. Yet a comparison of different farnesene synthases in an industrial process setup has not been reported. In this study we expressed three different farnesene synthase genes from plants in *S. cerevisiae* and successfully produced both, α- and β-farnesene. All enzymes showed high product specificity in our experiments. Only in case of farnesene synthase from *C. junos*, sesquiphellandrene was detected as byproduct. *S. cerevisiae* CEN.PK113-5D expressing α-farnesene synthase from *M. domestica* produced 4 mg/L in shake flask cultivation, which was 17 and 32% higher in comparison to the corresponding synthases from *C. junos* and *A. annua*, respectively. By using the same farnesene synthase in a *S. cerevisiae* strain optimized for sesquiterpene production (SCIST10), final titers of approximately 170 mg/L were attained in fed-batch cultivations using *RQ*-controlled exponential feeding. Similar to the results in shake flasks, farnesene synthase from *M. domestica* led to maximal titers in all strain backgrounds suggesting higher efficiency of this enzyme. However, when comparing the different synthases in the respective background strain (e.g. for SCIST07-09), the titer ratio clearly fluctuated, which could be explained by variations in the biomass yields Y_{SX}. Taking the product yields Y_{XFar} into consideration, a farnesene synthase with clearly superior efficiency could not be identified from these experiments. In general, performance of the fed-batch operation exerted a strong influence on the final product titers. As depicted in Table III, significant differences regarding the specific growth rates were observed between all strains, where a growth rate of 0.1 1/h was not attained. This was caused by the initial feed rate ν_0, which was chosen too low and would require elevated biomass yields to compensate. However, based on these results, biomass yields of 0.3–0.4 gDCW/g were even slightly reduced compared to previous experiments, where Y_{SX} amounted for 0.5 gDCW/g (Scalcinati et al., 2012b).

As described above, feed control by *RQ* was used to maintain fully aerobic respiration as ethanol accumulation was anticipated to cease at an *RQ* of 1. However, ethanol formation was still observed at the end of the cultivations, mostly for strains SCIST07-12. Several aspects may have played a role for this effect. Most likely, higher fluctuations of the *RQ*, which occurred at the end of the fermentations in particular, could have led to overfeeding and thereby to ethanol formation. On the other hand, fed-batch cultivations with exponential feeding are normally only performed until mass transfer becomes limiting. At this point, the exponential feed phase is terminated and the feed is set to a constant value to avoid stress responses (Villadsen and Patil, 2007). Although the

aeration rate was increased proportionally to the reaction volume, dissolved oxygen concentrations (*DO*) dropped below 20% in most experiments. As a result, partial limitation of oxygen may have contributed to ethanol formation as well. In addition, ethanol formation might have been directly related to down-regulation of *ERG9*, as insufficient feeding may have completely blocked ergosterol synthesis and thereby hindered growth. As a result, ethanol is produced for regeneration of NAD^+. This can be seen particularly from the growth profiles of strains SCIST10-12. When biomass concentration almost reached steady state after 24 h, ethanol accumulation set in. On the other hand, ethanol accumulation in SCIST12 cultures also serves as a possible explanation why similar final farnesene titers were obtained for SCIST09 and 12 despite the additional genome modifications in SCIST12. For these reasons, farnesene titers would likely have been higher than reported in Table III, if ethanol formation had been cut off completely. Nonetheless, in an industrial scenario, the cultivation could be continued after the feed phase to allow for ethanol depletion by *S. cerevisiae*.

Reconsidering the impact of the selected plant terpene synthases, none of the three candidates revealed outstanding efficiency during the fed-batch evaluation. This, however, does not allow for a general conclusion in relation to farnesene synthases. Although alignment of the amino acid sequences of the selected candidates using the BLAST algorithm did not score identities above 41%, greater genetic distances between the natural hosts may lead to identification of a synthase with superior efficiency. A different perspective on the efficiency of these enzymes might be obtained by comparing production of farnesene to other sesquiterpenes. We therefore used santalene as a second case study since it represents another sesquiterpene of great commercial interest, which requires a plant terpene synthase to catalyze the direct conversion of FPP to the desired product. By using α-santalene synthase from *C. lansium*, 92 mg/L could be produced in fed-batch cultivations with exponential feeding at a fixed growth rate of 0.6 1/h (Scalcinati et al., 2012a). Cultivation of strain SCIGS24 carrying a plasmid for expression of *tHMG1* and a codon optimized santalene synthase in fed-batch with *RQ*-controlled feeding resulted in final titers of 163 mg/L. This observation indicates similar efficiencies of the santalene and farnesene synthase. However, as the biomass yield was significantly higher compared to strains SCIST10-12, the product yield on substrate during the fed-batch phase amounted to only 0.006 g/gDCW, which represents a more than 50% decrease compared to the results on farnesene in the same strain background. An explanation for this reduction may be the cyclization involved in santalene production, which adds complexity to the reaction and influences the kinetics of the corresponding enzyme.

To conclude on the experimental setup, *RQ*-controlled exponential feeding was successfully implemented to evaluate the three selected farnesene synthases in strains that are differently optimized for sesquiterpene production. However, instead of indirectly targeting a certain substrate concentration by controlling the *RQ*, feed control using a glucose sensor might be even more precise (Beom Soo et al., 1994). Nevertheless, high cell densities were attained and ethanol accumulation could be kept to a minimum. Furthermore, a clear improvement in farnesene production was seen from strains SCIST04-06 to SCIST10-12. While similar titers were attained for santalene, the product yield on biomass was substantially lower in this case.

The authors wish to thank the Knut and Alice Wallenberg Foundation, FORMAS, the Swedish Research Council VR and the Novo Nordisk Foundation. We thank Dr. António Roldão for his assistance during the fed-batch experiments.

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