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Metabolic engineering of a *Saccharomyces cerevisiae* strain capable of simultaneously utilizing glucose and galactose to produce enantiopure (*2R,3R*)-butanediol

Jiazhang Lian [a,b], Ran Chao [a,b], Huimin Zhao [a,b,c,*]

[a] Department of Chemical and Biomolecular Engineering, University of Illinois at Urbana-Champaign, Urbana, IL 61801, United States
[b] Energy Biosciences Institute, Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana, IL 61801, United States
[c] Departments of Chemistry, Biochemistry, and Bioengineering, University of Illinois at Urbana-Champaign, Urbana, IL 61801, United States

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ABSTRACT

2,3-Butanediol (BDO) is an important chemical with broad industrial applications and can be naturally produced by many bacteria at high levels. However, the pathogenicity of these native producers is a major obstacle for large scale production. Here we report the engineering of an industrially friendly host, *Saccharomyces cerevisiae*, to produce BDO at high titer and yield. By inactivation of pyruvate decarboxylases (*PDC*s) followed by overexpression of *MTH1* and adaptive evolution, the resultant yeast grew on glucose as the sole carbon source with ethanol production completely eliminated. Moreover, the *pdc*- strain consumed glucose and galactose simultaneously, which to our knowledge is unprecedented in *S. cerevisiae* strains. Subsequent introduction of a BDO biosynthetic pathway consisting of the cytosolic acetolactate synthase (cyto*ILV2*), *Bacillus subtilis* acetolactate decarboxylase (Bs*AlsD*), and the endogenous butanediol dehydrogenase (*BDH1*) resulted in the production of enantiopure (*2R,3R*)-butanediol (*R*-BDO). In shake flask fermentation, a yield over 70% of the theoretical value was achieved. Using fed-batch fermentation, more than 100 g/L *R*-BDO (1100 mM) was synthesized from a mixture of glucose and galactose, two major carbohydrate components in red algae. The high titer and yield of the enantiopure *R*-BDO produced as well as the ability to co-ferment glucose and galactose make our engineered yeast strain a superior host for cost-effective production of bio-based BDO from renewable resources.

1. Introduction

Due to increasing concerns on sustainability, energy security, and global warming, intensive research has been devoted to microbial production of fuels and chemicals from renewable feedstocks such as lignocellulose biomass and marine macroalgae (Du et al., 2011; Nielsen et al., 2009). One such example is the biological production of 2,3-butanediol (BDO), an important chemical with extensive industrial applications (Celińska and Grajek, 2009; Ji et al., 2011; Savakis et al., 2013; Syu, 2001). Due to its low freezing point, BDO can be used as an antifreeze agent. A more important potential application is to produce 1,3-butanediene, the monomer of synthetic rubber. In addition, BDO derivatives, such as methyl ethyl ketone and diacetyl, have found applications in fuel and food industries (Celińska and Grajek, 2009). BDO exists in three isomeric forms, (*2S,3S*)-butanediol (*S*-BDO), *meso*-butanediol (*meso*-BDO), and (*2R,3R*)-butanediol (*R*-BDO). Many native hosts such as *Klebsiella* and *Enterobacter* species can accumulate BDO to high levels. Nevertheless, these native producers are pathogenic and synthesize a mixture of BDO stereoisomers, which prevents their commercial application (Ji et al., 2011). Therefore, development of an industrially friendly host for the production of BDO from renewable feedstock is highly desirable.

Saccharomyces cerevisiae has been successfully used in modern fermentation industry to produce a wide variety of products including ethanol, organic acids, amino acids, enzymes, and therapeutic proteins (Chen et al., 2013; Hong and Nielsen, 2012; Runguphan and Keasling, 2014). Various metabolic engineering strategies have been applied to redirect the metabolic flux from ethanol to the desired products (Abbott et al., 2009), among which the most straightforward strategy is to delete the alcohol dehydrogenases (*ADH*s, Fig. 1). Unfortunately, the resultant yeast strains suffer from accumulation of the toxic intermediates, acetaldehyde and acetate, as well as the residual production of ethanol as the major product due to the redundancy of *ADH*s in the *S. cerevisiae* genome (de Smidt et al., 2012; Ida et al., 2012). A recent effort to produce BDO by inactivating ADHs in

* Corresponding author at: Department of Chemical and Biomolecular Engineering, University of Illinois at Urbana-Champaign, Urbana, IL 61801, United States.
E-mail address: zhao5@illinois.edu (H. Zhao).

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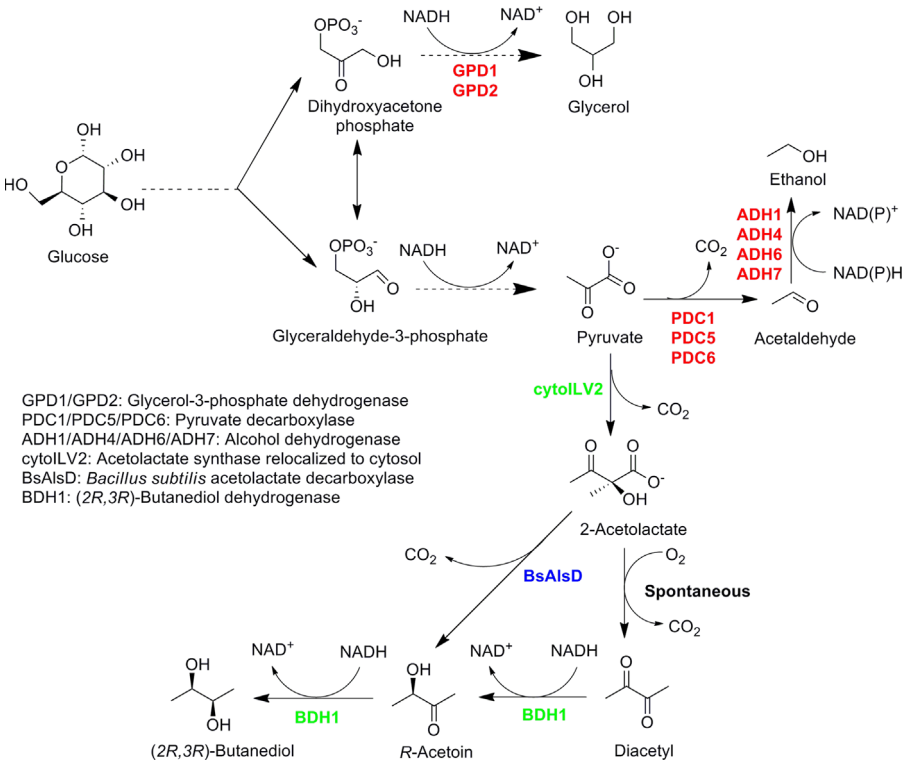

Fig. 1. Metabolic pathway for the synthesis of (*2R,3R*)-butanediol in the engineered *Saccharomyces cerevisiae* strain. The dashed arrows indicate several steps of enzymatic reactions. Genes in red represent the endogenous genes, which lead to the synthesis of major metabolites, such as ethanol and glycerol, and are targets to be inactivated to redirect the flux to BDO. As for the overexpressed BDO pathway, green genes are cloned from *S. cerevisiae*, while the blue one indicates the heterologous *AlsD* gene. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

S. cerevisiae led to low productivity and yield (Ng et al., 2012). Another strategy is to inactivate the upstream enzymes, pyruvate decarboxylases (*PDC*s, Fig. 1). The pyruvate decarboxylation activity results from the expression of three structural genes, *PDC1*, *PDC5*, and *PDC6*, whose expression are dependent on the transcription factor, PDC2 (Hohmann, 1993). However, the *pdc*- strain (either *pdc2*- or *pdc1-pdc5-pdc6*-) is notorious for its inability to grow on glucose as the sole carbon source and the requirement of C_2 (acetate or ethanol) supplementation to synthesize cytoplasmic acetyl-CoA (Flikweert et al., 1999). Inverse metabolic engineering revealed that an internal deletion in the *MTH1* coding sequence enabled the growth of *pdc*- strain on glucose (Oud et al., 2012; van Maris et al., 2004). MTH1 is a transcription factor involved in glucose sensing which can inhibit the expression of hexose transporter genes (*HXT*s), thus the uptake of glucose. The internal deletion increased the stability of MTH1 by removing the putative sites related to protein degradation (Oud et al., 2012), maintaining the intracellular glucose concentration to a low level and alleviating glucose repression.

Recently, the use of marine macroalgae as a renewable feedstock has attracted increasing attention mainly because the lack of lignin makes the hydrolysis of seaweed rather simple and straightforward (Wargacki et al., 2012; Wei et al., 2013). Among several different types of marine macroalgae, the red algae, such as *Gelidium amansii*, are known for high carbohydrate content and abundance in nature. The major carbohydrate components of red algae are glucose and galactose, which are mainly released from cellulose and agarose, respectively (Wi et al., 2009). To make a biorefinery process based on a renewable feedstock economically feasible, the producing host should be able to utilize all sugars efficiently and simultaneously (Kim et al., 2012b). However, catabolite repression is widely found in microorganisms, which means glucose is the preferred carbon source and the utilization of other carbon sources will be inhibited until glucose is depleted. As a result, sequential utilization or diauxic growth is observed during mixed sugar fermentation, leading to low yield and productivity of the final products (Vinuselvi et al., 2012). A cellobiose utilization pathway consisting of a cellodextrin transporter and a β-glucosidase was recently introduced into *S. cerevisiae* to overcome glucose repression, which enabled the consumption of cellobiose and galactose simultaneously (Ha et al., 2011). Nevertheless, the cellobiose consumption rate is much slower than that of glucose even after intensive pathway engineering (Du et al., 2012; Eriksen et al., 2013; Ha et al., 2013; Yuan and Zhao, 2013), making co-utilization of glucose and galactose still a preferred process.

In the present study, *S. cerevisiae* was engineered to produce enantiopure *R*-BDO at high titer and yield. To redirect the flux from ethanol, three *PDC* structural genes (*PDC1-PDC5-PDC6*) were deleted and growth on glucose was restored through the introduction of the internal truncated *MTH1* (*MTH1*T) followed by adaptive evolution. In the presence of a BDO biosynthetic pathway, efficient production of BDO was achieved by completely eliminating ethanol production (Fig. 1). Moreover, the engineered *pdc*- strain showed glucose-derepressed properties, enabling co-fermentation of glucose and galactose, two major carbohydrate components in red algae. Notably, this strain has the potential to be further engineered for efficient and simultaneous utilization of a mixture of sugars derived from other renewable resources, such as brown macroalgae and cellulosic biomass, for cost-effective production of fuels and chemicals.

2. Materials and methods

2.1. Strains, media, and cultivation conditions

All engineered strains used in this study are based on *S. cerevisiae* CEN.PK2-1C strain. *Escherichia coli* strain DH5α was used to maintain