Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in Biotechnology

Metabolic engineering of *Saccharomyces cerevisiae* for production of butanol isomers

Wesley Cardoso Generoso*, Virginia Schadeweg*, Mislav Oreb and Eckhard Boles

Saccharomyces cerevisiae has decisive advantages in industrial processes due to its tolerance to alcohols and fermentation conditions. Butanol isomers are considered as suitable fuel substitutes and valuable biomass-derived chemical building blocks. Whereas high production was achieved with bacterial systems, metabolic engineering of yeast for butanol production is in the beginning. For isobutanol synthesis, combination of valine biosynthesis and degradation, and complete pathway re-localisation into cytosol or mitochondria gave promising results. However, competing pathways, co-factor imbalances and FeS cluster assembly are still major issues. 1-Butanol production via the *Clostridium* pathway seems to be limited by cytosolic acetyl-CoA, its central precursor. Endogenous 1-butanol pathways have been discovered via threonine or glycine catabolism. 2-Butanol production was established but was limited by B_{12}-dependence.

Addresses
Institute of Molecular Biosciences, Goethe University Frankfurt, Max-von-Laue-Str. 9, 60438 Frankfurt am Main, Germany

Corresponding author: Boles, Eckhard (e.boles@bio.uni-frankfurt.de)

* Equal contribution.

Current Opinion in Biotechnology 2015, **33**:1–7

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

Edited by **Eleftherios Terry Papoutsakis** and **Jack T Pronk**

For a complete overview see the Issue and the Editorial

Available online 4th October 2014

http://dx.doi.org/10.1016/j.copbio.2014.09.004

Introduction

Among second-generation biofuels, butanol isomers 1-butanol, 2-butanol and isobutanol are regarded as suitable gasoline substitutes due to their high octane rating and low hygroscopicity [1]. Several review articles provide comprehensive overviews of efforts to engineer synthesis of butanol isomers in diverse host organisms (e.g. [2–8]). The scope of this review will be an actual overview of academic research dealing with production of butanol isomers in the yeast *S. cerevisiae* (Figure 1).

S. cerevisiae is successfully employed in various industrial processes due to its high robustness [9]. Whereas ethanol is its main product, yeast is also able to produce isobutanol and 1-butanol as byproducts (around 0.2 mg/g glucose). These properties make *S. cerevisiae* an attractive organism for metabolic engineering to improve butanol production. The maximum theoretical yields are 0.41 g/g glucose.

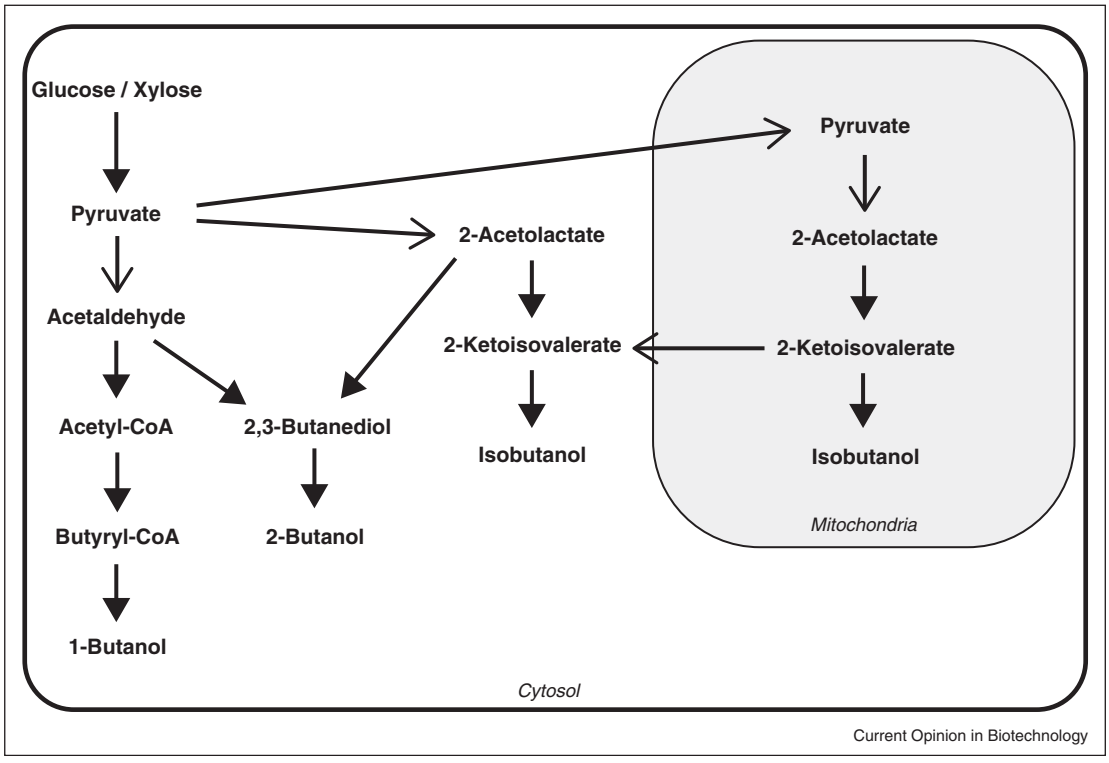
Isobutanol

Isobutanol biosynthesis occurs via the Ehrlich pathway [10], whereby 2-ketoisovalerate (KIV), an intermediate of valine biosynthesis (and degradation) is first decarboxylated by 2-ketoacid decarboxylase (Kdc) to isobutyraldehyde, which is reduced to isobutanol by alcohol dehydrogenase (Adh). In bacteria, overproduction of isobutanol can be achieved by concomitant overexpression of enzymes involved in valine anabolism and suitable Kdcs and Adhs [11]. In yeast, the situation is more complicated, because the proteins required for synthesis of KIV are located in mitochondria, while Kdc and Adh are cytosolic (Figure 1). First, pyruvate is imported into mitochondria and two molecules are condensed to acetolactate (ALAC) by acetolactate synthase (Als) Ilv2. Ilv6 acts as a valine-responsive regulator of Ilv2. In the subsequent step, ALAC is reduced to 2,3-dihydroxyisovalerate (DIV) by acetohydroxyacid reductoisomerase (Ahar) Ilv5. Finally, DIV is converted to KIV by dihydroxyacid dehydratase (Dhad) Ilv3, an iron sulfur (FeS) cluster containing enzyme. For isobutanol synthesis, KIV must then be transported into the cytosol.

In a first attempt to enhance synthesis of isobutanol in yeast, Chen *et al.* [12] overexpressed the mitochondrial part of the pathway (*ILV2*, *ILV5* and *ILV3* genes) and achieved six-fold increased isobutanol yields compared to wild-type levels. Additional overexpression of cytosolic branched-chain amino acid aminotransferase Bat2 further increased isobutanol production (Table 1), possibly by raising the availability of KIV and degrading valine, which exerts a negative feedback regulation on Ilv2 via Ilv6 [13].

In subsequent publications, valine synthesis and the Ehrlich pathway were short-circuited either in the cytosol or in mitochondria. Avalos *et al.* [14••] demonstrated that moving the complete pathway into mitochondria resulted in a substantial increase in isobutanol synthesis compared to the split pathway. Kdcs and Adhs were either overexpressed in the cytosol or imported into mitochondria by fusing them with an N-terminal targeting signal. The

Figure 1



Simplified scheme of metabolic pathways for production of butanol isomers in *Saccharomyces cerevisiae*. For sake of clarity, the scheme does not discriminate between native and heterologous enzymatic reactions and does not comprise the pathways for 1-butanol production from threonine and glycine. Filled-head arrows represent multiple enzymatic steps and open-head arrows represent direct reactions or transmembrane transports.

highest isobutanol titers were obtained with a completely mitochondrial pathway (Table 1). Based on the fact that Kdc and Adh enzymes showed an increased local concentration within mitochondria, the authors speculated that the smaller volume of the organelle might concentrate enzymes and substrates, favoring faster reaction rates. Unfortunately, no comparison with the full cytosolic pathway was included. Similarly, Yuan and Ching [15] developed a strain with the genes encoding a complete mitochondrial pathway integrated into the genome using a δ-integration system which resulted in 15 mg isobutanol per g glucose. Nevertheless, it must be considered that under industrially relevant fermentation conditions like anaerobiosis and high sugar concentrations, yeast cells contain only few poorly differentiated mitochondria [16].

A fully cytosolic pathway was established by Brat *et al.* [17••,18]. To re-localize Ilv2, Ilv5 and Ilv3 into the cytosol, various versions lacking mitochondrial targeting sequences were constructed. Only when the complete cytosolic pathway was overexpressed in the absence of a mitochondrial pathway and in the absence of valine, significantly increased isobutanol production was observed. After codon optimization of truncated *ILV* genes, isobutanol titers increased up to 630 mg/L in a *Δilv2* strain. On the other hand, two other groups achieved increased isobutanol titers by overexpressing cytosolic Ilv2, Ilv3, Ilv5 and Kdc proteins despite the presence of an intact mitochondrial pathway [19,20], or even with concomitant mitochondrial Ilv2 overexpression [21••].

Nevertheless, ethanol production remains a major competing pathway. Kondo *et al.* [22] showed that deletion of *PDC1*, the major pyruvate decarboxylase isoform, had a positive effect on isobutanol production. However, *pdc*-negative strains are auxotrophic for cytosolic C2-compounds, which necessitates further interventions, e.g. overexpression of the regulatory factor Mth1 [23] or introduction of a pyruvate-formate lyase [24]. Matsuda *et al.* [21••] showed that elimination of a subunit of the mitochondrial pyruvate dehydrogenase (Pdh) complex leads to increased isobutanol titers.

A further problem arises from redox cofactor imbalances as glycolysis yields NADH and Ahar (and some Adhs) require NADPH and *S. cerevisiae* does not naturally contain a transhydrogenase. Bastian *et al.* [25] achieved the theoretically maximal isobutanol yield in *Escherichia coli* by engineering NADH-dependent Ahar and Adh. Thus, introducing a mutated Ahar into yeast may be a promising strategy to further improve isobutanol production. Matsuda *et al.* [21••]

Table 1

Overview of representative yeast strains engineered to produce butanol isomers. The strain variants with best characteristics in each reference are given. Only the strategies where butanol isomers were produced from sugars are included. Note that titers shown do not take into account different fermentation times and conditions and should therefore not be directly compared. The yields (mg/g carbon source) represent a more reproducible characteristic but were not indicated in each reference. Where possible, the yields were calculated for the purpose of this review from the available data. Defined media may vary in their individual compositions of e.g. amino acids, vitamins and nucleobases. The fermentation conditions regarding oxygen supply are given according to the references and may not be consistently defined.

Strain background	Overexpressed genes	Pathway localization	Relevant genome modifications	Carbon source (g/L)	Medium properties	Fermentation conditions	Average Titer (mg L^{-1})/ Yield (mg g^{-1} C-source)	Reference
Isobutanol								
CEN.PK2-1C	*ILV2, ILV3, ILV5*	*Mitochondrial Ilv/ cytosolic Ehrlich pathway*	*None*	Glucose (40)	Defined	Aerobic	n.i./3.86	[12]
D452-2	*ILV2Δ55, ILV3Δ20, ILV5Δ34, ^{Ll}kivD*	*Full cytosolic pathway with parallel mitochondrial Ilv branch*	*None*	Glucose (40)	Complex	Microaerobic	151/3.78[a]	[19]
YPH499	*ILV2, ^{Ll}kivD, ADH6*	*Mitochondrial Ilv/ cytosolic Ehrlich pathway*	*Δpdc1*	Glucose (20)	Defined	Microaerobic	143/6.60	[22]
YPH499	*ILV2, ILV2Δ54, ILV3Δ41, ILV5Δ47, ^{Ll}kivD, ADH6*	*Full cytosolic pathway with parallel mitochondrial Ilv branch*	*None*	Glucose (20)	Defined	Microaerobic	63/2.10[b]	[20]
CEN.PK2-1C	*^{syn}ILV2Δ54, ^{syn}ILV3Δ19, ^{syn}ILV5Δ48*	*Full cytosolic pathway*	*Δilv2*	Glucose (40)	Defined	Aerobic	630/14.18	[17••]
BY4741 x Y3929 (diploid)	*ILV2, ILV3, ILV5, ARO10-TS, ^{Ll}adhA^{RE1}-TS*	*Full mitochondrial pathway with parallel cytosolic Ehrlich pathway*	*None*	Glucose (100)	Complex	Semi-aerobic	635/6.40	[14••]
CEN.PK2-1C	*^{syn}ILV2Δ54, ^{syn}ILV3Δ19, ^{syn}ILV5Δ48, ARO10, ADH2, TAL1, XKS1, ^{synCp}xylA*	*Full cytosolic pathway*	*Δilv2*	Xylose (20)	Defined	Aerobic	1.36/0.16	[18]
YPH499	*ILV2, ILV2Δ54, ILV3Δ41, ILV5Δ47, ^{Ll}kivD, ADH6, MAE1*	*Full cytosolic pathway with parallel mitochondrial Ilv branch*	*Δlpd1*	Glucose (100)	Defined	Microaerobic	1620/16.00	[21••]
1-Butanol								
BY4742	*ERG10, ^{Cb}hbd, ^{Cb}crt, ^{Sc}ccr, ^{Cb}adhE2*	*Cytosolic*	*None*	Galactose (20)	Defined	Semi-aerobic	2.5/n.i.	[38]

Table 1 (*Continued*)

Strain background	Overexpressed genes	Pathway localization	Relevant genome modifications	Carbon source (g/L)	Medium properties	Fermentation conditions	Average Titer (mg L^{-1})/ Yield (mg g^{-1} C-source)	Reference
CEN.PK113-11C	*ERG10*, ^{synCb}*hbd*, ^{synCb}*crt*, ^{synTd}*ter*, ^{synCb}*adhE2*, ^{synSe}*acs*^{L641P}, *ADH2, ALD6*	*Cytosolic*	*Δcit2*	Glucose (20)	Defined	Aerobic	16.3/n.i.	[40•]
YSG50	*None*	*Cytosol/ mitochondria*	*Δadh1 Δilv2*	Glucose (20)	Complex	Microaerobic	242.8/n.i.	[45••]
CEN.PK2-1C	*ERG10*, ^{cytoYl}*KR*, ^{cytoYl}*HTD*, ^{Ec}*EutE*, ^{Ca}*BdhB*	*Cytosolic*	*None*	Glucose (20)	Defined	Oxygen limited	20/n.i.	[41]
CEN.PK2-1C	^{Ca}*thl*, ^{Ca}*hbd*, ^{Cb}*crt*, ^{Td}*ter*, ^{Ec}*eutE*, ^{Ca}*bdhB*, ^{synSe}*acs*^{L641P}, ^{ec}*pdh*	*Cytosolic*	*Δadh1 Δadh4 Δgpd1 Δgpd2*	Glucose (20)	Defined	Oxygen limited	>100/n.i.	[42••]

Genes from *Clostridium acetobutylicum* (Ca), *Clostridium beijerinckii* (Cb), *Clostridium phytofermentans* (Cp), *Escherichia coli* (Ec), *Lactococcus lactis* (Ll), *Salmonella enterica* (Se), *Streptomyces collinus* (Sc), *Treponema denticola* (Td) and *Yarrowia lipolytica* (Yl) are indicated by prefixes in superscript. The suffix 'RE1' denotes a variant of AdhA from *Lactococcus lactis* with increased affinity for isobutyraldehyde and 'L641P' an acetylation-resistant mutant of the acetyl-CoA synthetase from *Salmonella enterica*; the prefix 'syn' indicates synthetic codon optimized genes; the prefix 'cyto' indicates cytosolic localization of truncated *Yarrowia lipolytica* proteins; the deleted N-terminal amino-acids of the cytosolic versions of Ilv proteins are indicated (ΔAA); fusion with an N-terminal mitochondrial targeting sequence (TS) is indicated; native *S. cerevisiae* gene forms are shown without a denominator.

n.i.: not indicated in the publication.

[a] The values were not indicated by the authors but calculated from the data.

[b] Recalculated from molar yield.

constructed a transhydrogenase-like shunt, in which pyruvate is converted cyclically to oxaloacetate, malate and back to pyruvate, simultaneously converting NADH to NADPH (Table 1).

Even with all these advances, the production of isobutanol seems to be limited by supply of KIV. Lee *et al.* [19] and Avalos *et al.* [14••] showed that addition of KIV could enhance isobutanol production significantly. The enzyme responsible for conversion of DIV to KIV, the DHAD Ilv3, possesses FeS clusters. In yeast, FeS proteins are present in mitochondria, cytoplasm and nucleus, although the initial assembly of this co-factor takes place inside mitochondria. Since the biogenesis of FeS clusters is a well-regulated process [26–28], a simple overexpression of a FeS protein especially in the cytosol could be meaningless. Attempts have been reported in patent applications [29,30] to enhance Dhad activity by modulating expression of components of the FeS assembly machinery, such as Atm1 (a transporter of FeS precursors from mitochondria to cytosol), Aft1/2 (transcription factors controlling iron utilization and homeostasis) and Grx proteins (glutathione-dependent oxidoreductases involved in biosynthesis of FeS). Also for other pathways, overexpression of FeS proteins in yeast has been demonstrated as a challenging step [31,32].

1-Butanol

Various *Clostridium* species are established 1-butanol producers in so-called acetone-butanol-ethanol (ABE) fermentations [33,34]. Titers of up to 13 g/L 1-butanol have been reported [35,36], but in fermentation processes Clostridia are difficult to handle [37]. Therefore, several approaches have been published to express variants of the pathway in other, more robust host organisms like yeasts. In this pathway, two molecules of acetyl-CoA are condensed to acetoacetyl-CoA, which is converted into 3-hydroxybutyryl-CoA and transferred into crotonyl-CoA. This is reduced via butyryl-CoA and butyraldehyde into 1-butanol (Figure 1). Steen *et al.* [38] compared enzymes taken from different organisms to convert acetyl-CoA into 1-butanol in *S. cerevisiae*. Expressing a combination of Erg10 (thiolase) from *S. cerevisiae*, Hbd (NADH-dependent 3-hydroxybutyryl-CoA dehydrogenase) and Crt (crotonase) from *C. beijerinckii*, Ccr (NADH-dependent crotonyl-CoA reductase) from *Streptomyces collinus* and AdhE2 (bifunctional butyraldehyde/butanol dehydrogenase) from *C. beijerinckii* resulted in 2.5 mg/L 1-butanol [38] (Table 1). Ccr was used as it turned out that the butyryl-CoA dehydrogenase from *C. beijerinckii* together with an electron transfer flavoprotein EtfAB did not improve 1-butanol production.

By replacing Ccr with an NADH-dependent trans-enoyl-CoA reductase (Ter) from *Treponema denticola*, which provides an irreversible reaction and therefore acts as a strong driving force [39], Krivoruchko *et al.* [40•] could further improve production up to 6.6 mg/L. In an attempt to implement a reversal of the β-oxidation cycle in yeast, Lian and Zhao [41] found several other suitable candidate enzymes for 1-butanol synthesis in yeast. Expression of these enzymes together with a CoA-acylating aldehyde dehydrogenase from *E. coli* and a butanol dehydrogenase from *C. acetobutylicum* resulted in titers of up to 20 mg/L. It was speculated that lower availability of cytosolic acetyl-CoA in *S. cerevisiae* as compared to *E. coli* is the main reason for lower 1-butanol titers in yeast.

To increase the availability of cytosolic acetyl-CoA, Krivoruchko *et al.* [40•] made use of a pyruvate dehydrogenase (Pdh) bypass by overexpressing an acetaldehyde dehydrogenase and an acetyl-CoA synthetase in a *cit2* mutant devoid of extramitochondrial citrate synthase activity. Indeed, 1-butanol titers increased from 6.6 mg/L to 16.3 mg/L. Moreover, as ethanol and glycerol are still major unwanted (by-) products in 1-butanol production and high levels of NADH would be a strong driving force, a yeast strain deleted for all its alcohol dehydrogenase (*ADH*) and glycerol-3-P dehydrogenase (*GPD*) genes seems to be a promising host strain [42••,43,44]. Expression of an improved 1-butanol pathway together with an acetyl-CoA producing cytosolic version of Pdh from *E. coli* and Pdh-bypass reactions in an *adh1/4 gpd1/2* mutant strain increased 1-butanol production to 120 mg/L [42••] (Table 1). However, functionality of the Pdh complex from *E. coli* was not confirmed by additional experiments.

An endogenous pathway for 1-butanol production dependent on threonine catabolism was characterized in *S. cerevisiae* [45••]. The authors discovered a new catabolic pathway from glucose via threonine to 1-butanol using the enzymes of leucine biosynthesis together with Kdcs and Adhs. In addition, this endogenous pathway was stimulated by deletion of *ADH1* and *ILV2*, resulting in up to 242.8 mg/L 1-butanol. Another endogenous pathway for butanol production in *S. cerevisiae* uses glycine as a substrate [46•]. When glycine was added as the sole nitrogen source, 1-butanol accumulated up to 92 mg/L. The pathway was shown to proceed from glycine to glyoxylate, which was condensed with butyryl-CoA to yield 3-ethylmalate. 3-Ethylmalate is oxidized to 2-ketovalerate and finally converted to 1-butanol via the Ehrlich pathway. However, it is not clear from where the butyryl-CoA originates. Nevertheless, as biofuel production from amino acids is economically not viable, threonine or glycine overproduction strains would be required with lower theoretical maximum yields.

2-Butanol

2-Butanol has the highest octane number among the butanol isomers. High-level production of 2-butanol has not yet been tested for bacterial systems but only recently for *S. cerevisiae*. A heterologous pathway was

introduced converting 2,3-butanediol to 2-butanol via 2-butanone [47•]. All three subunits of a B_{12}-dependent diol dehydratase from *Lactobacillus reuteri* and its two-subunit activating enzyme were expressed in equal amounts via a TEV-cleavage based system. After simultaneous expression of a secondary alcohol dehydrogenase from *Gordonia* sp., the yeast transformants could produce 4 mg/L 2-butanol from *meso*-2,3-butanediol. In order to ensure an ample supply of NADH a *gpd1 gpd2* double deletion strain unable to produce glycerol was used under anaerobic conditions. Moreover, production of 2-butanol was dependent on the supply of adenosylcobalamine to the medium. It was speculated that additional engineering of high level production of 2,3-butanediol [48,49] together with expression of a B_{12}-independent diol dehydratase could enable higher production levels of 2-butanol from glucose.

Conclusions and outlook

Production rates and yields for butanol isomers are still lower in *S. cerevisiae* compared to bacterial systems. While in bacteria the maximal theoretical yields (0.41 g/g glucose) have nearly been reached for isobutanol and 1-butanol, the yields reported for yeast are only less than 5% of the maximal values. However, as yeast cells have decisive advantages as industrial production organisms compared to bacteria [9], further pathway engineering is highly desirable. Nevertheless, although yeasts are less sensitive against butanol [50], tolerance will become a serious issue once higher titers are reached. González-Ramos *et al.* [51•] established that maintenance of protein integrity plays an essential role in butanol tolerance. Strains overexpressing genes involved in degradation systems of damaged proteins showed increased resistance to butanol. From a proteomic analysis of an evolved butanol tolerant yeast strain, Ghiaci *et al.* [52] concluded that upregulation of proteins involved in the mitochondrial ATP synthesizing machinery and glycerol biosynthesis seem to be beneficial for a successful adaptation to butanol stress. These studies show promising targets to engineer *S. cerevisiae* for improved tolerance when butanol titers increase to more than 15 g/L.

Acknowledgements

Financial support by CNPq (National Council for Scientific and Technological Development - Brazil) to WCG is gratefully acknowledged [Grant: 239395/2012-3].

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Savage N: **Fuel options: the ideal biofuel**. *Nature* 2011, **474**:9-11.

2. Dürre P: **Biobutanol: an attractive biofuel**. *Biotechnol J* 2007, **12**:1525-1534.

3. García V, Päkkilä J, Ojamo H, Muurinen E, Keiski RL: **Challenges in biobutanol production: how to improve the efficiency?** *Renew Sust Energ Rev* 2011, **2**:964-980.

4. Peralta-Yahya PP, Zhang F, Del Cardayre SB, Keasling JD: **Microbial engineering for the production of advanced biofuels**. *Nature* 2012, **7411**:320-328.

5. Kung Y, Runguphan W, Keasling JD: **From fields to fuels: recent advances in the microbial production of biofuels**. *ACS Synth Biol* 2012, **11**:498-513.

6. Lamsen EN, Atsumi S: **Recent progress in synthetic biology for microbial production of C3-C10 alcohols**. *Front Microbiol* 2012, **3**:196.

7. Lan EI, Liao JC: **Microbial synthesis of n-butanol, isobutanol, and other higher alcohols from diverse resources**. *Bioresour Technol* 2013, **135**:339-349.

8. Buijs NA, Siewers V, Nielsen J: **Advanced biofuel production by the yeast *Saccharomyces cerevisiae***. *Curr Opin Chem Biol* 2013, **3**:480-488.

9. Weber C, Farwick A, Benisch F, Brat D, Dietz H, Subtil T, Boles E: **Trends and challenges in the microbial production of lignocellulosic bioalcohol fuels**. *Appl Microbiol Biotechnol* 2010, **4**:1303-1315.

10. Hazelwood LA, Daran J-M, van Maris AJA, Pronk JT, Dickinson JR: **The Ehrlich pathway for fusel alcohol production: a century of research on *Saccharomyces cerevisiae* metabolism**. *Appl Environ Microbiol* 2008, **74**:2259-2266.

11. Atsumi S, Hanai T, Liao JC: **Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels**. *Nature* 2008, **7174**:86-89.

12. Chen X, Nielsen KF, Borodina I, Kielland-Brandt MC, Karhumaa K: **Increased isobutanol production in *Saccharomyces cerevisiae* by overexpression of genes in valine metabolism**. *Biotechnol Biofuels* 2011, **1**:21.

13. Pang SS, Duggleby RG: **Regulation of yeast acetohydroxyacid synthase by valine and ATP**. *Biochem J* 2001, **3**:749-757.

14. •• Avalos JL, Fink GR, Stephanopoulos G: **Compartmentalization of metabolic pathways in yeast mitochondria improves the production of branched-chain alcohols**. *Nat Biotechnol* 2013, **4**:335-341.
Moving the complete isobutanol pathway into mitochondria resulted in a substantial increase in isobutanol synthesis due to increased local concentrations of enzymes and substrates.

15. Yuan J, Ching CB: **Combinatorial assembly of large biochemical pathways into yeast chromosomes for improved production of value-added compounds**. *ACS Synth Biol* 2014 http://dx.doi.org/10.1021/sb500079f.

16. Watson K, Haslam JM, Linnane AW: **Biogenesis of mitochondria**. *J Cell Biol* 1970, **46**:88-96.

17. •• Brat D, Weber C, Lorenzen W, Bode HB, Boles E: **Cytosolic re-localization and optimization of valine synthesis and catabolism enables increased isobutanol production with the yeast *Saccharomyces cerevisiae***. *Biotechnol Biofuels* 2012, **1**:65.
Localization of the complete isobutanol pathway in the cytosol in the absence of a mitochondrial pathway and in the absence of valine enabled increased isobutanol yields.

18. Brat D, Boles E: **Isobutanol production from D-xylose by recombinant *Saccharomyces cerevisiae***. *FEMS Yeast Res* 2013, **2**:241-244.

19. Lee W, Seo S, Bae Y, Nan H, Jin Y, Seo J: **Isobutanol production in engineered *Saccharomyces cerevisiae* by overexpression of 2-ketoisovalerate decarboxylase and valine biosynthetic enzymes**. *Bioproc Biosyst Eng* 2012, **9**:1467-1475.

20. Matsuda F, Kondo T, Ida K, Tezuka H, Ishii J, Kondo A: **Construction of an artificial pathway for isobutanol biosynthesis in the cytosol of *Saccharomyces cerevisiae***. *Biosci Biotechnol Biochem* 2012, **11**:2139-2141.

21. •• Matsuda F, Ishii J, Kondo T, Ida K, Tezuka H, Kondo A: **Increased isobutanol production in *Saccharomyces cerevisiae* by**

eliminating competing pathways and resolving cofactor imbalance. *Microb Cell Fact* 2013, **1**:119.
Elimination of the competing pyruvate dehydrogenase activity and introduction of a transhydrogenase-like shunt to interconvert NADH and NADPH improved isobutanol production.

22. Kondo T, Tezuka H, Ishii J, Matsuda F, Ogino C, Kondo A: **Genetic engineering to enhance the Ehrlich pathway and alter carbon flux for increased isobutanol production from glucose by *Saccharomyces cerevisiae***. *J Biotechnol* 2012, **1**–**2**:32-37.

23. Oud B, Flores C, Gancedo C, Zhang X, Trueheart J, Daran J, Pronk JT, van Maris AJA: **An internal deletion in MTH1 enables growth on glucose of pyruvate-decarboxylase negative, non-fermentative *Saccharomyces cerevisiae***. *Microb Cell Fact* 2012, **11**:131.

24. Kozak BU, van Rossum HM, Benjamin KR, Wu L, Daran JG, Pronk JT, van Maris AJA: **Replacement of the *Saccharomyces cerevisiae* acetyl-CoA synthetases by alternative pathways for cytosolic acetyl-CoA synthesis**. *Metab Eng* 2014, **21**:46-59.

25. Bastian S, Liu X, Meyerowitz JT, Snow CD, Chen MMY, Arnold FH: **Engineered ketol-acid reductoisomerase and alcohol dehydrogenase enable anaerobic 2-methylpropan-1-ol production at theoretical yield in *Escherichia coli***. *Metab Eng* 2011, **3**:345-352.

26. Lill R, Mühlenhoff U: **Iron-sulfur protein biogenesis in eukaryotes: components and mechanisms**. *Annu Rev Cell Dev Bi* 2006, **22**:457-486.

27. Lill R, Mühlenhoff U: **Maturation of iron-sulfur proteins in eukaryotes: mechanisms, connected processes, and diseases**. *Annu Rev Biochem* 2008, **77**:669-700.

28. Netz DJA, Mascarenhas J, Stehling O, Pierik AJ, Lill R: **Maturation of cytosolic and nuclear iron-sulfur proteins**. *Trends Cell Biol* 2013, **25**:1-10.

29. Anthony LC, Maggio-Hall LA, Rothman SC, Tomb JF: **Increased heterologous Fe-S enzyme activity in yeast**. Patent: CA2735945 A1, April 1, 2010.

30. Dundon CA, Aristidou A, Hawkins A, Lies D, Albert LH: **Methods of increasing dihydroxy acid dehydratase activity to improve production of fuels, chemicals, and amino acids**. Patent: US8017376 B2, September 13, 2011.

31. Benisch F, Boles E: **The bacterial Entner-Doudoroff pathway does not replace glycolysis in *Saccharomyces cerevisiae* due to the lack of activity of iron-sulfur cluster enzyme 6-phosphogluconate dehydratase**. *J Biotechnol* 2014, **171**:45-55.

32. Carlsen S, Ajikumar PK, Formenti LR, Zhou K, Phon TH, Nielsen ML, Lantz AE, Kielland-Brandt MC, Stephanopoulos G: **Heterologous expression and characterization of bacterial 2-C-methyl-D-erythritol-4-phosphate pathway in *Saccharomyces cerevisiae***. *Appl Microbiol Biotechnol* 2013, **97**:5753-5769.

33. Huang H, Liu H, Gan Y: **Genetic modification of critical enzymes and involved genes in butanol biosynthesis from biomass**. *Biotechnol Adv* 2010, **5**:651-657.

34. Lee SY, Park JH, Jang SH, Nielsen LK, Kim J, Jung KS: **Fermentative butanol production by Clostridia**. *Biotechnol Bioeng* 2008, **2**:209-228.

35. Ezeji TC, Qureshi N, Blaschek HP: **Butanol fermentation research: upstream and downstream manipulations**. *Chem Rec* 2004, **5**:305-314.

36. Nielsen DR, Leonard E, Yoon S, Tseng H, Yuan C, Jones Prather KL: **Engineering alternative butanol production platforms in heterologous bacteria**. *Metab Eng* 2009, **4-5**:262-273.

37. Atsumi S, Cann AF, Connor MR, Shen CR, Smith KM, Brynildsen MP, Chou KJY, Hanai T, Liao JC: **Metabolic engineering of *Escherichia coli* for 1-butanol production**. *Metab Eng* 2008, **6**:305-311.

38. Steen EJ, Chan R, Prasad N, Myers S, Petzold CJ, Redding A, Ouellet M, Keasling JD: **Metabolic engineering of *Saccharomyces cerevisiae* for the production of n-butanol**. *Microb Cell Fact* 2008, **7**:36.

39. Bond-Watts BB, Bellerose RJ, Chang MCY: **Enzyme mechanism as a kinetic control element for designing synthetic biofuel pathways**. *Nat Chem Biol* 2011, **4**:222-227.

40. • Krivoruchko A, Serrano-Amatriain C, Chen Y, Siewers V, Nielsen J: **Improving biobutanol production in engineered *Saccharomyces cerevisiae* by manipulation of acetyl-CoA metabolism**. *J Ind Microbiol Biotechnol* 2013, **9**:1051-1056.
Krivoruchko *et al.* were the first to identify inefficient acetyl-CoA production as one bottleneck of 1-butanol production in yeast.

41. Lian J, Zhao H: **Reversal of the β-oxidation cycle in *Saccharomyces cerevisiae* for production of fuels and chemicals**. *ACS Synth Biol* 2014 http://dx.doi.org/10.1021/sb500243c.

42. •• Lian J, Si T, Nair NU, Zhao H: **Design and construction of acetyl-CoA overproducing *Saccharomyces cerevisiae* strains**. *Metab Eng* 2014, **24**:139-149.
1-Butanol production was improved by increasing flux toward cytosolic acetyl-CoA, the precursor metabolite for 1-butanol biosynthesis, and by partially deleting competing ethanol and glycerol pathways.

43. Ida Y, Hirasawa T, Furusawa C, Shimizu H: **Utilization of *Saccharomyces cerevisiae* recombinant strain incapable of both ethanol and glycerol biosynthesis for anaerobic bioproduction**. *Appl Microbiol Biotechnol* 2013, **11**:4811-4819.

44. Hirasawa T, Ida Y, Furuasawa C, Shimizu H: **Potential of a *Saccharomyces cerevisiae* recombinant strain lacking ethanol and glycerol biosynthesis pathways in efficient anaerobic bioproduction**. *Bioengineered* 2014, **2**:123-128.

45. •• Si T, Luo Y, Xiao H, Zhao H: **Utilizing an endogenous pathway for 1-butanol production in *Saccharomyces cerevisiae***. *Metab Eng* 2014, **22**:60-68.
Si *et al.* discovered an endogenous pathway for 1-butanol production in yeast originating from threonine catabolism and using the enzymes of the leucine biosynthesis pathway. Engineering of this pathway resulted in the highest 1-butanol titers reported for *S. cerevisiae* so far.

46. • Branduardi P, Longo V, Berterame NM, Rossi G, Porro D: **A novel pathway to produce butanol and isobutanol in *Saccharomyces cerevisiae***. *Biotechnol Biofuels* 2013, **1**:68.
Branduardi *et al.* discovered an endogenous pathway for isobutanol and 1-butanol production from glycine and butyryl-CoA.

47. • Ghiaci P, Norbeck J, Larsson C: **2-Butanol and butanone production in *Saccharomyces cerevisiae* through combination of a B12 dependent dehydratase and a secondary alcohol dehydrogenase using a TEV-based expression system**. *PLoS ONE* 2014, **7**:e102774.
The first report about the production of 2-butanol in yeast. To achieve equimolar expression of the individual subunits of two protein complexes a TEV-protease based expression system was used.

48. Ng CY, Jung M, Lee J, Oh M: **Production of 2,3-butanediol in *Saccharomyces cerevisiae* by in silico aided metabolic engineering**. *Microb Cell Fact* 2012, **11**:68.

49. Kim S, Seo S, Jin Y, Seo J: **Production of 2,3-butanediol by engineered *Saccharomyces cerevisiae***. *Bioresource Technol* 2013, **146**:274-281.

50. Fischer CR, Klein-Marcuschamer D, Stephanopoulos G: **Selection and optimization of microbial hosts for biofuels production**. *Metab Eng* 2008, **6**:295-304.

51. • González-Ramos D, van den Broek M, van Maris AJA, Pronk JT, Daran JG: **Genome-scale analyses of butanol tolerance in *Saccharomyces cerevisiae* reveal an essential role of protein degradation**. *Biotechnol Biofuels* 2013, **1**:48.
By combining a screening of the yeast gene deletion collection, gene overexpression, and genome analysis of evolutionary engineered 1-butanol tolerant strains, it was shown that protein degradation plays an important role in tolerance.

52. Ghiaci P, Norbeck J, Larsson C: **Physiological adaptations of *Saccharomyces cerevisiae* evolved for improved butanol tolerance**. *Biotechnol Biofuels* 2013, **1**:101.