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DOI
10.1016/j.copbio.2013.03.023

Publication date
2013

Document Version
Final published version

Published in
Current Opinion in Biotechnology

Available online at www.sciencedirect.com

SciVerse ScienceDirect

Current Opinion in Biotechnology

Metabolic engineering of yeast for production of fuels and chemicals

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Microbial production of fuels and chemicals from renewable carbohydrate feedstocks offers sustainable and economically attractive alternatives to their petroleum-based production. The yeast *Saccharomyces cerevisiae* offers many advantages as a platform cell factory for such applications. Already applied on a huge scale for bioethanol production, this yeast is easy to genetically engineer, its physiology, metabolism and genetics have been intensively studied and its robustness enables it to handle harsh industrial conditions. Introduction of novel pathways and optimization of its native cellular processes by metabolic engineering are rapidly expanding its range of cell-factory applications. Here we review recent scientific progress in metabolic engineering of *S. cerevisiae* for the production of bioethanol, advanced biofuels, and chemicals.

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Current Opinion in Biotechnology 2013, **24**:398–404

This review comes from a themed issue on **Energy biotechnology**

Edited by **Eric Toone** and **Han de Winde**

For a complete overview see the Issue and the Editorial

Available online 20th April 2013

0958-1669/$ – see front matter,

http://dx.doi.org/10.1016/j.copbio.2013.03.023

Introduction

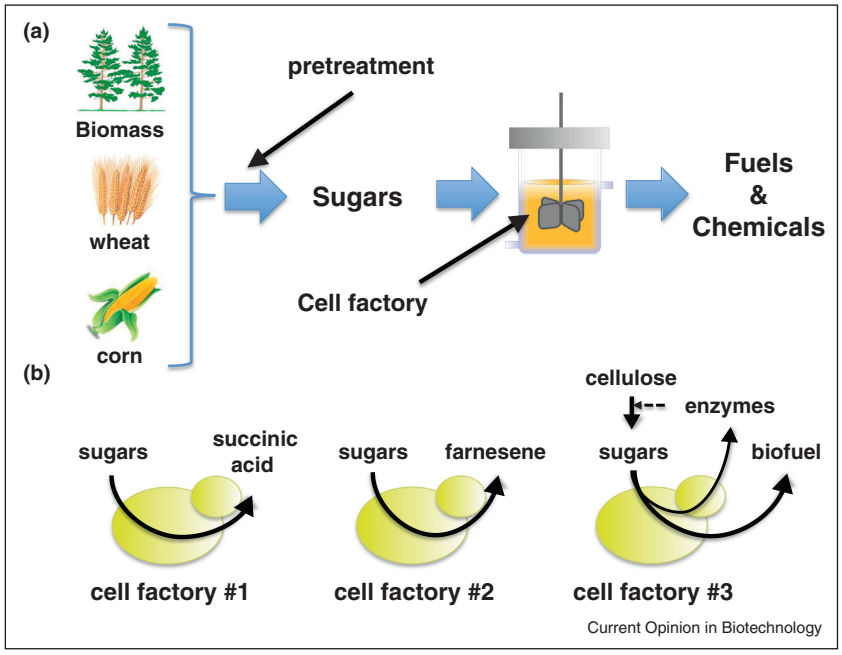
Global consumption of liquid transportation fuels amounts to about 2.9 TW and these fuels are currently mainly derived from petroleum, whereas biofuels only account for 2.7% of the total transportation energy (Key World Energy Statistics 2011; URL: http://www.iea.org). The use of petroleum for transportation results in emission of more than 5 Gt CO_2, and this represents a major contribution to the total green house gas (GHG) emissions. Currently by far the dominant biofuel is ethanol, which is being produced at 75 billion liters annually, with the majority being produced in the USA (50 billion liters) with corn as the major feedstock. The remainder of the production is concentrated in Brazil with sugar cane as feedstock. The use of biofuels is much debated due to the high costs of the corn-based process as well as the limited reduction in GHG emission by this process compared with petroleum [1]. Several predictions, however, show an increasing role of biofuels [2,3]. This is due to future production of second generation ethanol with biomass as feedstock. Another reason being the production of advanced biofuels that have improved fuel properties compared with ethanol. Such advanced biofuels will not only be cost competitive with petroleum but will also substantially reduce GHG emissions [1].

Future production of biofuels will take place in biorefineries, which may be retrofitted corn-ethanol plants, or new, dedicated plants for processing biomass. In biorefineries different types of feedstock will be processed to sugars that are subsequently converted into the desired products through microbial fermentation (Figure 1). At the heart of biorefineries is the fermentation process, in which microbial biocatalysts ensure conversion of sugars into the fuel or chemical to be produced. In order to ensure flexibility in biorefineries, industry is highly interested in so-called platform cell factories. Owing to its role in bioethanol production, the yeast *Saccharomyces cerevisiae* is already the most intensively applied microbial cell factory. In addition, robustness under process conditions, genetic accessibility and a strong fundamental knowledge base in physiology and systems biology [4,5,6•,7•] contribute to its current popularity as a 'general purpose' metabolic engineering platform [6•]. Novel synthetic biology methods, based on the unsurpassed efficiency of homologous recombination in *S. cerevisiae*, contribute to a further tremendous acceleration of genetic modification in this yeast [7•]. Here we will review the recent advances in metabolic engineering of *S. cerevisiae* for its use as a platform cell factory for the production of ethanol from conventional and lignocellulosic feedstocks and of advanced biofuels and chemicals.

First generation bioethanol production: status and perspectives

Considerable efforts have been made to minimize or completely abolish formation of glycerol, the major by-product during current bioethanol production. During anaerobic growth of *S. cerevisiae*, glycerol serves as an essential electron sink for reoxidizing reduced redox cofactors (NADH) generated in biosynthesis. Glycerol formation can be prevented or reduced by deleting one or both genes encoding cytosolic NADH-dependent glycerol-3-phosphate dehydrogenases, *GPD1* and *GPD2* [8]. However, a double deletion renders cells unable to grow anaerobically. Deletion of, for example, *GPD2*,

Figure 1



Overview of a biorefinery. **(a)** Different types of feedstock, that is, corn, biomass and agricultural waste, are processed to generate sugars. These sugars are subsequently converted into fuels and chemicals by a biocatalyst, for example, the yeast *S. cerevisiae*. **(b)** By metabolic engineering different yeast strains can be generated and this allows for production of a range of different products using the same infrastructure, that is, a plug-and-play solution. Yeast can also be engineered to express enzymes that can degrade the polymers present in the feedstock, and hereby it is possible to reduce the overall processing costs, as enzymes used for degradation of starch, cellulose or lignocellulose are quite expensive. A process where yeast is secreting enzymes for polymer degradation is referred to as a consolidated bioprocess.

results in an increased ethanol yield and decreased glycerol formation, but severely hampers growth and ethanol productivity [9]. Alternative approaches aim at engineering cellular redox metabolism to reduce formation of cytosolic NADH. Nissen *et al.* [10] deleted the NADPH-dependent glutamate dehydrogenase, *GDH1*, while overexpressing *GLN1* and *GLT1* (encoding glutamine synthetase and glutamate synthase), respectively. The resulting ammonium assimilation pathway consumed NADH as well as ATP and led to a reduction in glycerol yield by 38% while the yield of ethanol was increased by 10%. Reducing energy conservation in alcoholic fermentation of sugars can by itself increase the ethanol yield in *S. cerevisiae*, as illustrated by Basso *et al.* [11]. These authors substituted the dominant extracellular invertase with a cytoplasmic version relying on proton-symport for transport of sucrose into the cell. The increased energy expenditure was shown to be compensated by an increased flux toward ethanol. An alternative approach to reduce both the surplus of cytosolic NADH and produce less ATP has been done by replacing the natural glyceraldehyde-3-phosphate dehydrogenase with a non-phosphorylating, $NADP^+$-dependent counterpart (GAPN) from *Bacillus cereus* or *Streptococcus mutans* [12–15]. All these attempts were successful in reducing the glycerol yield and increasing the ethanol yield. Zhang *et al.* [15] took this one step further and combined expression of $NADP^+$ dependent GAPN with introduction of novel NADH-reoxidizing pathways. They used either a NAD^+ dependent fumarate reductase or an acetaldehyde dehydrogenase and in both cases impressive ethanol yields above 95% of the theoretical maximum were reported [15]. An earlier redox engineering study by Guadalupe Medina *et al.* [16•] demonstrated that expression of an acetylating NAD^+-dependent acetaldehyde dehydrogenase from *Escherichia coli* enabled acetate-dependent, glycerol-negative anaerobic growth of a *gpd1Δgpd2Δ* mutant. Since the resulting strain converted acetate into ethanol, this concept may be valuable for conversion of acetate-containing biomass-based feedstocks. Jain *et al.* [17] expressed alternative oxidoreductase genes for consumption of excess NADH in a *gpd1Δgpd2Δ* background which partly restored the ability to grow under anaerobic conditions.

A problem associated with reduced ability to produce glycerol in *S. cerevisiae* is that the osmosensitivity as well as the general robustness is reduced [18]. Maintenance of an osmotolerant phenotype is crucial when moving toward using high or very high gravity fermentations, which are attractive in terms of productivity and titer, and hence lower capital and energy requirements. Efforts

have therefore been made to improve stress resistance even when glycerol formation is hampered, for example, by selected genetic manipulations followed by genome shuffling methods to increase osmotolerance as well as ethanol and heat tolerance [14,19,20]. By this strategy it has been shown that it is indeed possible to decrease glycerol formation concomitant with an increase in fermentation rate and ethanol yield. Guo *et al.* [13] used a more targeted approach where they combined *GPD1* deletion with expression of GAPN and overexpression of the trehalose synthesis genes *TPS1* and *TPS2* and hereby obtained a robust, high-yielding strain. This could be of interest since trehalose is also a stress protectant and there is a correlation between accumulation of this compound and thermotolerance as well as ethanol tolerance of *S. cerevisiae* strains [20,21]. Furthermore, overexpression of *TPS1* in *S. cerevisiae* will result in enhanced thermotolerance [22], which is interesting as it enables, firstly, reduced energy requirements for the subsequent distillation process and secondly, a closer correspondence of the optimum temperatures for enzymes used in the saccharification process and the optimum temperature for fermentation in so-called SSF processes (simultaneous saccharification and fermentation) [23].

Conversion of lignocellulosic feedstocks

Engineering of *S. cerevisiae* for second-generation bioethanol production has long focused on conversion of D-xylose and L-arabinose, two abundant sugars in lignocellulosic hydrolysates. Expression of heterologous, isomerase-based pathways, combined with overexpression of the non-oxidative pentose-phosphate pathway and, in the case of arabinose, a suitable transporter [24], has yielded strains whose ethanol yields on pentoses equal those on glucose [25,26]. A growing number of xylose isomerases can now be functionally expressed in *S. cerevisiae* and several companies are implementing pentose-fermenting yeast strains in large-scale processes. Engineering *S. cerevisiae* for fermentation of galacturonic acid, a key compound in pectin-rich feedstocks such as sugar-beet pulp and citrus peel, is still in its infancy. Enzymes from fungal and bacterial galacturonate pathways have been functionally expressed [27,28] but alcoholic fermentation of galacturonate has not yet been reported.

Although stoichiometric challenges are solved, the highest reported rates of pentose fermentation by *S. cerevisiae* strains are still below those of glucose fermentation [26,29,30]. Moreover, during the batch-wise conversion of sugar mixtures that are often used in industrial ethanol production, pentoses are typically only converted after glucose exhaustion [31,32]. Only under glucose limitation, such as in chemostats or in fed-batch fermentations, are these glucose and pentoses co-consumed [33]. This suboptimal kinetics prolongs process times and augments the sensitivity to inhibitors such as acetic acid [33]. A specially designed evolutionary engineering protocol based on repeated batch cultivation was successfully applied to accelerate utilization of mixtures of glucose, xylose and arabinose [31]. Mechanistically, research on pentose fermentation kinetics increasingly focuses on pentose transport. The *S. cerevisiae* hexose transporters that are capable of transporting xylose and arabinose have low maximum pentose-uptake rates and their superior affinity for glucose effectively blocks pentose transport while glucose is present. An intensive search is underway to identify and express eukaryotic pentose transporters that combine favourable kinetics for pentoses with inertness toward glucose. While promising transporters have been identified [24,34,35], this has not yet led to breakthroughs in fermentation kinetics.

Optimization of biomass hydrolysis should ultimately prevent inhibition of yeast performance by furfural and hydroxymethylfurfural, which are formed by heating of sugars at low pH. In contrast, acetic, formic, and ferulic acids are integral parts of plant biomass. Significant improvement of yeast tolerance to acetic acid has been achieved by evolutionary engineering [30]. Promising recent developments include exploration of biodiversity among *Saccharomyces* strains by comparative genomics and reverse engineering of tolerance phenotypes [36,37] and the reductive conversion of acetic acid into ethanol by engineered strains [16•].

The 'holy grail' of current yeast bioethanol research is to efficiently express all enzymes required for feedstock hydrolysis, currently a decisive cost factor in lignocellulosic biotechnology, in *S. cerevisiae*. This concept of 'consolidated bioprocessing' is vigorously pursued [38,39]. High-level expression of cellobiohydrolases has been demonstrated [40•], as well as functional expression of heterologous genes for uptake and hydrolysis or phosphorolytic cleavage of cellobiose [41–43]. Moving this promising field beyond the demonstration of slow conversion of model cellulose compounds raises confounding scientific challenges related to high-level cellulase expression and protein burden in anaerobic, energetically compromised yeast cultures [44], interaction of yeast cells with solid substrates, and functional assembly of hydrolase enzyme complexes.

Advanced biofuels

There have been several studies on production of advanced biofuels using *E. coli* as cell factory, for example, for production of 1-butanol [45], fatty acyl ethyl esters (FAEEs) [46], and alkanes [47]. There are, however, only few studies on production or advanced biofuels using *S. cerevisiae* as a cell factory.

Steen *et al.* [48] engineered yeast to produce 1-butanol, but the titers were much lower than what has been obtained with *E. coli* (a few mg/L compared with g/L for *E. coli*). There is, however, much interest in

Figure 2

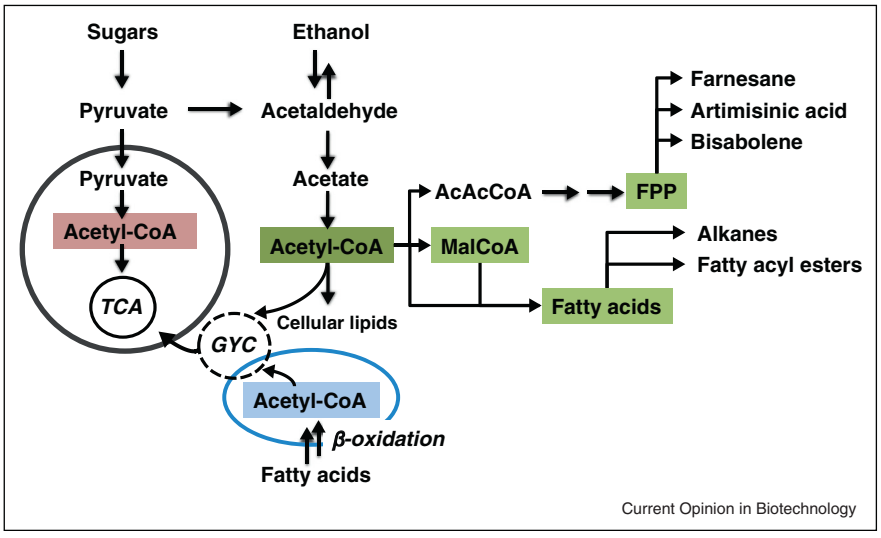


Illustration of the concept of cell factory platforms. A strain with efficient production of farnesyl pyrophosphate (FPP) can be used for production of a range of different sesquiterpenes and a strain with efficient production of acetyl-CoA can be used for production of both sesquiterpenes and fatty acid derived biofuels. The compartmental issue of acetyl-CoA metabolism is also illustrated pointing to the difficulties with engineering the central carbon metabolism of *S. cerevisiae*. *Abbreviations*: TCA, tricarboxylic acid cycle; GYC, glyoxylate cycle; AcAcCoA, acetoacetyl-CoA; MalCoA, Malonyl-CoA; FPP, farnesyl pyrophosphate.

commercial (iso-)butanol production using *S. cerevisiae* as a cell factory as demonstrated by a range of patents and patent applications by Butalco, Butamax Advanced Biofuels (a joint venture of BP and Dupont) and Gevo [6•], and several of these patents report productivities that far surpass productivities reported by *E. coli*. Butanols have a number of advantages as biofuels compared with bioethanol, that is, a higher energy density, better blending into gasoline, and less hydroscopic [49].

Other interesting advanced biofuels are longer-chain hydrocarbons and FAEEs that can be used as diesel or jet fuels. The company LS9 is actively pursuing production of alkanes and FAEEs, but they rely on *E. coli* as a cell factory platform [46,47]. Their process is based on secretion of the fuels to the extracellular medium, which in comparison to the current production by re-esterification of plant oils has decreased by-product formation, improved overall energetic yields, and the ability to use non-food crops as feedstock [50]. Recently Shi *et al.* demonstrated production of FAEEs in yeast by expression of heterologous wax synthases [51]. The company Amyris is developing yeast cell factories for the production of farnesene — a non-cyclic sesquiterpene that can be chemically converted into farnesane, which has obtained EPA certification for blends up to 35% with petroleum diesel. In this process the company is taking advantage of their earlier work on developing a process for production of the anti-malarial precursor, artemisinic acid, which is another sesquiterpene derived from the same precursor farnesyl pyrophosphate (FPP) [52,53•]. This is a very good example of how a cell factory platform that through metabolic engineering has a high flux toward FPP, can be used for production of different types of products (Figure 2). It is also interesting to note that many of these advanced biofuels are derived from acetyl-CoA, which makes it interesting to develop a platform yeast cell factory that has efficient supply of this precursor metabolite [54] (see Figure 2).

Chemicals

The rapidly expanding variety of chemicals produced by engineered *S. cerevisiae*, ranging from commodity chemicals such as 1,2-propanediol, through ascorbic acid to fine chemicals such as resveratrol, or valancene, have recently been comprehensively reviewed by Hong and Nielsen [6•]. Seen from a different perspective, the engineered product range of *S. cerevisiae* encompasses compounds that are closely linked to primary metabolism as well as molecules whose formation from yeast central metabolites requires the functional expression of entire heterologous and/or synthetic pathways.

Production of C4-dicarboxylic acids (such as malate and succinate) is a prominent example of the category of products that are closely linked to primary metabolism and illustrates many of the current conceptual developments in yeast metabolic engineering. Production of these acids at low pH avoids the formation of gypsum in product recovery, which strongly benefits process economy. A first challenge in the conversion of glucose to non-native products is the elimination of ethanol as a

by-product. Deletion of the three structural genes encoding pyruvate decarboxylase and subsequent introduction of a mutated *MTH1* allele, enables growth of Pdc^- yeast strains at high glucose concentrations without the formation of ethanol [55]. Other key factors in the successful metabolic engineering of *S. cerevisiae* for production of C4-dicarboxylic acids include redox-driven product formation, engineering of carboxylation reactions and functional expression of heterologous product exporters [56–58]. For robust, high-yield production, product pathways should ideally yield a small surplus of ATP to meet cellular maintenance requirements. In malate and succinate production, this requires replacement of the native pyruvate carboxylase by more energy-efficient carboxylation reactions. These and selected other free-energy-coupling reactions in *S. cerevisiae* have recently been reviewed [59]. Optimization of energy coupling in pathway design is strongly supported by integration of thermodynamic information in genome-scale metabolic network models [60].

Isoprenoids form a good example of a class of compounds whose production by metabolically engineered *S. cerevisiae* requires functional expression and/or deregulation of multi-enzyme pathways leading from central metabolism to complex products. Initially developed for production of the plant-derived antimalarial precursor artemisinic acid [53•], a thoroughly optimized strain platform was used to produce other products, such as farnesene and squalene, from FPP as mentioned above [61]. The same concepts have been applied to efficiently produce different plant sesquiterpenes that can be used as perfumes and fine fragrances [62]. Supply of precursors and specifically cytosolic acetyl-Coenzyme A is essential for high-level formation of isoprenoids and many other industrially relevant products. Consequently, the native metabolism of acetyl-CoA in *S. cerevisiae* is being thoroughly investigated [63] and heterologous pathways, such as the *Aspergillus nidulans* phosphoketolase pathway [64], have been expressed in *S. cerevisiae* to increase acetyl-CoA supply to product pathways.

Perspectives

The ongoing implementation of engineered yeast strains in large-scale processes for production of second generation ethanol (BP/Verenium, DSM/Poet, Dupont/Danisco, Mascoma and others), succinic acid (DSM/Roquette and Bio-Amber/Cargill), butanols (Butamax and Gevo), and isoprenoid-derived chemicals (Amyris, Firmenich and others) illustrates the firm anchoring of yeast metabolic engineering in modern industrial biotechnology. The coming years will show whether key constraints in the physiology of *S. cerevisiae* can be addressed by remodelling of its core cellular machinery or whether use of non-*Saccharomyces* yeasts is a more attractive means of meeting specific process requirements for certain products. The latter approach has already been implemented in industry (Cargill) via the application of an engineered acid-resistant yeast species for industrial production of lactic acid.

Acknowledgements

We thank Harmen van Rossum for his assistance with the layout of the manuscript. We acknowledge funding for our research in this field from Vetenskapsrådet, FORMAS, the Novo Nordisk Foundation, Swedish Energy Agency, Nordic Energy Research, BE-Basic, The Netherlands Genomics Initiative and NWO.

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