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Engineering of carboligase activity reaction in *Candida glabrata* for acetoin production

Shubo Li [a,c], Nan Xu [a,c], Liming Liu [a,b,c,*], Jian Chen [a,b]

[a] State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi 214122, China
[b] The Key Laboratory of Industrial Biotechnology, Ministry of Education, Jiangnan University, Wuxi 214122, China
[c] The Laboratory of Food Microbial-Manufacturing Engineering, Jiangnan University, Wuxi 214122, China

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ABSTRACT

Utilization of *Candida glabrata* overproducing pyruvate is a promising strategy for high-level acetoin production. Based on the known regulatory and metabolic information, acetaldehyde and thiamine were fed to identify the key nodes of carboligase activity reaction (CAR) pathway and provide a direction for engineering *C. glabrata*. Accordingly, alcohol dehydrogenase, acetaldehyde dehydrogenase, pyruvate decarboxylase, and butanediol dehydrogenase were selected to be manipulated for strengthening the CAR pathway. Following the rational metabolic engineering, the engineered strain exhibited increased acetoin biosynthesis (2.24 g/L). In addition, through *in silico* simulation and redox balance analysis, NADH was identified as the key factor restricting higher acetoin production. Correspondingly, after introduction of NADH oxidase, the final acetoin production was further increased to 7.33 g/L. By combining the rational metabolic engineering and cofactor engineering, the acetoin-producing *C. glabrata* was improved stepwise, opening a novel pathway for rational development of microorganisms for bioproduction.

1. Introduction

As a member of the C4-compound family, acetoin has been defined as one of the potential top 30 sugar-derived chemical building blocks by the US Department of Energy(Werpy et al., 2004), and is attracting increasing attention because of its wide applications in industries, such as dietary supplements, cosmetics, pharmaceuticals, and chemicals(Liu et al., 2011b; Singh and Krishnan, 1959; Xiao and Xu, 2007). With increasing concerns about food security, energy, and environment, microbial fermentation has gained renewed interest owing to low-cost raw materials, mild process conditions, and higher safety and purity of products. Currently, high levels of acetoin production and yield were obtained by microbial fermentation (Table 1). However, with regard to bacterial hosts, such as *Lactococcus lactis*, *Bacillus subtilis*, *Klebsiella pneumonia*, and *Bacillus amyloliquefaciens*, acetoin is only a minor byproduct in mixed acid fermentation (Ji et al., 2013). Furthermore, as some of these bacteria were potentially pathogenic (Yu and Saddler, 1983) or show poor growth and low tolerance to acidity, osmotic stress, and high glucose levels (Sun et al., 2012; Zhang et al., 2012), application of bacterial hosts for acetoin production at an industrial scale is limited. For example, *B. amyloliquefaciens* has been reported to exhibit higher productivity (1.42 g/L/h) and titer (51.2 g/L), but low tolerance to high glucose levels (120 g/L) (Zhang et al., 2012). To overcome these limitations, many researchers have shifted their interest to yeasts owing to their superior properties. In yeasts, two major pathways for acetoin biosynthesis have been identified (Fig. 1) (Romano and Suzzi, 1996). When compared with the non-enzymatic decarboxylation (NOD) reaction, the carboligase activity reaction (CAR) may be a better option for acetoin production using pyruvate decarboxylase (PDC; EC 4.1.1.1) as the key enzyme (Chen and Jordan, 1984; Wu et al., 2000). As a thiamin diphosphate (ThDP)-dependent enzyme, PDC can catalyze the irreversible non-oxidative decarboxylation of pyruvate to form acetaldehyde-TPP complex, and can also catalyze an aldol-type condensation reaction between "active acetaldehyde" and free acetaldehyde to form acetoin. Therefore, PDC and acetaldehyde were the key factors of CAR pathway for acetoin production, and some strategies for increasing acetoin production have been presented in the literature (Cambon et al., 2006; Remize et al., 1999). For example, Ehsani et al. reported that overexpression of *GPD1* and deletion of *ALD6* in *Saccharomyces cerevisiae* could enrich the availability of acetaldehyde to yield 5.9 g/L of acetoin (Ehsani et al., 2009).

The haploid multi-vitamin-auxotroph yeast, *Candida glabrata* (*Torulopsis glabrata*), can only produce 0.045 g/L of acetoin owing to inhibition of pyruvate decarboxylation and intense non-enzymatic oxidative decarboxylation (NOD). However, it is also an attractive candidate for microbial acetoin production, and has

* Corresponding author at: State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi 214122, China. Fax: +86 510 85917875.
E-mail address: mingll@jiangnan.edu.cn (L. Liu).

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Table1
Comparison of acetoin production by different microorganisms.

Strain	Titer (g/L)	Productivity [g/(L·h)]	Yield (g/g)	References
bacteria				
Enterobacter cloacae	< 14	< 0.29	< 0.28	(Gupta et al., 1978)
L. lactis	9.28	0.19	0.20	(Kaneko et al., 1990)
Psenibacillus polymyxa	9.24	0.19	0.19	(Nakashimada et al., 1998)
Bacillus licheniformis	40.7	1.13	0.41	(Liu et al., 2011a)
Serratia marcescens	75.2	1.88	0.36	(Sun et al., 2012)
B. amyloliquefaciens	51.2	1.42	0.43	(Zhang et al., 2012)
K. pneumoniae	25.9	0.32	0.06	(Ji et al., 2013)
yeast				
S. cerevisiae	5.9	0.05	0.03	(Ehsani et al., 2009)
S. cerevisiae	9.5	0.11	0.05	(Cambon et al., 2006)
C. glabrata	7.33	0.11	0.07	This study

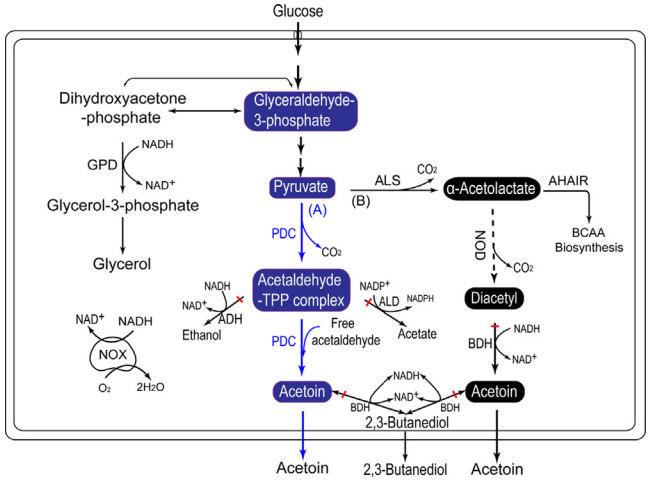

Fig. 1. The biosynthetic pathway for acetoin production from pyruvate in *C. glabrata*: (1) enhancement of CAR pathway (A, blue arrows); (2) non-enzymatic spontaneous reaction pathway (B, black arrows). The enzymes that catalyze the pathways are: pyruvate decarboxylase (PDC), alcohol dehydrogenase (ADH), acetaldehyde dehydrogenase (ALD), acetolactate synthase (Ilv2+Ilv6, ALS), acetohydroxyacid reductoisomerase (AHAIR), butanediol dehydrogenase (BDH), non-enzymatic oxidative decarboxylation (NOD), glycerol-3-phosphate dehydrogenase (GPD), NOX (NADH oxidase), and branched chain amino acid (BCAA). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

been extensively used in other industrial applications, including pyruvate and α-ketoglutarate production (Liu et al., 2007a, b). *C. glabrata* is robust, could grow on a variety of substrates, has high acid tolerance, and provides a large quantity of precursor (pyruvate) for acetoin biosynthesis, with the advantage of well-established cofactor regulation (Li et al., 2002). Thus, an efficient approach to achieve high-level acetoin production in *C. glabrata* is to modify the CAR pathway. The reconstructed *C. glabrata* *i*NX804 genome-scale metabolic model (GSMM) (Xu et al., 2013), with sequenced genome that is well characterized both genetically and phenotypically, along with strategies for genetic optimization, could facilitate engineering of *C. glabrata* with relative ease as well as high efficiency and directionality. By integrating these advantages, *C. glabrata* can be exploited as a suitable and promising host for biotechnological production of acetoin through *in silico* aided metabolic engineering.

In this study, *C. glabrata*, a pyruvate-overproducing strain, was used as a model system to study the CAR pathway for acetoin production. To this end, based on the known regulatory and metabolic information, the study aimed to increase precursor availability and PDC activity through rational metabolic engineering, and block the reduction of acetoin using the genetic knockouts. In addition, to further improve acetoin production, *in silico* simulation and intracellular redox balance analysis were carried out to predict and identify the target genes for metabolic engineering. By combining these strategies, the capacity to produce acetoin was improved stepwise, and high-level acetoin production was successfully realized in *C. glabrata*.

2. Materials and methods

2.1. Strains and plasmids

Escherichia coli JM109 was used for plasmid constructions. The *C. glabrata* strains used in this study were generated from *C. glabrata* CCTCC M202019. The details of the plasmids and strains used were presented in Table 2.

2.2. Construction of C. glabrata deletion strains

Gene deletion in *C. glabrata* was accomplished by using the transcription activator-like effector nucleases (TALENs). The TALENs were designed using 240 N-terminal and 63 C-terminal truncations (Zhang et al., 2011), and the repeat variable di-residue (RVD) of each repeat was designed following the general rule of TALEN recognition (NG, HD, NI, and NN recognize T, C, A, and G, respectively) with six exceptions. The designed amino acid sequences of TALENs were converted to DNA sequences that corresponded to the codon usage in *C. glabrata* and the sequences were synthesized. Subsequently, two vectors (pAD and pAL) containing the DNA sequences were constructed using a commercial service (Beijing ComWin Biotech Co., Ltd.). To construct the engineered strain *C. glabrata*-Δ*adh* (C-Δ*adh*), the *ADH* gene was disrupted by introducing pAD. After that, the pAD was intentionally eliminated from the strain by subculturing in the absence of selection pressure. To construct *C. glabrata*-Δ*adh*-Δ*ald* (C-Δ*adh*-Δ*ald*), the *ALD* gene was deleted by introducing pAL.

The deletion of *BDH* gene was performed according to the method previously described (Zhou et al., 2009), in which the *bdh* gene was replaced with *arg8*. The Δ*bdh*::*arg8* cassette was constructed by fused PCR and transformed into *C. glabrata* by electroporation. The transformants were selected and confirmed by colony PCR.

2.3. Plasmid construction and transformation

The plasmids used and constructed in this study were listed in Table 1 and briefly described in this section. The *PDC1* gene was amplified from the genomic DNA of *S. cerevisiae* with the primers PDC-F-*Bam*HI and PDC-R-*Xho*I. The amplified fragment