Metabolic Engineering and Synthetic Biology in *Saccharomyces cerevisiae* for Isobutanol Production

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Introduction

The inability of the oil supply to meet the world demand fosters the need for research on environmentally-friendly production of biofuels. Yeast are the logical option rather than prokaryotes due to their tolerance to pH and robustness against inhibition and toxic compounds. Metabolic engineering and synthetic biology tools are proving crucial in the design of biofuel-producing microorganisms with high yields from raw material. In fact, important companies are filing patents with their advances in the race for such microbial cell factory. This work reviews the most relevant advances in the field at laboratory scale, describing the importance of the techniques used and the current limitations of industrial-scale production.

Why isobutanol?

Although production of bioethanol is by far the most advanced of all biofuels, isobutanol is promising likewise due to its many advantages, including [1]:

- Higher energy density
- Reduced distribution costs
- Less hygroscopy and corrosiveness
- Easier storage and transportation
- Adaptability to existing distribution networks

These characteristics make isobutanol an attractive and promising alternative, encouraging worldwide research on its synthesis.

Relevant advances

Three approaches to isobutanol production enhancement have been selected and studied in this work:

- **1 -** Screening of heterologous enzymes more suitable than those found in the *S. cerevisiae* endogenous Ehrlich pathway along with their overexpression and deletion of PDC-1 [2].
- **2 -** Re-localization of the whole Ehrlich pathway into the cytosol [3].
- **3 -** Re-localization of the whole Ehrlich pathway into the mitochondria [4].

The table below displays the results obtained in these experiments.

Table 1. Values of isobutanol yield, productivity and isobutanol/glucose yield in each of the studied articles.

		Final [isobutanol] (mg/L)	Productivity (mg/L·h)	Isobutanol/glucose yield (mg/g)
1	Pathway engineering only	143	1.2	6.6
2	Cytosolic re-localization	630	6.6	14.86
3	Mitochondrial re-localization	635	6.4	6.7

The best results are obtained combining synthetic biology techniques with metabolic engineering (cases **2** and **3**). More specifically, the best results are obtained in case **2**. Nevertheless, the results might have been better in case **3** if techniques such as codon optimization had been used.

Isobutanol synthesis: the Ehrlich pathway

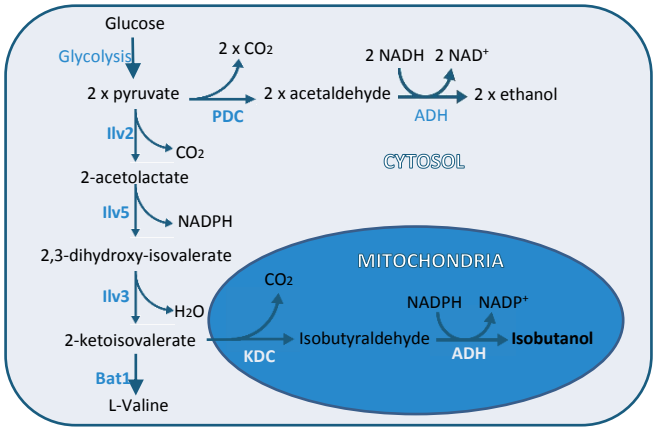

Figure 1. Representation of the reactions comprising the Ehrlich pathway in yeast and the enzymes involved. [1]

Isobutanol is produced normally in *S. cerevisiae* as part of the valine metabolism in the so-called **Ehrlich pathway**, the steps of which are split between cytosol and mitochondria. This pathway is the basis for research on isobutanol production enhancement.

Patents

Companies strive for monopolizing the isobutanol market niche and patent filing is the best way to achieve this. Published patents in the recent years for isobutanol production include:

- Use of a yeast with an endogenous isobutanol pathway (**Butalco**) [5] or a heterogeneous pathway (**Butamax**) with overexpression of its enzymes [6].
- Specific proteins which allow high isobutanol yields, such a bacterial dyhydroxyacid dehydratase (**Butamax**) [7], a yeast with enhanced expression of a NADP-dependent isocitrate dehydrogenase (**Toyota Motor Corporation**) [8] or a yeast with a transport protein (**Gevo**) [9].
- Modified yeasts with reduced amino acid metabolism or with enhanced tolerance towards butanol (**Butamax**) [10].

Synthetic biology techniques

LoxP-Cre recombinase

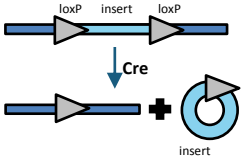

Figure 2. Gene deletion in the LoxP-recombinase system.

The **LoxP/Cre recombinase** system allows the deletion or insertion of a gene. Cre is a LoxP site-specific recombinase. In gene deletion, Cre recognises and cleaves LoxP sites and the ends are rejoined.

Selectable markers

Common selectable markers include **antibiotic resistances** such as geneticin resistance (*kanMX*) and **auxotrophic markers**, which include *URA3* as shown in figure 3.

Codon optimization

When expressing a recombinant gene, the **codon usage** of the strain, which is the relative abundance of tRNA isoacceptors, must be taken into account. Thus, codon optimization of the gene sequence is common in heterologous expression.

Methods for overexpression

- Use of **2μ plasmids** is a common method to overexpress a gene at a mRNA level. These are shuttle vectors able to replicate in both *Escherichia coli* and *S. cerevisiae*. The 2μ replication origin is responsible for their high copy number.
- **Promoters** and **terminators** are often modified in order to maximize protein yield. Common promoters include *PGK1* (phosphoglycerate kinase 1) and *ADH1* (alcohol dehydrogenase 1).

Modular vectors

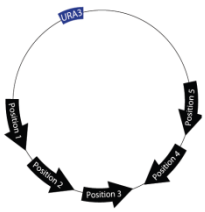

Figure 3. Basic scheme of a modular vector in which each position represents a different cassette. [4]

Modular or multigenic vectors enable the insertion of multiple gene expression cassettes in different orientations into a single plasmid. Up to an entire metabolic pathway can be introduced in the host microorganism via a single vector.

This is feasible thanks to unique restriction sites which flank all expression cassettes. Only a "**scar**" composed by two non-functional restriction sites is left after the insertion.

Conclusions

Industrial butanol production is still at its infancy, as there are many question marks remaining.

- Several **bottlenecks** must be resolved, such as **by-product accumulation** and maintenance of the **redox balance**.
- The host would ideally have resistance to **isobutanol toxicity and high growth rates** to prevent contamination **.**
- **No industrial strains** have been tested yet, and glucose is the only **carbon source** tested.

The ideal approach would be a **consolidated bioprocess** in which the yeast secretes enzymes for lignocellulosic biomass usage as carbon source, as achieved in the case of bioethanol.

Relevant bibliography

[1] Schirmer, M. A. R. A. New microbial fuels: a biotech perspective. *Curr. Opin. Microbiol.* 274–281 (2009).
[2] Kondo, T. *et al.* Genetic engineering to enhance the Ehrlich pathway and alter carbon flux for increased isobutanol production from glucose by Saccharomyces cerevisiae. *J. Biotechnol.* 159, 32–37 (2012).
[3] Brat, D., Weber, C., Lorenzen, W., Bode, H. B. & Boles, E. Cytosolic re-localization and optimization of valine synthesis and catabolism enables inseased isobutanol production with the yeast Saccharomyces cerevisiae. *Biotechnol. Biofuels* **5,** 65 (2012).
[4] Avalos, J. L., Fink, G. R. & Stephanopoulos, G. Compartmentalization of metabolic pathways in yeast mitochondria improves the production of branched-chain alcohols. *Nat. Biotechnol.* 31, 335–41 (2013).
[5] Patent US 2011/0053235. Butalco GmbH. March 2011
[6] Patent US 2007/092957. Butamax TM Advanced Biofuels. January 2013
[7] Patent US 2014/0051137 Butamax TM Advanced Biofuels. February 2014.
[8] Patent EP 2 446 043. Toyota Motor Corporation. May 2012.
[9] Patent US 2013/0302868. Gevo Inc. November 2013.
[10] Patent US 2010/0167363. Butamax TM Advanced Biofuels. July 2010.