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Metabolic Engineering 8 (2006) 102–111
METABOLIC ENGINEERING
www.elsevier.com/locate/ymben

# In silico aided metabolic engineering of *Saccharomyces cerevisiae* for improved bioethanol production

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Received 15 March 2005; received in revised form 7 September 2005; accepted 14 September 2005
Available online 10 November 2005

**Abstract**

In silico genome-scale cell models are promising tools for accelerating the design of cells with improved and desired properties. We demonstrated this by using a genome-scale reconstructed metabolic network of *Saccharomyces cerevisiae* to score a number of strategies for metabolic engineering of the redox metabolism that will lead to decreased glycerol and increased ethanol yields on glucose under anaerobic conditions. The best-scored strategies were predicted to completely eliminate formation of glycerol and increase ethanol yield with 10%. We successfully pursued one of the best strategies by expressing a non-phosphorylating, $NADP^+$-dependent glyceraldehyde-3-phosphate dehydrogenase in *S. cerevisiae*. The resulting strain had a 40% lower glycerol yield on glucose while the ethanol yield increased with 3% without affecting the maximum specific growth rate. Similarly, expression of GAPN in a strain harbouring xylose reductase and xylitol dehydrogenase led to an improvement in ethanol yield by up to 25% on xylose/glucose mixtures.


*Keywords:* *Saccharomyces cerevisiae*; Genome-scale model; Redox metabolism; Non-phosphorylating $NADP^+$-dependent glyceraldehydes-3-phosphate dehydrogenase; Ethanol; Glycerol; Xylose

## 1. Introduction

Many industrially important biotech products are derived directly from the central carbon metabolism, e.g., lactic acid, ethanol and citric acid. These products represent annual revenues exceeding US$14 billion, and there is a constant effort to improve the yield and productivity of processes leading to these products (Bailey, 1991, 1999; Papoutsakis, 1998; Stephanopoulos, 2002). Therefore, there have been many attempts to redirect the flux through the central carbon metabolism, but most of these attempts have been unsuccessful due to the tight control of flux in this part of the metabolism (Schaaff et al., 1989; Smits et al., 2000). One interesting avenue for redirecting fluxes is to express heterologous enzymes. However, a major obstacle in this avenue is the lack of efficient design methods and particularly the ability to predict the function of heterologous enzymes in a heterologous host. Here, we demonstrate the successful use of a genome-scale metabolic network model for in silico aided metabolic engineering for an improved network of the central carbon metabolism in *Saccharomyces cerevisiae*. A previously published genome-scale metabolic model of *S. cerevisiae* (Förster et al., 2003) was used and subjected to genome modifications. Particularly, a single gene insertion analysis with reactions taken from the KEGG database (http://www.genome.jp/kegg/ligand.html) has been conducted that led to the identification of novel targets that may allow an improvement in ethanol production.

Ethanol is by far the largest fermentation product with annual sales exceeding 10 billion US$, and the production of this compound also represents a good model system for optimization of the flux through the glycolytic pathway, and thereby the production of other biotech products. Ethanol is most commonly produced by anaerobic fermentations with *S. cerevisiae*. Under anaerobic conditions, *S. cerevisiae* produces only four major products from glucose: $CO_2$, ethanol, biomass and glycerol. In order to increase the overall conversion yield, there is an interest in

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1096-7176/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.ymben.2005.09.007

redirecting the flow of carbon going to biomass or glycerol towards ethanol. Some of the carbon flowing to biomass can be redirected towards ethanol by increasing the consumption of ATP for biomass production or reducing the amount of ATP formed in association with ethanol production (Nissen et al., 2000b). The flux to glycerol is quite substantial, and if the carbon flow towards glycerol could be redirected towards ethanol, one may obtain an increase in the ethanol yield of up to 10% (Nissen et al., 2000a). Deletion of the structural genes in glycerol biosynthesis (Bjorkqvist et al., 1997; Eriksson et al., 1995; Larsson et al., 1993; Nissen et al., 2000a) is not a successful strategy, since the maximum specific growth rate is severely lowered in such strains (Nissen et al., 2000a; Valadi et al., 1998). Furthermore, during anaerobic growth, formation of glycerol is necessary for growth of *S. cerevisiae* (Bjorkqvist et al., 1997) as the formation of this by-product plays an important role in maintaining the redox balance within the cells. Under anaerobic conditions, NADH originating from the production of organic acids and biomass can only be re-oxidized to $NAD^+$ by formation of glycerol (van Dijken and Scheffers, 1986), since respiration is not possible and the formation of ethanol is a redox-neutral process (see Fig. 1). However, there are several possible metabolic engineering strategies for redirecting the flux of carbon from glycerol flux towards ethanol. Efficient strategies involve engineering of reactions involving the co-factors NADH and/or NADPH in the cell. The different strategies can be grouped into: (1) substitution of NADPH-oxidizing reactions in biomass formation with NADH-oxidizing reactions (Fig. 2A), (2) substitution of $NAD^+$-reducing reactions in biomass formation by $NADP^+$-reducing reactions (Fig. 2B), (3) introduction of a reaction which either directly or via a cycle converts NADH into NADPH (Fig. 2C) or (4) substitution of the glycerol production with production of ethanol, which has a net oxidation of NADH (Fig. 2D).

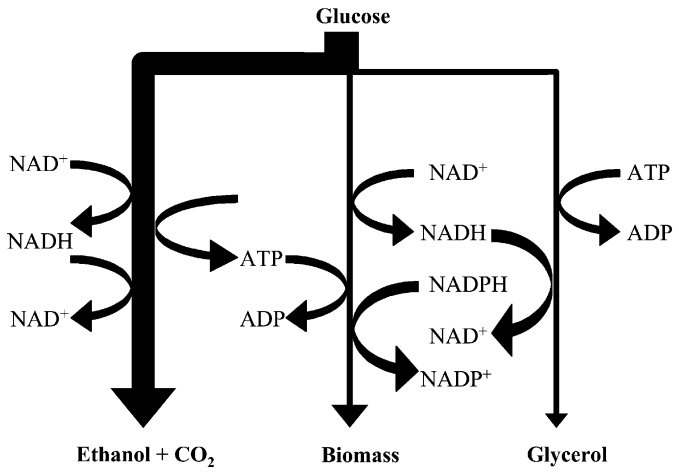


Fig. 1. Overview of main fluxes under anaerobic conditions in *S. cerevisiae* and their co-factor requirement.

The strategy of substituting an NADPH-oxidizing reaction in biomass synthesis (Fig. 2A) has previously been pursued successfully by changing the co-factor requirements associated with ammonia assimilation (Nissen et al., 2000b). Deletion of the NADPH-dependent glutamate dehydrogenase gene *GDH1* and overexpression of the NADH-dependent glutamate dehydrogenase gene *GDH2* resulted in a 30% reduction in glycerol production, while overexpression of both the glutamate synthase gene *GLT1* and the glutamine synthase gene *GLN1* in the *GDH1*-deleted mutant resulted in 38% reduction in glycerol yield and a 10% increase in ethanol yield (Nissen et al., 2000b). Despite the success of this strategy, it does require biomass growth for proper function, and industrial media often contain amino acids as nitrogen source, which will reduce its effect considerably.

We therefore looked at a number of alternative strategies for engineering of the redox metabolism in *S. cerevisiae* following the various approaches described above. In order to score these strategies, we used the reconstructed genome-scale metabolic model (Förster et al., 2003) for in silico characterization of the different strategies proposed to identify the most optimal strategy in terms of reducing glycerol formation and increasing the ethanol yield on glucose.

## 2. Materials and methods

### 2.1. General computational protocol for in silico prediction of strain performance

The effect of genome modifications (gene insertion, gene overexpression and/or gene deletion) on cellular behaviour (biomass, ethanol and glycerol yield) was simulated using a previously described genome-scale metabolic model for *S. cerevisiae* (Förster et al., 2003). In brief, this model is a stoichiometric model that allows the simulation of steady-state behaviour. Its current version contains 584 unique metabolites and 1175 enzymatic reactions. In the published version, initially no regulatory information has been included. The design of stoichiometric flux models relies on metabolite balancing, and the assumption of steady state leads to a linear equation system, which may be expressed in matrix notation as Eq. (1)

$$Sv = 0, \tag{1}$$

where $S$ is a matrix with all the stoichiometric coefficients, i.e., the stoichiometric coefficient for metabolite $i$ in the $j$th reactions is given as element $S_{ij}$, and $v$ is a vector of reaction rates (or fluxes) with the element $v_j$ corresponding to the flux of the $j$th reaction (Stephanopoulos et al., 1998). The complete model can be found at http://www.cpb.dtu.dk/models/yeastmodel.html. Linear programming was applied and an objective function $Z$ was defined, such as minimization of glucose uptake, which is equivalent to maximization of biomass yield (for a review