Improvement of bisabolene production in *Saccharomyces cerevisiae* through metabolic engineering

Fernando Foncillas, Clara - Universitat Autònoma de Barcelona, 2015

Universitat Autònoma de Barcelona

Objectives

- The aim of this work is to describe and to improve the production of bisabolene in *Saccharomyces cerevisiae* using metabolic engineering.
- Several metabolic engineering techniques and the metabolic engineering cycle are described.

What are bisabolene and bisabolane?

Bisabolene is the immediate precursor of bisabolane, a fully reduced monocyclic sesquiterpene which can be obtained by its chemical hydrogenation. Bisabolane can be a biosynthetic alternative to D2 diesel fuel due to the comparable cold properties, cetane number and carbon length. An hypothetical economic analysis which estimated ~$6 per gal (more expensive than current D2 diesel) assumed a break-even price of sugar close to $0.10/lb, being $1.76/kg the final cost.

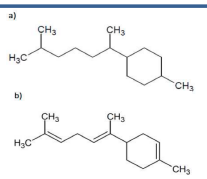
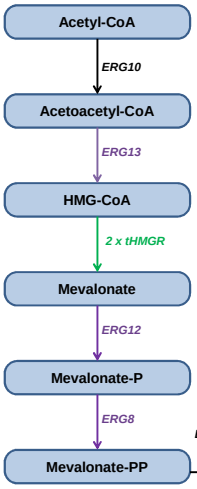


Figure 1. Chemical structures of bisabolane (a) and bisabolene (b).

Bisabolene production in *S. cerevisiae*

Production of bisabolene in an engineered *S. cerevisiae* strain was carried out in two steps [1]:



***A. S. cerevisiae* as an FPP overproduction platform.** In this strain the mevalonate pathway was modified:

1. Overexpression of *tHMGR*, a truncated HMG-CoA reductase, and integration of an additional copy.

2. Overexpression of *ERG20*, the FPP synthase.

3. Overexpression of *upc2-1*, the global transcription regulator of the sterol pathway.

4. Downregulation of *ERG8*, the squalene synthase.

δ-integration vectors, which allow sequential insertion of multiple cloned genes, were used to integrate overexpressed genes into the chromosome. These plasmids were constructed using restriction enzymes and the overexpressed genes were under control of galactose promoter. pRS-ERG9 was constructed to downregulate *ERG9*, which was placed under control of a methionine repressible promoter (P_{MET3}). All strains were transformed using the lithium acetate method.

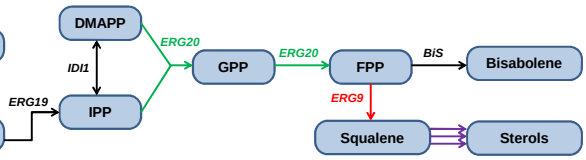


Figure 2. Representation of the engineered mevalonate pathway for bisabolene production (Adapted from [2] and [3]).

B. Adaptation of *S. cerevisiae* for the production of bisabolene. Five bisabolene synthases (BiS) known in the literature were screened for high bisabolene titers, and the highest production was achieved using the bisabolene synthase from *Abies grandis*.

Plasmid pRS425-Leu2d::BiS was constructed using pRS425, a yeast episomal plasmid (Yep), as a backbone. *Leu2d* allele was used, which increases plasmid copy number in *S. cerevisiae* due to its weakened expression. This plasmid contains the 2μ origin of replication, which increases copy number too, and the REP3 and FRT sequences necessary for high copy propagation in yeast. It also contains the pMB1 origin of replication, then it can be used in *E. coli*.

The bisabolene synthases genes were amplified from their respective templates using their respective primers and then digested with *Nhe*I and *Xho*I restriction sites to be cloned into the vector. Once screened, the bisabolene synthase with the highest production was codon optimized.

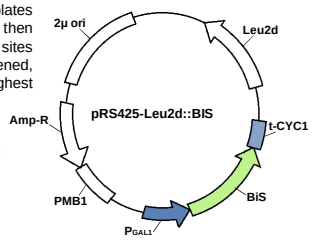


Figure 3. Plasmid containing BiS under control of the Gal1 promoter (Adapted from [4]).

Gene overexpressed:
- *tHMGR*
- *ERG20*
- *upc2-1*

Gene downregulated:
- *ERG9*

Genes indirectly upregulated:
- *ERG13*
- *ERG12*
- *ERG8*
- Other ergosterol genes.

The metabolic engineering cycle and techniques

Metabolic engineering is an interdisciplinary field which comprises computational modelling, genetics, biochemistry, molecular biology, etc. Its main purpose is to redirect the metabolic fluxes to meet the industrial and medical needs.

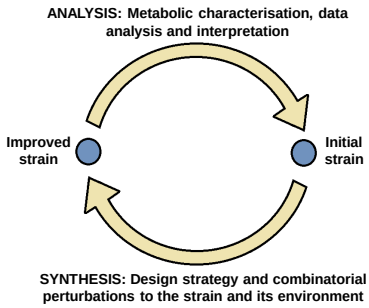


Figure 4. The metabolic engineering cycle (Adapted from [5]).

Analysis

Its aim is finding what can have failed, understanding the reasons of this failure and identifying possible targets. It contains three main points:

1. Fermentation physiology.
2. Metabolic pathway analysis:
   a) Identification of metabolic network structure.
   b) Quantification of fluxes: FBA.
   c) Identification of control structures: MBA.
3. Analytical techniques such as DNA microarrays, northern blot and mass spectrometry are used to calculate gene, protein and metabolite expression levels. GC and HPLC are used to identify these metabolites.

Synthesis

Its goal is to design a proper genetic strategy and to apply it to the initial strain in order to improve it. Improvements can be applied at three different levels:

a) DNA/gene content: using suitable strains and vectors.

b) Transcription efficiency: Utilising transcription factors and promoters of different strength.

c) mRNA and translation efficiency: It can be modulated by antisense RNA and codon usage adaptation. It also takes into account protein abundance by controlling its concentration.

Design of Experiments (DOE) can be useful to design a genetic strategy. It uses several parameters previously studied, which provide information about the best parameter combination.

S. cerevisiae as a chassis organism

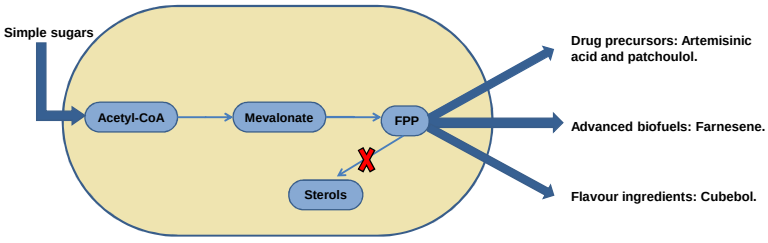


Figure 5. Representation of *S. cerevisiae* as a chassis organism for the production of different terpenoid products.

S. cerevisiae is an organism highly preferred by the industry since it can withstand high osmotic pressure and reduced pH compared to bacteria. In addition, the high efficiency of homologous recombination in this species has facilitated targeted manipulations within chromosomes. It is also important its classification as GRAS (Generally Regarded As Safe) by the U.S. Food and Drug Administration (FDA).

This microorganism can be seen as a chassis for the production of different terpenoid products depending on the genetic design applied. The introduction of a sesquiterpene synthase to this engineered microorganism has enabled the production of a variety of sesquiterpenes such as drug precursors (artemisinic acid and patchoulol), terpene-based advanced biofuels (farnesene) and flavour ingredients (cubebol).

Conclusions and future perspectives

- Preliminary tests of bisabolane showed that its properties make it a promising biosynthetic alternative to D2 diesel fuel. Bisabolane can be obtained by chemical hydrogenation of bisabolene, its immediate precursor.
- *S. cerevisiae* was engineered to produce bisabolene through metabolic engineering, which could be used to improve this production. In this work laboratory strains were used, so it would be necessary to achieve a strain suitable for large scale production to produce a economically viable biofuel. It was already proved that bisabolene presents no toxicity to *S. cerevisiae*.
- A great advance would be adapting *S. cerevisiae* to lignocellulosic substrates, since these are cheaper feedstock. The ultimate goal would be the complete microbial production of bisabolane, which would require the reduction of terpenes *in vivo*.

References

[1]. Peralta-Yahya, P.P. *et al.* Identification and microbial production of a terpene-based advanced biofuel. *Nature Communications.* 2011, vol. 2, no. 483.
[2]. Ro, D-K. *et al.* Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature*. 2006, vol 440.
[3]. Kampranis, S.C.; Makris, A.M. Developing a yeast cell factory for the production of terpenoids. *Computational and Structural Biotechnology Journal*. 2012, vol. 3, no. 4
[4]. Ro, D-K. *et al.* Induction of multiple pleiotropic drug resistance genes in yeast engineered to produce an increased level of anti-malarial drug precursor, artemisinic acid. *BMC Biotechnology*. 2008, vol. 8, no. 83.
[5]. Stephanopoulos, G.; Alper, H. And Moxley, J. Exploiting biological complexity for strain improvement through systems biology. *Nature Biotechnology*, 2004, vol. 22, no. 10.