Developing a yeast cell factory for the production of terpenoids

Satirios C. Kampranis *, Antonios M. Makris *

Abstract: Technological developments over the past century have made microbes the work-horses of large scale industrial production processes. Current efforts focus on the metabolic engineering of microbial strains to produce high levels of desirable end-products. The arsenal of the contemporary metabolic engineer contains tools that allow either targeted rational interventions or global screens that combine classical approaches with -omics technologies. Production of terpenoids in S. cerevisiae presents a characteristic example of contemporary biotechnology that integrates all the variety of novel approaches used in metabolic engineering. Terpenoids have attracted significant interest as pharmaceuticals, flavour and fragrance additives, and, more recently, biofuels. The ongoing metabolic engineering efforts, combined with the continuously increasing number of terpene biosynthetic enzymes discovered will enable the economical and environmentally friendly production of a wide range of compounds.

Introduction

Since antiquity, microbial fermentation processes have been extensively used for the processing of foods and the production of beverages, while technological developments over the past century made microbes the work-horses for large scale industrial production processes. Since the 1980s in particular, significant advances in genetic engineering have converted microbes to “cell factories” for the production of a diverse range of important chemical compounds. Manipulation of microbial metabolism holds major advantages, since microbes offer an environmentally friendly means to efficiently convert cheap raw materials like glucose, sucrose, and biomass derived materials into high value chemicals and fuels. Saccharomyces cerevisiae is an organism highly preferred by the industry, as it can withstand high osmotic pressure and reduced pH compared to bacteria [1]. Currently, there is continuous development and improvement of yeast strains for the production of high levels of desirable end products. The pace of strain development has accelerated as new tools for metabolic engineering manipulations are introduced. The overall approach for generating high production strains is currently based on a number of complementary approaches that include: a) the upregulation of desirable biosynthetic pathways, b) the suppression of pathways that drain resources or precursors (competing pathways), c) the introduction of exogenous genes or biosynthetic pathways, and d) the development of methodologies to alleviate stress and/or toxicity caused by the production of high levels of the product or of an undesirable intermediate. The arsenal of the contemporary metabolic engineer contains tools that allow for either targeted rational interventions that introduce changes in the strain’s genotype based on past knowledge of the biosynthetic machinery and its regulation, or global screens that combine classical approaches of strain evolution through adaptation and selection with -omics approaches that can globally assess changes leading to desirable outputs.

Efforts to produce terpenoids in S. cerevisiae are characteristic of the variety of novel approaches used for strain improvement. Terpenoids and isoprenoids are an important class of secondary metabolites contributing more than 50,000 compounds to the rich chemical diversity of natural product structures [2]. Members of this group have attracted commercial interest as flavour and fragrance additives in the food and cosmetic industry. One such example is sclareol, an industrially important diterpene used by the fragrance industry [3,4]. Many terpenoids also possess pharmaceutical properties and are currently used in clinical practice. Among them taxol, a diterpene from yew, which has successfully been established as a major antineoplastic agent, and artemisinin, a sesquiterpene lactone, which is an effective antimalarial agent [5-10]. Recently, attention has also focused on microbially produced terpenes as biofuels [11-13]. Terpenoids are biosynthesized from two C₅ precursors, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) [14]. In yeast and mammals, IPP originates from acetyl-CoA through the intermediate mevalonic acid (MVA). IPP then gives rise through the action of prenyltransferase enzymes to the higher order building blocks, geranyl pyrophosphate (GPP; C₁₀), farnesyl pyrophosphate (FPP; C₁₅) and geranylgeranyl pyrophosphate (GGPP; C₂₀) [14]. In yeast, most of the pathway output in the form of FPP is utilized for the biosynthesis of sterols. Sterols are essential structural and regulatory components of eukaryotic cell membranes. Ergosterol, the main sterol in yeast, is responsible for structural membrane features such as fluidity and permeability, in a similar way as cholesterol is in human cells [15]. The pathway has been extensively studied for several years, since it functioned as a model for understanding human disease caused by high cholesterol levels [16] and is target of an important class of antifungal compounds [16]. Extensive knowledge on the biosynthesis and regulation of the pathway provided the first set of targeted interventions to increase the pool of the intermediates GPP, FPP, and GGPP, which are the substrates of terpene synthases.

The terpene hydrocarbon scaffolds are generated by the action of mono-, sesqui-, and diterpene synthases that catalyze multi-step
Figure 1. Synopsis of terpene biosynthesis in yeast indicating the genes involved and the metabolic engineering interventions employed. Upregulated yeast genes indicated in green, downregulated yeast genes in red. Genes whose products have been fused or attached to a synthetic protein scaffold are denoted with superscripted (f). Enzymes with product yield or specificity improved or altered by protein engineering are indicated with superscripted (e). (CPP, copalylyl diphosphate; CDS, copalylyl diphosphate synthase; DTS, diterpene synthase; MTS, monoterpane synthase; SeACSL614P, Salmonella enterica acetyl-CoA synthase mutant L614P; AtoB, acetoacetyl-CoA synthase/thiolase).

Targeted interventions in the yeast MVA pathway to increase the terpene substrate pool

**HMGR**
HMGR-CoA reductase (3-hydroxy-3-methyl-glutaryl-CoA reductase; HMGR) is the key enzyme of the MVA pathway. The HMGR-catalyzed reaction produces mevalonic acid from HMGR-CoA by reduction with NADPH (Figure 1). Statins, a class of top-selling drugs for lowering cholesterol, target HMGR [31]. Yeast possesses two enzymes of HMGR, Hmg1p and Hmg2p, which share a similar structure to the mammalian and plant counterparts composed of an N-terminal eight-helix spanning domain, a linker, and a C-terminal catalytic domain. Between the two enzymes Hmg1p is considered quite stable whereas Hmg2p undergoes mevalonate products-induced degradation [32]. A variety of early studies had pointed out that increased levels of pathway products led to degradation of Hmg proteins, lowering production levels [33,34]. As a first step towards sterol-overproducing strains, Polakowski and co-workers used a deregulated version of Hmg1p [35]. The gene was truncated and the soluble tHmg1p catalytic domain was overexpressed, causing accumulation of a large amount of squalene [35]. Overexpression of tHMGI was applied to improve amorphadiene levels produced in yeast [9]. It has since become a basic tool for intervention to the MVA pathway, either by expressing tHMGI epistemally or by integrating it into the chromosome [10,25,36,37]. Alternatively, our lab used a stable variant of Hmg2p which harbors a point mutation substituting Lysine 6 by an Arginine (K6R), thus rendering the enzyme resistant to ubiquitination. Expression of HMG2 (K6R) had equivalent effects in enhancing monoterpane and sesquiterpene production [21]. The extent by which proper subcellular localization of Hmg proteins on the perinuclear membrane of the engineered strains may affect their function is not yet clear. However, we have recently identified a set of gene perturbations which lead to increased stabilization of the endogenous Hmg proteins and to dramatic improvements in sesquiterpene production that could not be achieved by tHmg2p overproduction alone (Ignea, submitted).

**ERG9**
The ERG9 gene codes for squalene synthase, the enzyme that joins two FPP moieties to form squalene in the first committed step
of the sterol biosynthetic pathway. Squalene formation is the major
draining route of isoprenoid substrates, and as such its suppression
is desirable. Since deletion of ERG9 is lethal, complete elimination of
squalene synthesizing activity is not feasible. Studying Hmg2p
regulation, Gardner and Hampton [38], used a tunable MET3
promoter (P_{MET3}) to replace the endogenous ERG9 promoter.
Addition of methionine in the medium at concentrations >0.5 mM
suppressed the transcription of ERG9 [32]. The same approach was
taken by Ro et al. [9], who used ERG9 suppression, under conditions of
tHMG1 overexpression, to achieve an additional 2-fold improvement in the levels of amorphaiden production. Suppression of ERG9 through the use of a P_{MET3} promoter was also used in an
effort to increase production of the sesquiterpenes cubebol, valencene
and patchoulol [33]. In this study, the introduction of the P_{MET3}
promoter was less efficient than anticipated and a concomitant
increase in farnesol formation, as by-product, was observed. This
raised the issue of tightness of P_{MET3} regulation due to consumption of
methionine in the medium [20]. A recent study addressed this issue
by testing fusions of the P_{MET3} to the lacZ gene and monitored β-
galactosidase activity of the strain growing under different regiments
of methionine supplementation [24]. In the cultures that contained
L-methionine, LacZ activity was initially very low. However, at about
mid-exponential phase, it started to increase and rapidly reached levels
measured in the non-repressed cultures [24]. To provide a tighter
control of ERG9 expression, the glucose regulated promoters P_{HXT1}
and P_{HXT2} were tested, aiming to maintain moderate expression of
ERG9 during exponential growth and maximal repression during
glucose limited conditions in a batch cultivation process. P_{HXT1} was
shown to be efficient in downregulating ERG9 expression under
glucose limiting conditions. An alternative to using promoters with
varying strengths and inducibility was based on posttranscriptional
mechanisms controlling mRNA stability [39]. A set of hairpin
sequence modules recognized by the RNAase III enzyme Rnt1p can
be inserted at the 3′UTR of a gene to regulate transcript stability.
With decreasing transcript levels, a similar decrease in protein l
be inserted at the 3′UTR of a gene to regulate transcript stability.

**ERG20**

The Erg20p enzyme catalyzes the condensation of IPP and
DMAPP to form GPP and subsequently FPP, which is the major
product. ERG20 overexpression was used to increase amorphaiden production but its effect was limited to a 10% increase in total yield
[9]. A small increase in sesquiterpene production was also observed
when one allele of ERG20 was controlled by the strong P_{GAL1} [21].
The ratio of GPP:FPP produced in vitro by a cell extract of wild type
cells was 25:75. A mutation was identified in lysine 197 of Erg20p
(K197E) which suppressed the second part of the catalysis resulting
in reversed ratios 70:30 GPP:FPP [41]. This shift towards GPP
formation was used to enhance monoterpene formation in yeast cells.
Expression of geraniol synthase in an ERG20 (K197G) background resulted
in improved production in geraniol productivity [42].

**ACS**

Shiba et al. addressed the bottleneck in the supply of acetyl-CoA
to the mevalonate pathway, and showed that overproduction of
acetalddehyde dehydrogenase ALD6 and introduction of a
heterologous acetyl-CoA synthase variant from *Salmonella enterica*
(L641P) together with tHMG1 expression achieved substantial
improvements in amorphaiden production [43].

**UPC2**

Upc2p and Ecm22p are two highly homologous zinc cluster
proteins regulating a number of ERG genes in the yeast ergosterol
biosynthetic pathway. They positively regulate transcription by
binding to sterol response elements in the promoters of the target
genes. The upc2-1 mutant contains a single amino acid change
(G888D) within the activation domain of the protein [44,45].
Overexpression of upc2-1 by itself appears to exert modest effects on
amorphaiden production which become more pronounced in combination with tHMG1 and P_{MET3-ERG9} [9-11].

**IDI1**

Encodes for IPP isomerase, catalyzing the conversion of IPP to its
isomer DMAPP. ERG20 adds one molecule of IPP to DMAPP
to form GPP. In the case of monoterpene production, when a rich GPP
pool is required, IDI1 overexpression significantly enhanced
monoterpene titer [21].

**BTS1**

Encodes for geranylgeranyl diphosphate synthase. The enzyme
uses FPP and IPP to synthesize the C20 GGPP substrate used for
ubiquinone biosynthesis and geranylgeranylation of proteins for
membrane attachment. GGPP is the substrate for diterpene and
carotenoid biosynthesis. Overexpression of BTS1 has been combined
with CrtYB and CrtI from *Xanthophyllomyces dendrorhous*
to generate β-carotene and with CrtS to produce astaxanthin [46,47].
Bts1p has been fused to Erg20p to improve product yields for
geranylgeraniol [48,49] and mitiradiene [25]. When the crtE gene,
which encodes for the GGPP synthase of *X. dendrhorous*, was co-
expressed with CrtYB and CrtI, significant improvement over BTS1
was observed [47]. This does not appear to be due to species
specificity of GGPPS, since we observed a comparably high
production upon co-expression of CrtYB and CrtI with the plant
derived *Cistus ceticus* GGPPS1 [50] (unpublished observations).

**LPP1, DPP1**

Inhibition of squalene synthase (SQS) in mammalian species was
shown to lead to conversion of FPP to farnesol. Farnesol
accumulation was also seen in yeast strains treated with zaragozin acid,
a natural inhibitor of SQS [51]. It has been postulated that
dephosphorylation of FPP and GGPP may be a mechanism to alleviate
the potentially toxic effects of substrate accumulation. Lpp1p and
Dpp1p, two enzymes initially identified as phosphatidic acid
hydrolases were shown to also dephosphorylate isoprenoid phosphates
[52]. Deletion of DPP1 was reported to result in a modest increase of
the sesquiterpene α-santalene and a 24% drop in farnesol
accumulation [24]. However, other studies aiming at high
sesquiterpene production did not observe significant improvements
[53,54]. Still, when LPPI and DPPI were overexpressed fused to BTSI, they exerted a strong positive effect on geranylgeraniol production, with DPPI exerting the strongest effect, yielding 2.9-fold higher levels of GGGOH than simple co-expression of the genes [49].

Protein engineering to improve product yield and to expand the chemical diversity

The primary goals of protein engineering studies to date have been to increase product yield and to interfere with the cyclization chemistry of the terpene synthases so as to either produce enzymes with higher specificity or to derive new products out of a specific enzyme.

Improving product yield – Based on the previous observation that the two main bottlenecks in terpene biosynthesis in E. coli are caused by the poor activities of the HMGR and the terpene synthase [55], Yoshikuni and coworkers developed a methodology based on adaptive evolution to improve the catalytic efficiency of these two enzymes [56]. By comparing the sequences of a large number of central metabolic enzymes, they noticed that Gly and Pro residues were significantly less frequently mutated than other amino acids in E. coli enzymes, suggesting that these residues may be more essential. They applied this finding in the truncated form of HMGR and in an inefficient humulene synthase. By switching non-conforming Gly and Pro residues to match the consensus sequence, and by saturation mutagenesis of the glycines and prolines that did not align, an engineered version of tHMGR (tHMGR-G9), which contains nine mutated residues, exhibited a 2.5- to 3-fold increase in the production of mevalonate compared to the wild-type tHMGR. Similarly, HUM-G6 (an engineered version of humulene synthase bearing six such substitutions) improved sesquiterpene production by 80 fold. Integration of the tHMGR-G9 and HUM-G6 mutants into the same host resulted into a three- to four-fold improvement in growth, leading to an overall improvement in sesquiterpene production by nearly 1000 fold [56].

Using a colorimetric assay based on the production of lycopene in E. coli as a high-throughput screening method, Leonard and coworkers applied a saturation mutagenesis approach to isolate improved Taxus canadensis GGPPS variants [57]. The double mutant S239C/G295D improved levopimaradiene production by 1.7-fold. To further improve levopimaradiene production, fifteen residues constituting the binding pocket of levopimaradiene synthase (LPS) were selected based on homology modeling. Perturbation of these residues using phylogeny-based mutation, saturating mutagenesis, and combination of the beneficial mutations improved enzyme productivity by up to 10-fold (some combinations also showed an improvement in product specificity, albeit with lower than maximal productivity) [57].

Improving product specificity – To investigate whether a promiscuous terpene cyclase can be engineered to have improved product selectivity, Yoshikuni and coworkers employed γ-humulene synthase from Abies grandis, an enzyme with an extremely promiscuous product profile, synthesizing more than 50 different sesquiterpene products. Using homology modeling, they identified 19 residues which were subsequently altered by saturation mutagenesis. By systematically combining different beneficial mutations, a collection of more specific terpene synthases was constructed, including an E-β-farnesene synthase, a siberene synthase, a β-bisabolene synthase, a longifolene synthase and an α-longipinene synthase [58].

Expanding product diversity - Until recently, there has been insufficient evidence to support the conclusion that a modified terpene synthase scaffold can produce entirely novel compounds, rather than altered levels of compounds already made by the parent scaffold. Although the large scale mutagenesis of A. grandis γ-humulene synthase mentioned above yielded mutant synthases with narrower product specificity, it failed to produce structures not synthesized by the wild-type enzyme [58]. However, this approach used a mathematical model to predict changes that would improve specificity for certain compounds and this may have influenced this screen against the identification of new entities. The first evidence for the ability of a terpene synthase scaffold to acquire a new specificity came through a concentric mutagenesis approach aiming to interconvert the activities of tobacco 5-epi-abietolcholene synthase (TEAS) and Hyoscyamus muticus prennaspirodiene synthase (HPS). In this study, a combination of second tier residue alterations enabled the production of epi-abietolcholene by HPS, an activity not present in the wild-type enzyme [59]. Our work on the engineering of Salvia terpene synthases extended this finding, by demonstrating that a monoterpene synthase can be modified to produce sesquiterpenes by a single amino acid substitution [60]. In the same study, the Salvia pomifera sabine synthase 1 (Sp-SabS1) was modified to produce 1,8-cineole and a-terpinneol, two products not made by the wild-type enzyme. Subsequent studies extended these observations to diterpene synthases, where single residue substitutions converted an isokaurene synthase into a specific pimaradiene synthase [61], or a levopimaradiene/abietadiene synthase into an isopimaradiene synthase [62]. The limited sequence-space explored in these experiments makes it possible that significant novel activity can be produced using more thorough mutagenesis approaches.

Metabolic channeling by fusion enzymes

The generation of fused enzymes has been proposed as a means to minimize losses of intermediate products through diffusion, degradation, or utilization by rival enzymes. This could be especially important when exogenous enzymes, such as terpene synthases (TPSs), are used. Fusions of Erg20p to TPSs were tested for the production of patchoulol and exhibited higher yields than the separate enzymes [53]. Not all fusions appear to result in a net gain, since some enzymes are also sensitive to their placement in the fusion. We noted that a C-terminal fusion of EFYF to a sesquiterpene synthase (STS) caused a significant reduction in enzymatic activity. Similarly, no improvement was seen when farnesyl diphosphate synthase (FPPS) was fused at the C-terminus of patchoulol synthase (PTS), in contrast to the inverse fusion (FPPS-PTS) [53]. Expanding this system, a dual fusion strategy was employed by Zhou and colleagues for the production of the diterpene miltiadene. The BtsI protein was fused to the N-terminus of Erg20p to drive efficient GGPP production, while copalyl diphosphate (CPP) synthase was appended to the C-terminus of miltiadene synthase to provide the latter with ample adequate CPP substrate [25]. A parallel approach fused FPPS and STS genes to the COX4 mitochondrial targeting sequence, aiming to target the pathway to the organelles where FPP pools are naturally present. When combined with cytosolic tHMGR overexpression, an extra boost in sesquiterpene production was observed [63].

An alternative approach to fusion proteins was applied to the mevalonate pathway by attachment of protein interaction domains and their respective peptide ligands. Expression of yeast HMGR and HMG-CoA synthase (HMGS) attached to the SH3 domain and the SH3 ligand respectively in E. coli improved mevalonate yields [64]. The protein interaction domains were also built into a synthetic
Production of terpenoids in yeast

Coping with stress

Increased production of terpenes, especially oxidized compounds is expected to impose stress on the cellular machinery. Examination of cells producing artemisinic acid for induction of pleiotropic drug resistance revealed high induction of the ABC transporters PDR5, PDR15, YOR1, and SNQ2 [65]. Identification of plant-pathway components which participate in biosynthesis and transport of terpenoids could be an additional means to relieve the stress imposed on the yeast cell by high production. We previously identified a plant HSP90 by two-hybrid assays, which interacted with the monoterpene cineole synthase (SfCinS1). Co-expression of the HSP90 and SfCinS1 in yeast cells contributed to a modest, albeit consistent, improvement in cineole production [21]. Presumably additional plant genes can be tested for their contribution to terpene biosynthesis in yeast cells.

Global approaches to strain improvement

The development of -omics technologies and advanced modeling tools has enabled global approaches which could illuminate the genetic basis of phenotypic diversity. Such an approach was applied to assess the differences in ergosterol production between widely used lab strains. One such strain is CEN.PK113-7D which is becoming a strain of choice for terpenoid production. The strain was fully sequenced, annotated and compared to the 288c reference genome to identify single nucleotide polymorphisms (SNP) which could be informative on the nature of changes taken place in the ergosterol pathway. Focusing only on metabolic genes, 85 out of 219 SNPs were encoding for amino acid changes (non-synonymous or non-silent) [66]. A number of SNPs were identified in the genes ERG8 (phosphomevalonate kinase), ERG9 and HFA1 (mitochondrial acetyl co-enzyme A carboxylase catalyzing the production of malonyl-CoA in fatty acid biosynthesis). Co-expressed with β-amyrin synthase with combinations of these variants led to a 5-fold improvement in amyrin production [67].

Another computational approach based on a minimization of metabolic adjustments algorithm (MOMA), identified GDH1 as a possible target which could shift the metabolic flux towards the ergosterol pathway. The gene encodes a glutamate dehydrogenase involved in ammonium metabolism in yeast and requires NADPH for its function. The conversion of HMG-CoA to mevalonate is an NADPH requiring step, thus deletion of GDH1 was postulated to be beneficial for carbon flux through the mevalonate pathway by increasing the pool of available NADPH for HMGR. In yeast there are two other glutamate dehydrogenase enzymes encoded by GDH2 and GDH3. GDH3 appears to have arisen from genome duplication of GDH1, while GDH2, unlike the other two, is an NADH-dependent enzyme. Deletion of GDH1 in cells expressing cubebol synthase led to approximately 85% increase in the final titer [68]. However, despite the presence of the other two GDH1 enzymes, gdh1 causes severe growth impediments [68]. An approach to suppress but not eliminate protein levels could be effective without the side effects. Recently, we tested heterozygous deletions of GDH1 alone or in combinations with other haploinsufficiencies and observed a modest but consistent increase in sesquiterpene production (Ignea, submitted). Being complementary to approaches focused on the biosynthetic components, as the production yield increases, suppression of GDH1 may become more important.

The dramatic reductions in sequencing costs and the rapid development of new computational tools will contribute to classical strain improvement approaches using evolution and adaptation by providing feedback on the genetic changes that take place and cannot be assessed by metabolic flux models or rational design approaches.

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Competing Interests:
The authors have declared that no competing interests exist.

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