Metabolic Engineering 11 (2009) 328–334

Contents lists available at ScienceDirect

Metabolic Engineering

journal homepage: www.elsevier.com/locate/ymben

Enhancing sesquiterpene production in *Saccharomyces cerevisiae* through *in silico* driven metabolic engineering

Mohammad A. Asadollahi [a,b], Jérôme Maury [a,1], Kiran Raosaheb Patil [a], Michel Schalk [c], Anthony Clark [d], Jens Nielsen [a,*]

[a] Center for Microbial Biotechnology (CMB), Department of Systems Biology, Building 223, Technical University of Denmark (DTU), DK-2800 Kgs. Lyngby, Denmark
[b] Biotechnology Group, Faculty of Advanced Sciences and Technologies, University of Isfahan, Isfahan 81746-73441, Iran
[c] Firmenich SA, Corporate R&D Division, P.O. Box 239, CH–1211 Geneva 8, Switzerland
[d] Firmenich Inc., Corporate R&D Division, P.O. Box 5880, Princeton, New Jersey 08543, USA

ARTICLE INFO

Article history:
Received 18 March 2009
Received in revised form
25 June 2009
Accepted 13 July 2009
Available online 18 July 2009

Keywords:
In silico metabolic engineering
Saccharomyces cerevisiae
Isoprenoid
Sesquiterpene
Cubebol
Glutamate dehydrogenase
Flux balance analysis
Minimization of metabolic adjustments
NADPH availability

ABSTRACT

A genome-scale metabolic model was used to identify new target genes for enhanced biosynthesis of sesquiterpenes in the yeast *Saccharomyces cerevisiae*. The effect of gene deletions on the flux distributions in the metabolic model of *S. cerevisiae* was assessed using OptGene as the modeling framework and minimization of metabolic adjustments (MOMA) as objective function.

Deletion of NADPH-dependent glutamate dehydrogenase encoded by *GDH1* was identified as the best target gene for the improvement of sesquiterpene biosynthesis in yeast. Deletion of this gene enhances the available NADPH in the cytosol for other NADPH requiring enzymes, including HMG-CoA reductase. However, since disruption of *GDH1* impairs the ammonia utilization, simultaneous over-expression of the NADH-dependent glutamate dehydrogenase encoded by *GDH2* was also considered in this study.

Deletion of *GDH1* led to an approximately 85% increase in the final cubebol titer. However, deletion of this gene also caused a significant decrease in the maximum specific growth rate. Over-expression of *GDH2* did not show a further effect on the final cubebol titer but this alteration significantly improved the growth rate compared to the *GDH1* deleted strain.

1. Introduction

Metabolic engineering has significantly facilitated rational design of cell factories in order to obtain desired cellular phenotypes (Nielsen, 2001). Since the emergence of metabolic engineering as an independent scientific discipline over a decade ago, there have been numerous successful examples of metabolic engineering for improved production of endogenous or heterologous products, extension of substrate range used by microorganism, introduction of new catalytic activities for degradation of toxic compounds and manipulation of cell properties (Nielsen, 2001; Stephanopoulos et al., 1998). These accomplishments in most cases were achieved through alteration of gene expressions or introduction of new genes by taking the advantage of molecular biology tools.

Metabolic engineering of cell factories for heterologous production of isoprenoids is a prominent example showing how metabolic engineering strategies can be applied for improving a certain phenotype (for review see Maury et al., 2005). Application of metabolic engineering strategies has allowed production of up to 480 mg/L amorphadiene (Newman et al., 2006), 102 mg/L lycopene (Yoon et al., 2006) and 503 mg/L β-carotene (Yoon et al., 2007) in engineered *Escherichia coli* strains. There are also several studies on the engineering of the mevalonate pathway in yeast strains to enhance isoprenoid production (Asadollahi et al., 2008; Engels et al., 2008; Jackson et al., 2003; Kirby et al., 2008; Lindahl et al., 2006; Miura et al., 1998a, 1998b; Ro et al., 2006; Shiba et al., 2007; Shimada et al., 1998; Takahashi et al., 2007; Yamano et al., 1994).

The metabolic engineering strategies applied to the yeast strains were mainly relying on the manipulation of the classical targets in the mevalonate pathway such as over-expression of *HMG1* (Engels et al., 2008; Jackson et al., 2003; Kirby et al., 2008; Ro et al., 2006; Shimada et al., 1998; Verwaal et al., 2007), disruption or down-regulation of *ERG9* (Asadollahi et al., 2008; Ro et al., 2006; Shimada et al., 1998; Takahashi et al., 2007) and over-expression of *ERG20* (Ro et al., 2006). There are only a few studies on alteration of genes

* Corresponding author. Current address: Systems Biology, Department of Chemical and Biological Engineering, Chalmers University of Technology, Kemivägen 10, SE-412 96 Gothenburg, Sweden. Fax: +46 31 772 38 01.
E-mail address: nielsenj@chalmers.se (J. Nielsen).
[1] Current address: Fluxome Sciences A/S, Diplomvej 378, DK-2800 Kgs. Lyngby, Denmark.

1096-7176/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.ymben.2009.07.001

outside the mevalonate pathway, e.g. engineering of the pyruvate dehydrogenase bypass (Shiba et al., 2007), over-expression of semi-dominant mutant allele of a global transcription factor regulating sterol biosynthesis in yeast, *upc2-1* (Engels et al., 2008; Jackson et al., 2003; Ro et al., 2006) and removing a phosphatase activity encoded by *DPP1* (Takahashi et al., 2007). In order to establish yeast as a platform for large scale production of isoprenoids, in addition to the genes directly involved in the mevalonate pathway, it is necessary to deregulate the pathway through manipulation of genes outside the pathway. However, this is a difficult task as it needs a comprehensive knowledge about all pathways and genes functioning in the cell and their interactions with each other, which is currently limited due to the immense complexity and non-linearity of interactions in the metabolic network and insufficient available information.

Nevertheless, the explosion of data generated by annotated genome sequences has encountered metabolic engineering with new challenges in the post-genomic era mainly concerning exploiting the wealth of information for improving cellular phenotypes (Alper and Stephanopoulos, 2004). As such, this abundance of data has led to reconstruction of several genome-scale metabolic networks (Covert et al., 2001) including *E. coli* (Edwards and Palsson, 2000; Reed et al., 2003), *Haemophilus influenzae* (Edwards and Palsson, 1999) and *S. cerevisiae* (Förster et al., 2003). Current genome scale models are, however, mainly stoichiometric and comprise a set of stoichiometric reactions to describe the biochemical reactions in the system (Patil et al., 2004). These genome scale models, together with some simplifying assumptions, can be used to predict the cellular metabolic behavior under certain steady-state growth conditions using constraint-based models such as flux balance analysis (FBA) (Kauffman et al., 2003). FBA is widely used for evaluating the feasible flux distributions in a metabolic network at steady-state conditions and assuming maximum growth as objective function. Adaptation of metabolic network for optimal growth is logical for wild type strains as a consequence of evolutionary pressure during million of years, but may not hold for the mutant strains constructed in the laboratory. Minimization of metabolic adjustments (MOMA) between a wild-type and a knockout mutant could therefore be a more realistic objective function for assessing the flux distributions in the metabolic networks of deletion mutants (Segrè et al., 2002). The prediction power of FBA/MOMA simulations can effectively be used for metabolic engineering by formulating a bi-level programming framework for suggesting gene deletions leading to over-production of a certain biochemical compound (Burgard et al., 2003). The *in silico* gene knockout using MOMA simulations has been successfully used for improving the biosynthesis of lycopene (Alper and Stephanopoulos, 2004; Alper et al., 2005) and L-valine (Park et al., 2007) in the engineered *E. coli* strains.

In this study, we sought to assess the effect of gene deletions on the flux distributions in the stoichiometric model of *S. cerevisiae* (Förster et al., 2003) in order to identify target genes whose deletions can improve sesquiterpene production in yeast. OptGene (Patil et al., 2005) which is an extension of OptKnock (Burgard et al., 2003) was used as a modeling framework and MOMA as objective function to identify the gene knockouts leading to enhanced sesquiterpene production. OptGene uses a genetic algorithm method to search for the global optimal solution and thereby demands less computational time and allows optimization of non-linear objective functions (Patil et al., 2005).

2. Materials and methods

2.1. In silico computations

Farnesyl diphosphate (FPP), which is precursor for sesquiterpenes, is one of the intermediates in the endogenous mevalonate pathway in yeast. Conversion of FPP to sesquiterpenes catalyzed by sesquiterpene synthases was introduced into the stoichiometric model of *S. cerevisiae* (Förster et al., 2003). This model consisted of 1175 metabolic reactions and 584 unique metabolites.

The gene knockouts leading to enhanced sesquiterpene biosynthesis were found using the OptGene algorithm (Patil et al., 2005) and MOMA as objective function. Thus, the knockouts leading to higher sesquiterpene yields were selected and further tested experimentally.

2.2. Strain construction

The plasmid pIP025 was constructed by cloning GFTpsC (a sesquiterpene synthase isolated from *Citrus paradisi* and encoding for a cubebol synthase) in a pESC-URA vector (Stratagene) under control of the *GAL1* promoter using *BamH*I and *Xho*I restriction sites. Over-expression of the catalytic domain of HMG-CoA reductase (*tHMG1*) was performed using gap repair technique taking advantage of the high efficiency homologous recombination in yeast. The *tHMG1* gene was amplified from the genomic DNA of *S. cerevisiae* CEN.PK113-7D using the primers 5′tctggcgaagaattgttaattaagagctcaTTAGGATTTAATGCAGGTGACGG-AC3′ and 5′cactaaagggcggccgcactagtatcgatgACT***ATG***GACCAATT-GGTGAAAACTG3′. The lower case letters in the primers show the homologous overhang used for gap repair. The truncated coding region encodes for the HMG-CoA reductase lacking the 530N-terminal residues. The PCR conditions were in accordance with the Phusion™ Hot Start High-Fidelity DNA Polymerase (Finnzymes Oy, Espoo, Finland). pESC-URA plasmid was digested with *Cla*I and *Sac*I restriction enzymes. The PCR product and the digested expression vector were separated by gel electrophoresis and gel purified using the QIAEX® II Gel extraction kit (Qiagen, Hilden, Germany). Homologous recombination of PCR product into the digested expression vector after transformation of CEN.PK113-5D with both fragments resulted in pIP026 plasmid.

The plasmid pIP028 was obtained by cloning GFTpsC in pIP026 plasmid under control of the *GAL1* promoter using *BamH*I and *Xho*I restriction sites.

In order to construct the strains YIP-00-07 and YIP-00-08, the CEN.MS1-10C T1 and CEN.PK113-13D strains were mated together on YPD plates. The next day some of the large colonies (the large colonies are more likely to be diploid) were picked and placed on an YPD master plate. After growing one day on this plate, the cells were replica plated onto a sporulating plate. After 3 days at 30 °C, a small bit of cells from the sporulation plate was taken off with a sterile toothpick and mixed with 10 μl of sterile distilled water. Ten microliters of 5 mg/ml Zymolyase was added to the mixture and incubated at room temperature for 10 min. The reaction was stopped by placing the samples on ice and adding 150 μl of sterile H_2O. Spores were dissected, separated with a glass needle under microscope, and grown on YPD medium for 3 days at 30 °C. In the next step the tetrads were tested for the desired genotypes. Disruption of *URA3* was confirmed by growing the strains on SC (synthetic complete medium containing 2% glucose) and SC-ura (synthetic complete medium without uracil). Over-expression of *GDH2* was also verified by growing the strains on YPD plates supplemented with 300 mg/L G418.

To identify the mating type, the haploid strains on the dissection plate were mated with mating tester strains. Those plates were replica plated onto sporulation plates and incubated at 30 °C for 3–4 days. The ability of spores to fluoresce under UV lamp at 302 nm wavelength, made it possible to see which strain was originally *MAT a* and which was *MAT* α.

Table 1
Strains used in this study.

Strain	Genotype	Plasmid	Plasmid description	Reference
YIP-00-03 (CEN.PK113-5D)	*MAT***a** *MAL2-8^c SUC2 ura3-52*	None		Peter Kötter[a]
CEN.PK113-13D	*MAT***α** *MAL2-8^c SUC2 ura3-52*			Peter Kötter[a]
CEN.MS1-10C T1	*MAT***a** *MAL2-8^c SUC2 gdh1(209,1308)::loxP gdh2::P_{PGK}-GDH2-KanMX3*	None		dos Santos et al. (2003)
YIP-00-08	*MAT***a** *MAL2-8^c SUC2 ura3-52 gdh1(209,1308)::loxP*	None		This study
YIP-00-07	*MAT***a** *MAL2-8^c SUC2 ura3-52 gdh1(209,1308)::loxP gdh2::P_{PGK}-GDH2-KanMX3*	None		This study
YIP-0C-04	*MAT***a** *MAL2-8^c SUC2 ura3-52*	pIP025	pESC-URA 2μ *URA3* P_{GAL1}- GFTpsC	This study
YIP-0C-06	*MAT***a** *MAL2-8^c SUC2 ura3-52 gdh1(209,1308)::loxP*	pIP025	pESC-URA 2μ *URA3* P_{GAL1}- GFTpsC	This study
YIP-0C-05	*MAT***a** *MAL2-8^c SUC2 ura3-52 gdh1(209,1308)::loxP gdh2::P_{PGK}-GDH2-KanMX3*	pIP025	pESC-URA 2μ *URA3* P_{GAL1}- GFTpsC	This study
YIP-MC-11	*MAT***a** *MAL2-8^c SUC2 ura3-52 gdh1(209,1308)::loxP gdh2::P_{PGK}-GDH2-KanMX3*	pIP028	pESC-URA 2μ *URA3* P_{GAL1}-GFTpsC P_{GAL10}-*tHMG1*	This study

[a] Institut für Mikrobiologie, der Johann Wolfgang Goethe-Universität, Frankfurt am Main, Germany.

Deletion of *GDH1* in the strains was verified by colony PCR using the primers GGTTTGGGCAGGGAAATGTC and GTCCAATCAGCAGAGAGAAG. Thus, the strains YIP-00-07 and YIP-00-08 were obtained. Transformation of these strains with either pIP025 or pIP028 plasmid resulted in YIP-0C-05, YIP-MC-11 and YIP-0C-06 strains. Table 1 lists the strains used in this study.

2.3. Media for batch cultivations

A defined minimal medium as described by Verduyn et al. (1992) containing 20 g/L of galactose as the sole carbon source was used for all batch fermentations. The media had the following compositions: 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 metal solution; 1 ml/L vitamin solution and 50 μl/L synperonic antifoam.

Galactose was autoclaved separately from the rest of the medium and subsequently added to the fermenter, as was the case for the vitamin solution that was added after filter sterilization.

2.4. Batch fermentations

Batch fermentations were carried out in well-controlled 5 L in-house manufactured glass bioreactors with a working volume of 4 L. The bioreactors were equipped with two disk-turbine impellers and 4 baffles to ensure proper mixing. The pH was controlled between 4.95 and 5.05 by automatic addition of 2 M NaOH. The temperature was kept constant at 30 °C. The air flow was 4 L/min (1 vvm) and was sterilized by filtration and the off gas passed through a condenser. Carbon dioxide and oxygen concentrations in the off-gas were determined by a Brüel & Kjær acoustic gas analyzer (Brüel & Kjær, Nærum, Denmark). Batch fermenters were inoculated to an initial OD_{600} of 0.02 from a liquid preculture. For *in situ* solvent extraction of the sesquiterpenes produced and to limit losses through the outlet gas, 300 mL of dodecane was added aseptically to the media at OD_{600} of 1 ± 0.1.

2.5. OD and dry weight determinations

The OD of samples was determined at 600 nm in duplicate using a Hitachi U-1100 spectrophotometer. Dry weight measurement was achieved by using 0.45 μm pore-size nitrocellulose filters (Sartorius AG, Göttingen, Germany) according to the method described by Dynesen et al. (1998).

2.6. Analysis of galactose and extracellular metabolites

Determination of galactose and extracellular metabolites was performed using HPLC, as described previously (Asadollahi et al., 2008).

2.7. Analysis of sesquiterpenes in the organic layer

Samples from organic layer were centrifuged for 5 min at 3500 rpm and subsequently analyzed by GC-MS to determine the level of sesquiterpenes during the course of fermentation as described (Asadollahi et al., 2008).

3. Results

3.1. In silico metabolic engineering

The reaction for conversion of the precursor FPP to sesquiterpenes was added to the original genome-scale model of *S. cerevisiae* (Förster et al., 2003). The intracellular fluxes of the resulting model were calculated for the knockout mutants subject to MOMA using OptGene (Patil et al., 2005) to find the genes whose deletion could increase the flux towards sesquiterpenes. The MOMA theory is based on the hypothesis that the intracellular flux distribution for mutants can be better approximated by assuming that the fluxes tend to maintain minimum distance from their wild-type value. The wild-type flux distribution is often a reflection of cellular fitness, e.g. growth, and can be estimated using FBA. In the current case, FBA was used to obtain the reference strain (YIP-0C-04) flux distribution to be used as *wild-type* flux map for MOMA calculations. Ethanol, glycerol and sesquiterpene production rates were constrained to the experimentally observed values and maximization of growth was used as an objective function (alternatively, one can fix the growth rate to the experimentally observed value and use minimization of substrate uptake rate as an objective function for FBA (Oliveira et al., 2005)). OptGene suggested deletion of *GDH1* coding

glutamate dehydrogenase as the best target gene for the further experimental work. For this mutant, OptGene predicted ~10 fold increase in the flux towards sesquiterpene production (2% of the maximum theoretical value), with 15% reduction in the specific growth rate.

Glutamate dehydrogenase encoded by *GDH1* is involved in ammonium metabolism in yeast. Once imported in the cell, ammonium is incorporated into amino group of glutamate and amide group of glutamine which serve as nitrogen source for 85% and 15%, respectively, of the total nitrogen containing compounds in the cell (Magasanik and Kaiser, 2002). There are two metabolic routes for the biosynthesis of glutamate as the main source of nitrogen in the cell (Fig. 1) (ter Schure et al., 2000). In the major route in *S. cerevisiae*, ammonium reacts with α-ketoglutarate to form glutamate. This reaction is catalyzed by NADPH-dependent glutamate dehydrogenase encoded by *GDH1* (Moye et al., 1985). The second pathway which is a two-step process (GS-GOGAT) is mediated by glutamine synthetase (GS) and glutamate synthase (GOGAT) encoded by *GLN1* and *GLT1*, respectively (Magasanik, 2003).

There are two other glutamate dehydrogenases in *S. cerevisiae* encoded by *GDH2* and *GDH3*. In contrary to *GDH1*, which uses NADPH as cofactor, *GDH2* is NADH-dependent. However, Gdh2p (NADH-dependent glutamate dehydrogenase) has much lower activity than Gdh1p (NADPH-dependent glutamate dehydrogenase) in normal cell when ammonium is used as the sole nitrogen source (Nissen et al., 1997). *GDH3* also codes an NADPH-dependent glutamate dehydrogenase activity and it was shown that the cells lacking both *GDH1* and *GLT1* are still able to grow and are not completely glutamate auxotroph whereas the triple *GDH1 GDH3 GLT1* mutants were glutamate auxotroph suggesting a physiological role for *GDH3*. Nevertheless, simultaneous deletion of *GDH1* and *GDH3* did not affect the cell phenotype compared to the single *GDH1* deletion, which implies NADPH-dependent *GDH1* and the GS-GOGAT pathway are the major routes for ammonium assimilation (Avendaño et al., 1997).

A substantial amount of NADPH in the cell is used as cofactor for the reaction catalyzed by NADPH-dependent glutamate dehydrogenase (*GDH1*) (dos Santos et al., 2003). Therefore, if *GDH1* is deleted there will be more NADPH available for other

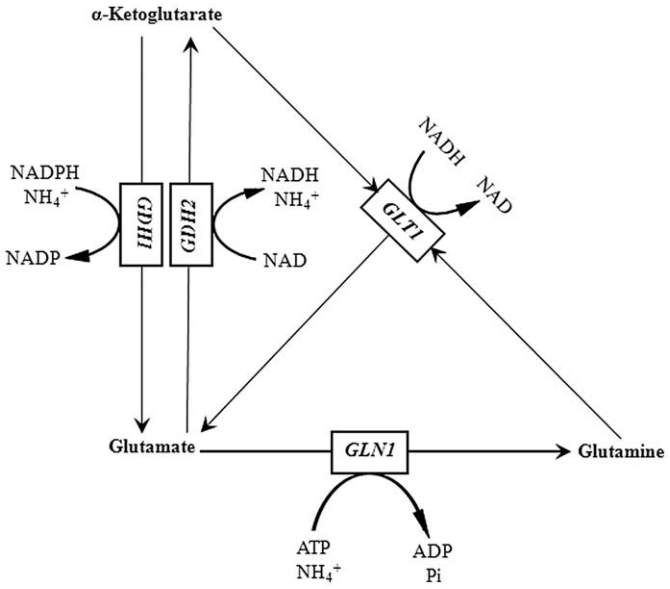

Fig. 1. Ammonium assimilation pathway in *S. cerevisiae*. The formation of glutamate via *Glt1* results in the formation of two moles of glutamate per mole of glutamine and α-ketoglutarate consumed.

Table 2
Maximum specific growth rates on galactose (μ_{max}), yields of ethanol on galactose ($Y_{EtOH/S}$), and yields of glycerol on galactose ($Y_{Glycerol/S}$) for the characterized strains.

Strain	μ_{max} (h^{-1})	$Y_{EtOH/S}$ (g/g)	$Y_{Glycerol/S}$ (g/g)
YIP-0C-04	0.20	0.138	0.015
YIP-0C-06	0.07	0.133	0.006
YIP-0C-05	0.13	0.142	0.008
YIP-MC-11	0.15	0.120	0.013

NADPH requiring reactions in the cell. In the third step of the mevalonate pathway in yeast, HMG-CoA is converted to mevalonate by the action of HMG-CoA reductase. This reaction is considered as a major flux controlling step in the mevalonate pathway (Veen and Lang, 2004) and requires NADPH as cofactor. Thus, the deletion of *GDH1*, suggested by *in silico* metabolic engineering could be beneficial for the carbon flux through the mevalonate pathway by increasing the pool of NADPH available for the HMG-CoA reductase. However, since disruption of *GDH1* impairs the ammonia utilization, simultaneous over-expression of the NADH-dependent glutamate dehydrogenase, *GDH2*, was also considered in this study.

3.2. Effect of alterations on specific growth rates and yields

The strains were grown in 5 L batch two phase fermenters. Table 2 summarizes the specific growth rates and yields of ethanol and glycerol on galactose for the characterized strains. Deletion of *GDH1* severely affected the specific growth rate of yeast strains. However, over-expression of *GDH2* restored the growth to some extent. Manipulation of the nitrogen metabolism did not affect the yield of ethanol substantially but there was a marked decrease in the yield of glycerol.

3.3. Sesquiterpene production

The strains manipulated in the ammonium assimilation pathway were characterized for sesquiterpene production in 5 L two phase batch fermenters (Fig. 2). Table 3 demonstrates the final titer and yield of sesquiterpenes for the cubebol producing yeast strains. In Table 3 total sesquiterpenes were calculated using the known percentage of cubebol in the mixture produced by the multi-product enzyme, cubebol synthase (Asadollahi et al., 2008). Deletion of *GDH1*, as was predicted by *in silico* modeling, increased cubebol production substantially. Over-expression of *GDH2* did not further enhance cubebol production but as mentioned above it resulted in an increased specific growth rate in the YIP-0C-05 compared to the YIP-0C-06 strain. Over-production of the catalytic domain of HMG-CoA reductase (YIP-MC-11 strain) further improved cubebol production as it was expected.

4. Discussion

Metabolic engineering of cell factories in order to improve a cellular phenotype, e.g. over-production of a biochemical compound, often requires manipulation of several genes in the cell. However, as the metabolic pathways are highly inter-connected, manipulation of a gene will often have secondary effects on other pathways and subsequently on the cell behavior. Therefore there has been a paradigm shift in metabolic engineering from focusing only on one gene related with the pathway of interest to the entire metabolic networks. However, because of the complicated regulation, lack of information on the kinetics of enzymes and

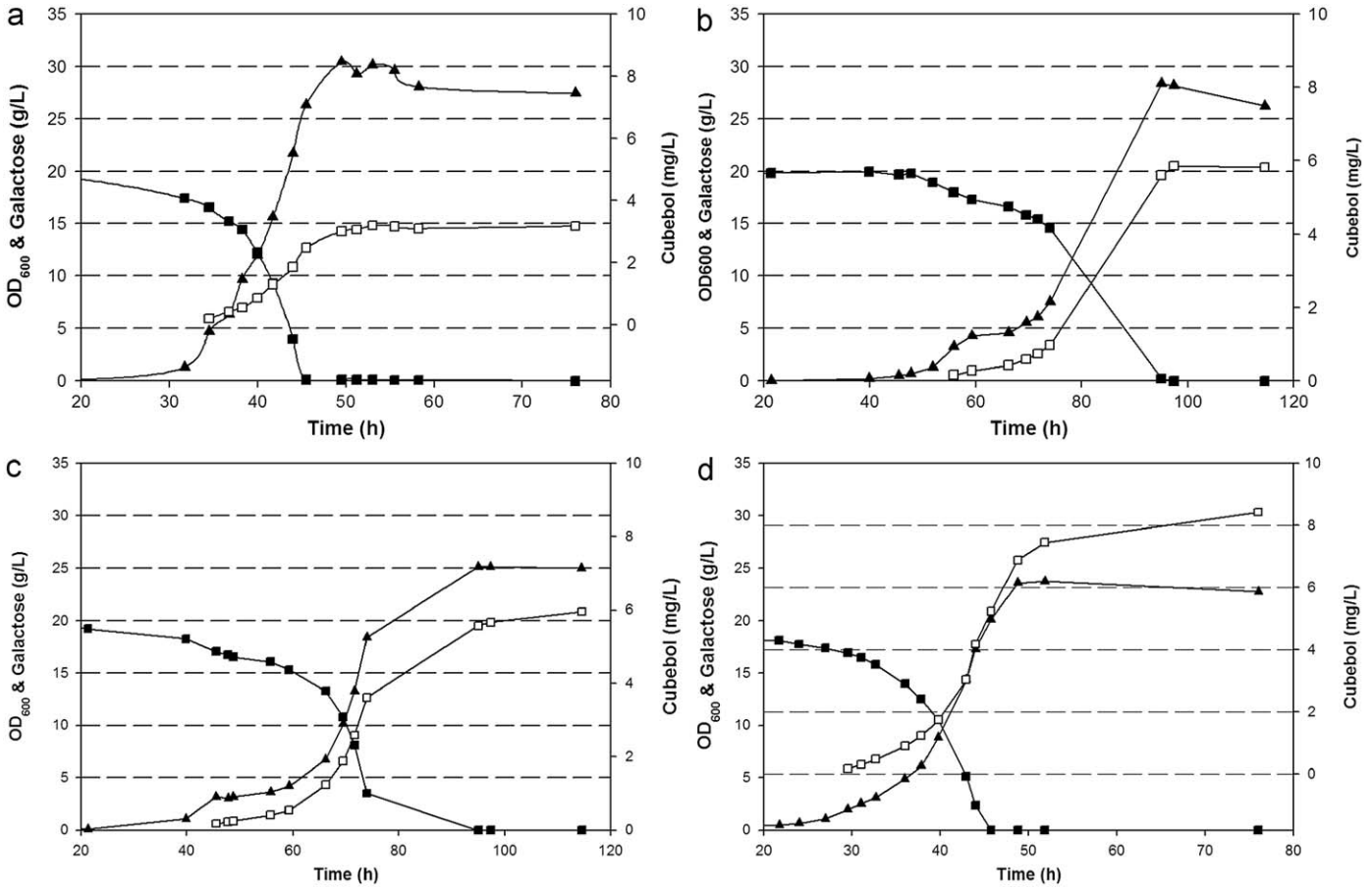

Fig. 2. Profiles of cubebol (□), galactose (■) and OD_{600} (▲) as a function of time for the cubebol producing yeast strains (a) YIP-0C-04; (b) YIP-0C-06; (c) YIP-0C-05; (d) YIP-MC-11. Cells were grown in 5 L batch fermenters containing 4 L minimal medium and 20 g/L galactose.

Table 3
Final concentrations and yields of sesquiterpenes for the different yeast strains.

Yeast strain	Cubebol (mg/L)	Total sesquiterpenes (mg/L)	Yield of cubebol on biomass (mg/g DW)	Yield of cubebol on galactose (mg/g galactose)
YIP-0C-04	3.2	11.3	0.43	0.15
YIP-0C-06	5.9	20.9	0.83	0.26
YIP-0C-05	6.0	21.3	0.81	0.28
YIP-MC-11	8.4	30.1	0.83	0.24

Cells were grown in 5 L batch fermenters containing 4 L minimal medium and 20 g/L galactose.

little information on the mechanism of interaction of pathways, it is difficult to predict the minimum genetic modifications in order to exploit the maximum capacity of cell for a desired phenotype. Despite their limited predictive power (Gombert and Nielsen, 2000), genome scale models that are primarily stoichiometric models, can be used to suggest required changes in the genotype for a desired phenotype. This strategy has been used with success for identifying new target genes for heterologous production of lycopene in *E. coli* (Alper and Stephanopoulos, 2004; Alper et al., 2005).

In this study, we used the genome-scale reconstructed metabolic network of *S. cerevisiae* (Förster et al., 2003) to identify new target genes for improved production of sequiterpenes in yeast. To our knowledge, this is one of the early examples of the use of MOMA approach for identifying metabolic engineering targets. Disruption of NADPH-dependent glutamate dehydrogenase encoded by *GDH1* was identified as the best target. Deletion of this gene will enhance the available NADPH in the cytosol for other NADPH requiring enzymes, including HMG-CoA reductase. In addition to the disruption of *GDH1*, over-expression of the NADH-dependent glutamate dehydrogenase encoded by *GDH2* was also pursued to restore efficient utilization of ammonium by cells. Manipulation of the ammonium assimilation pathway has been carried out for the purpose of improving ethanol production through reducing glycerol formation, the main by-product in anaerobic yeast fermentation. Thus, deletion of *GDH1* increased the ethanol yield by 8% and reduced the glycerol yield by 38%. The increased NADH consumption and ATP formation by the alternative GS-GOGAT pathway was the reason for these changes (Nissen et al., 2000). Likewise, this strategy enhanced ethanol production on xylose (Roca et al., 2003). Deletion of glutamate dehydrogenase (*gdhA*) in lycopene producing *E. coli* also led to a

13% improvement in lycopene accumulation (Alper and Stephanopoulos, 2004; Alper et al., 2005). In an alternative strategy for improving NADPH availability, the NAD-dependent glyceraldehydes 3-phosphate dehydrogenase (GAPDH) in *E. coli* was replaced with a NADP-dependent enzyme from *Clostridium acetobutylicum*. This strategy was shown to have a great impact on the lycopene biosynthesis in the engineered *E. coli* strains (Martinez et al., 2008). Expression of non-phosphorylating NADP-dependent glyceraldehyde 3-phosphate dehydrogenase from *Streptococcus mutans* in *S. cerevisiae* also resulted in a 40% lower glycerol yield and 3% higher ethanol yield on glucose while the specific growth rate was not affected (Bro et al., 2006).

Deletion of *GDH1* in the current study led to an approximately 85% increase in the final cubebol titer. However, deletion of this gene also caused a significant decrease in the maximum specific growth rate. This is in consistence with earlier reports on the reduced growth rate of yeast strains on glucose as a consequence of *GDH1* deletion in both anaerobic (Nissen et al., 2000) and aerobic (dos Santos et al., 2003) conditions. The OptGene-predicted reduction in the specific growth rate of the *GDH1* deletion mutant was considerably lower than the experimentally observed (15% vs. 65%). Moreover, the observed improvement in the sesquiterpene production was moderate compared to the predicted 10-fold improvement. These differences are consequence of the fact that stoichiometric models do not take into account the kinetic and thermodynamic constraints on the possible flux changes (apart from *a priori* imposed reaction directionality constraints). The model thereby assumes that the flux through Gdh2 can compensate for *GDH1* deletion. More generally, no reactions in the model are assumed to be limited by the availability of enzymes. Indeed, the observed specific growth rate of the *GDH2* over-expression mutant (YIP-0C-05) is much closer to the predicted value ($0.13\,h^{-1}$ vs. $0.17\,h^{-1}$). The observed improvement in the sesquiterpene yield attests the validity of the fundamental assumptions underlying the here-used modeling procedure for identifying metabolic engineering target. The physiological data obtained from this study can be used to impose additional constraints on the model for the next round of metabolic engineering.

Over-expression of *GDH2* did not show a further effect on the final cubebol titer but the YIP-0C-05 strain had a higher maximum specific growth rate compared to the YIP-0C-06 strain. However, the maximum specific growth rate of the YIP-0C-05 strain was still lower than that of YIP-0C-04 while in other studies over-expression of *GDH2* completely restored the specific growth rate on glucose to the wild-type level (Nissen et al., 2000; dos Santos et al., 2003). Although over-expression of *GDH2* enables cells to utilize ammonium as nitrogen source more efficiently, the modification of ammonium assimilation pathway changes the $NADPH/NADP^+$ ratio significantly. This could in turn have secondary effects such as channeling isocitrate into the glyoxylate cycle (Satrustegui et al., 1983) or reduced flux through the pentose-phosphate pathway (dos Santos et al., 2003).

The glycerol yields for both YIP-0C-06 and YIP-0C-05 strains were also significantly lower than that of YIP-0C-04 strain but there was not a marked change in ethanol yields. The surplus NADH in the cell is reoxidized to NAD^+ to avoid imbalances in the $NAD^+/NADH$ ratio. Since deletion of *GDH1* reroutes the formation of glutamate to the other NADH-dependent pathways there will be less free NADH and subsequently less glycerol formation (Nissen et al., 2000). Deletion of *GDH1* in the YIP-0C-06 strain leads to the formation of glutamate via the GS-GOGAT pathway accompanied by higher ATP consumption, which is presumably compensated by more ethanol formation (Nissen et al., 2000). However, in aerobic conditions the oxidative phosphorylation pathway is active and the slightly higher ATP consumption for growth does therefore not have a pronounced effect (dos Santos et al., 2003).

Formation of 100 g yeast biomass growing on ammonium as nitrogen source requires 931 mmol of NADPH (Bruinenberg et al., 1983). In this study, the highest yield of cubebol on biomass was 0.83 mg/g DW (2.96 mg total sesquiterpenes/g DW). Assuming the molecular weight of 222 for all sesquiterpenes formed by the cubebol synthase this yield is equivalent to 0.0133 mmol total sesquiterpenes/g DW. Since biosynthesis of each mmol sesquiterpene requires 6 mmol of NADPH consumed as cofactor by the HMG-CoA reductase, the highest NADPH requirement is 8 mmol/100 g DW. This is less than 1% of total NADPH requirement in the cell and therefore the question arises how enhancing NADPH availability leads to improved sesquiterpene production. We speculate that the higher available pool of NADPH would thermodynamically favor the NADPH-consuming reactions towards NADPH utilization and $NADP^+$ generation to balance $NADPH/NADP^+$ ratio. However, if the flux towards the mevalonate pathway is increased, at some point the availability of NADPH could also be a limiting factor for HMG-CoA reductase.

Acknowledgments

The authors gratefully acknowledge Firmenich and FTP for funding the project. Mohammad A. Asadollahi acknowledges Iranian Ministry of Science, Research and Technology (MSRT) for awarding his Ph.D. fellowship. Dr. Laurent Daviet (Firmenich SA, Corporate R&D Division) is thanked for his valuable comments and suggestion on the manuscript.

References

Alper, H., Stephanopoulos, G., 2004. Metabolic engineering challenges in the post-genomic era. Chem. Eng. Sci. 59, 5009–5017.

Alper H., JinY.–S., Moxley, J.F., Stephanopoulos, G., 2005. Identifying gene targets for the metabolic engineering of lycopene biosynthesis in *Escherichia coli*. Metab. Eng. 7, 155–164.

Asadollahi, M.A., Maury, J., Møller, K., Nielsen, K.F., Schalk, M., Clark, A., Nielsen, J., 2008. Production of plant sesquiterpenes in *Saccharomyces cerevisiae*: effect of *ERG9* repression on sesquiterpene biosynthesis. Biotechnol. Bioeng. 99, 666–677.

Avendaño, A., Deluna, A., Olivera, H., Valenzuela, L., Gonzalez, A., 1997. *GDH3* encodes a glutamate dehydrogenase isozyme, a previously unrecognized route for glutamate biosynthesis in *Saccharomyces cerevisiae*. J. Bacteriol. 179, 5594–5597.

Bro, C., Regenberg, B., Förster, J., Nielsen, J., 2006. *In silico* aided metabolic engineering of *Saccharomyces cerevisiae* for improved bioethanol production. Metab. Eng. 8, 102–111.

Bruinenberg, P.M., van Dijken, J.P., Scheffers, A., 1983. A theoretical analysis of NADPH production and consumption in yeasts. J. Gen. Microbiol. 129, 953–964.

Burgard, A.P., Pharkya, P., Maranas, C.D., 2003. Optknock: a bilevel programming framework for identifying gene knockout strategies for microbial strain optimization. Biotechnol. Bioeng. 84, 647–657.

Covert, M.W., Schilling, C.H., Famili, I., Edwards, J.S., Goryanin, I.I., Selkov, E., Palsson, B.Ø., 2001. Metabolic modeling of microbial strains *in silico*. Trends Biochem. Sci. 26, 179–186.

dos Santos, M.M., Thygesen, G., Kötter, P., Olsson, L., Nielsen, J., 2003. Aerobic physiology of redox-engineered *Saccharomyces cerevisiae* strains modified in the ammonium assimilation for increased NADPH availability. FEMS Yeast Res. 4, 59–68.

Dynesen, J., Smits, H.P., Olsson, L., Nielsen, J., 1998. Carbon catabolite repression of invertase during batch cultivations of *Saccharomyces cerevisiae*: the role of glucose, fructose, and mannose. Appl. Microbiol. Biotechnol. 50, 579–582.

Edwards, J.S., Palsson, B.Ø., 1999. Systems properties of the *Haemophilus influenzae* Rd metabolic genotype. J. Biol. Chem. 274, 17410–17416.

Edwards, J.S., Palsson, B.Ø., 2000. The *Escherichia coli* MG1655 *in silico* metabolic genotype: its definition, characteristics, and capabilities. Proc. Natl. Acad. Sci. USA 97, 5528–5533.

Engels, B., Dahm, P., Jennewein, S., 2008. Metabolic engineering of taxadiene biosynthesis in yeast as a first step towards Taxol (Paclitaxel) production. Metab. Eng. 10, 201–206.

Förster, J., Famili, I., Fu, P., Palsson, B.Ø., Nielsen, J., 2003. Genome-scale reconstruction of the *Saccharomyces cerevisiae* metabolic network. Genome Res. 13, 244–253.

Gombert, A.K., Nielsen, J., 2000. Mathematical modelling of metabolism. Curr. Opin. Biotechnol. 11, 180–186.
Jackson, B.E., Hart-Wells, E.A., Matsuda, S.P.T., 2003. Metabolic engineering to produce sesquiterpenes in yeast. Org. Lett. 5, 1629–1632.
Kauffman, K.J., Prakash, P., Edwards, J.S., 2003. Advances in flux balance analysis. Curr. Opin. Biotechnol. 14, 491–496.
Kirby, J., Romanini, D.W., Paradise, E.M., Keasling, J.D., 2008. Engineering triterpene production in *Saccharomyces cerevisiae*-β-amyrin synthase from *Artemisia annua*. FEBS J. 275, 1852–1859.
Lindahl, A.-L., Olsson, M.E., Mercke, P., Tollbom, Ö., Schelin, J., Brodelius, M., Brodelius, P.E., 2006. Production of the artemisinin precursor amorpha-4,11-diene by engineered *Saccharomyces cerevisiae*. Biotechnol. Lett. 28, 571–580.
Magasanik, B., Kaiser, C.A., 2002. Nitrogen regulation in *Saccharomyces cerevisiae*. Gene 290, 1–18.
Magasanik, B., 2003. Ammonia assimilation by *Saccharomyces cerevisiae*. Eukaryot. Cell 2, 827–829.
Martinez, I., Zhu, J., Lin, H., Bennett, G.N., San, K.-Y., 2008. Replacing *Escherichia coli* NAD-dependent glyceraldehydes 3-phosphate dehydrogenase (GAPDH) with a NADP-dependent enzyme from *Clostridium acetobutylicum* facilitates NADPH dependent pathways. Metab. Eng. 10, 352–359.
Maury, J., Asadollahi, M.A., Møller, K., Clark, A., Nielsen, J., 2005. Microbial isoprenoid production: an example of green chemistry through metabolic engineering. Adv. Biochem. Eng. Biotechnol. 100, 19–51.
Miura, Y., Kondo, K., Shimada, H., Saito, T., Nakamura, K., Misawa, N., 1998a. Production of lycopene by the food yeast, *Candida utilis* that does not naturally synthesize carotenoid. Biotechnol. Bioeng. 58, 306–308.
Miura, Y., Kondo, K., Saito, T., Shimada, H., Fraser, P.D., Misawa, N., 1998b. Production of carotenoids lycopene, ß-carotene, and astaxanthin in the food yeast *Candida utilis*. Appl. Environ. Microbiol. 64, 1226–1229.
Moye, W.S., Amuro, N., Rao, J.K., Zalkin, H., 1985. Nucleotide sequence of yeast *GDH1* encoding nicotinamide adenine dinucleotide phosphate-dependent glutamate dehydrogenase. J. Biol. Chem. 260, 8502–8508.
Newman, J.D., Marshall, J., Chang, M., Nowroozi, F., Paradise, E., Pitera, D., Newman, K.L., Keasling, J.D., 2006. High-level production of amorpha-4,11-diene in a two-phase partitioning bioreactor of metabolically engineered *Escherichia coli*. Biotechnol. Bioeng. 95, 684–691.
Nielsen, J., 2001. Metabolic engineering. Appl. Microbiol. Biotechnol. 55, 263–283.
Nissen, T.L., Schulze, U., Nielsen, J., Villadsen, J., 1997. Flux distributions in anaerobic, glucose-limited continuous cultures of *Saccharomyces cerevisiae*. Microbiology 143, 203–218.
Nissen, T.L., Kielland-Brandt, M.C., Nielsen, J., Villadsen, J., 2000. Optimization of ethanol production in *Saccharomyces cerevisiae* by metabolic engineering of the ammonium assimilation. Metab. Eng. 2, 69–77.
Oliveira, A.P., Nielsen, J., Förster, J., 2005. Modeling *Lactococcus lactis* using a genome-scale flux model. BMC Microbiol. 5, 39.
Park, J.H., Lee, K.H., Kim, T.Y., Lee, S.Y., 2007. Metabolic engineering of *Escherichia coli* for the production of L-valine based on transcriptome analysis and *in silico* gene knockout simulation. Proc. Natl. Acad. Sci. USA 104, 7797–7802.
Patil, K.R., Åkesson, M., Nielsen, J., 2004. Use of genome-scale microbial models for metabolic engineering. Curr. Opin. Biotechnol. 15, 64–69.
Patil, K.R., Rocha, I., Förster, J., Nielsen, J., 2005. Evolutionary programming as a platform for *in silico* metabolic engineering. BMC Bioinformatics 6, 308.
Reed, J.L., Vo, T.D., Schilling, C.H., Palsson, B.Ø., 2003. An expanded genome-scale model of *Escherichia coli* K-12 (iJR904 GSM/GPR). Genome Biol. 4, R54.
Ro, D.-K., Paradise, E.M., Ouellet, M., Fisher, K.J., Newman, K.L., Ndungu, J.M., Ho, K.A., Eachus, R.A., Ham, T.S., Kirby, J., Chang, M.C.Y., Withers, S.T., Shiba, Y., Sarpong, R., Keasling, J.D., 2006. Production of the antimalarial drug precursor artemisinic acid in engineered yeast. Nature 440, 40–943.
Roca, C., Nielsen, J., Olsson, L., 2003. Metabolic engineering of ammonium assimilation in xylose-fermenting *Saccharomyces cerevisiae* improves ethanol production. Appl. Environ. Microbiol. 69, 4732–4736.
Satrustegui, J., Bautista, J., Machado, A., 1983. NADPH/$NADP^+$ ratio: regulatory implications in yeast glyoxylic acid cycle. Mol. Cell. Biochem. 51, 123–127.
Segrè, D., Vitkup, D., Church, G.M., 2002. Analysis of optimality in natural and perturbed metabolic networks. Proc. Natl. Acad. Sci. USA 99, 15112–15117.
Shiba, Y., Paradise, E.M., Kirby, J., Ro, D.-K., Keasling, J.D., 2007. Engineering of the pyruvate dehydrogenase bypass in *Saccharomyces cerevisiae* for high-level production of isoprenoids. Metab. Eng. 9, 160–168.
Shimada, H., Kondo, K., Fraser, P.D., Miura, Y., Saito, T., Misawa, N., 1998. Increased carotenoid production by the food yeast *Candida utilis* through metabolic engineering of the isoprenoid pathway. Appl. Environ. Microbiol. 64, 2676–2680.
Stephanopoulos, G., Aristodou, A., Nielsen, J., 1998. Metabolic Engineering: Principles and Methodologies. Academic Press, San Diego.
Takahashi, S., Yeo, Y., Greenhagen, B.T., McMullin, T., Song, L., Maurina-Brunker, J., Rosson, R., Noel, J.P., Chappell, J., 2007. Metabolic engineering of sesquiterpene metabolism in yeast. Biotechnol. Bioeng. 97, 170–181.
ter Schure, E.G., van Riel, N.A., Verrips, T., 2000. The role of ammonia metabolism in nitrogen catabolite repression in *Saccharomyces cerevisiae*. FEMS Microbiol. Rev. 24, 67–83.
Veen, M., Lang, C., 2004. Production of lipid compounds in the yeast *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. 63, 635–646.
Verduyn, C., Postma, E., Scheffers, W.A., van Dijken, J.P., 1992. Effect of benzoic acid on metabolic fluxes in yeasts: a continuous-culture study on the regulation of respiration and alcoholic fermentation. Yeast 8, 501–517.
Verwaal, R., Wang, J., Meijnen, J.-P., Visser, H., Sandmann, G., van den Berg, J.A., van Ooyen, A.J.J., 2007. High-level production of beta-carotene in *Saccharomyces cerevisiae* by successive transformation with carotenogenic genes from *Xanthophyllomyces dendrorhous*. Appl. Environ. Microbiol. 73, 4342–4350.
Yamano, S., Ishii, T., Nakagawa, M., Ikenaga, H., Misawa, N., 1994. Metabolic engineering for production of ß-carotene and lycopene in *Saccharomyces cerevisiae*. Biosci. Biotechnol. Biochem. 58, 1112–1114.
Yoon, S.-H., Lee, Y.-M., Kim, J.-E., Lee, S.-H., Lee, J.-H., Kim, J.-Y., Jung, K.-H., Shin, Y.-C., Keasling, J.D., Kim, S.-W., 2006. Enhanced lycopene production in *Escherichia coli* engineered to synthesize isopentenyl diphosphate and dimethylallyl diphosphate from mevalonate. Biotechnol. Bioeng. 94, 1025–1032.
Yoon, S.-H., Park, H.-M., Kim, J.-E., Lee, S.-H., Choi, M.-S., Kim, J.-Y., Oh, D.-K., Keasling, J.D., Kim, S.-W., 2007. Increased β-carotene production in recombinant *Escherichia coli* harboring an engineered isoprenoid precursor pathway with mevalonate addition. Biotechnol. Prog. 23, 599–605.