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Engineered *Kluyveromyces marxianus* for pyruvate production at elevated temperature with simultaneous consumption of xylose and glucose

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HIGHLIGHTS

- A strain producing pyruvate without ethanol/glycerol as byproducts was constructed.
- Pyruvate was produced from xylose by the engineered strain.
- Xylose and glucose were simultaneously consumed for pyruvate production.
- Xylitol accumulation was reduced via cofermentation of xylose and glucose.

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ABSTRACT

Xylose and glucose from lignocellulose are sustainable sources for production of pyruvate, which is the starting material for the synthesis of many drugs and agrochemicals. In this study, the pyruvate decarboxylase gene (*KmPDC1*) and glycerol-3-phosphate dehydrogenase gene (*KmGPD1*) of *Kluyveromyces marxianus* YZJ051 were disrupted to prevent ethanol and glycerol accumulation. The deficient growth of *PDC* disruption was rescued by overexpressing mutant *KmMTH1-ΔT*. Then pentose phosphate pathway and xylitol dehydrogenase *SsXYL2-ARS* genes were overexpressed to obtain strain YZB053 which produced pyruvate with xylose other than glucose. It produced 24.62 g/L pyruvate from 80 g/L xylose with productivity of 0.51 g/L/h at 42 °C. Then, xylose-specific transporter *Sc*GAL2-N376F was overexpressed to obtain strain YZB058, which simultaneously consumed 40 g/L glucose and 20 g/L xylose and produced 29.21 g/L pyruvate with productivity of 0.81 g/L/h at 42 °C. Therefore, a platform for pyruvate production from glucose and xylose at elevated temperature was developed.

1. Introduction

Pyruvate, a product of glycolysis, is at an important branching point in the metabolism of carbohydrates in yeast. The commercial demand for pyruvate has been increasing due to its use as a starting material for the synthesis of many drugs and agrochemicals and as a component of animal cell culture media. It is also a key substrate for the enzymatic production of amino acids such as L-tryptophan, L-tyrosine, and L-dihydroxyphenylalanine (L-DOPA) (Wang et al., 2005, 2012; Zhu et al., 2010). Lately, pyruvate has been used as the key metabolic precursor of the second-generation biofuels isobutanol and 3-methyl-1-butanol (Causey et al., 2004; Johnson and Beckham, 2015; Zhu et al., 2008).

Commercial pyruvate is produced by chemical and fermentative processes. Numerous routes for the chemical synthesis of pyruvate have been reported. The classical chemical route that produces pyruvate by dehydration and decarboxylation of tartaric acid is currently implemented on an industrial scale. This process is simple enough to realize but not cost-effective because of the energy-intensive pyrolysis of tartaric acid (Causey et al., 2004; Xu et al., 2008). Some chemists have attempted to produce pyruvate from lactate by oxidative dehydrogenation. Nonetheless, this method requires esterification of lactic acid and hydrolysis of the produced alkyl pyruvate besides oxidative dehydrogenation. Because of the formation of CO_2 via C–C bond fission, it seems difficult to achieve high yields of pyruvate directly from lactic acid using chemical catalysts (Causey et al., 2004; Xu et al., 2008). The processes mentioned above are all energy-intensive and require the use of heavy metals; both of these approaches are controversial with regards to environmental protection and sustainable process development. Fermentative methods for pyruvate synthesis that

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are both efficient and cost-effective currently play a dominant role in pyruvate production (Causey et al., 2004; Xu et al., 2008).

Xylose is among the primary products of hydrolysis of lignocellulosic biomass and the second most abundant fermentable material. A major obstacle for the conversion of saccharified cellulosic biomass into biofuel or other value-added chemicals is the problem with efficient utilization of D-xylose. Thus, microbial strains that can utilize glucose and xylose simultaneously and efficiently are crucial for exploitation of lignocellulosic biomass as a feedstock (Hu et al., 2011). In contrast to the efficient glucose fermentation in yeast, D-xylose fermentation is challenging (Nduko et al., 2013; Zhang et al., 2013).

The optimal temperature for cellulase used in biomass saccharification is 45–50 °C, and hence a higher temperature is preferred in the simultaneous saccharification and fermentation (SSF) of biomass (Alvira et al., 2010; Hickert et al., 2013; Suriyachai et al., 2013). Moreover, the use of thermophilic and thermotolerant microbes has certain advantages over the use of mesophiles, e.g., solvent tolerance, savings of energy through reduced cooling costs, higher saccharification and fermentation rates, easier stripping of products from the broth, and minimal risk of contamination (Zeng et al., 2013). *Kluyveromyces marxianus*, a "generally regarded as safe" (GRAS) microorganism, has been attracting increasing interest because of its thermotolerance, high growth rate, and a broad substrate spectrum including xylose (Fonseca et al., 2008). *K. marxianus* can grow at 45 °C with growth rate of 0.58–0.63 h^{-1} (Banat and Marchant, 1995; Zhang et al., 2011). Even at 45 °C, *K. marxianus* can produce 5.1 g/L ethanol form 10 g/L glucose, while most yeast species cannot survive at this temperature (Banat et al., 1996; Zhang et al., 2011).

In this study, recombinant *K. marxianus* strains were constructed to produce pyruvate (from glucose and xylose as carbon sources) at an elevated temperature. The pyruvate decarboxylase gene (*KmPDC*) was disrupted to block the ethanol-producing pathway; *MTH1* and its mutants were expressed to improve the growth of the *KmPDC-null* strain (Fig. 1). The *KmGPD1* gene was disrupted to reduce formation of the byproducts of glycerol and acetate production (Fig. 1). The xylitol dehydrogenase gene (*XYL2*) and some genes of the phosphate pentose pathway were overexpressed to enhance the xylose assimilation. Finally, a xylose-specific transporter was overexpressed to overcome the glucose repression in the sugar transport pathway. The fermentation ability of *K. marxianus* strains was evaluated using glucose, xylose, and a glucose + xylose mixture.

2. Materials and methods

2.1. Reagents and microorganisms

All chemicals used here were of analytical grade or higher. D-Glucose, D-xylose, and yeast nitrogen base without amino acids (YNB) were acquired from Sangon Biotech Co. (Shanghai, China). Restriction enzymes and modifying enzymes were acquired from Thermo Fisher Scientific (West Palm Beach, Florida, USA). The yeast extract and peptone were purchased from Oxoid Ltd. (Basingstoke, Hampshire, England). *K. marxianus* NBRC1777 was obtained from the NITE Biological Resource Center (Tokyo, Japan). *K. marxianus* YHJ010 is a *TRP1*, *LEU2*, and *URA3* auxotrophic strain derived from NBRC1777 (Hong et al., 2007). The synthetic dropout (SD) medium (6.7 g/L YNB without amino acids, 20 g/L glucose) supplemented with appropriate amino acids was used to select the transformants. The yeast extract-peptone-dextrose (YPD) medium (10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose) was used to aerobically culture *K. marxianus* strains. To prepare solid plates of each medium, 15 g/L agar was added. *Escherichia coli* XL10-Gold was used for cloning and was grown in a lysogeny broth (LB) medium.

2.2. Plasmids and strains

The plasmids used in this study are summarized in Table 1. Briefly, plasmid pZB009 was constructed for the expression of the *K. marxianus MTH1* gene (*KmMTH1*, GenBank: BAO39532.1); pZB012 and pZB013 were constructed for the expression of mutant proteins *KmMTH1-A66P* and *KmMTH1-ΔT*. pZB014 was constructed for the expression of the *Saccharomyces cerevisiae MTH1* gene (*ScMTH1*, GenBank: NP_010563.3); pZB015 and pZB016 were constructed for the expression of mutants *ScMTH1-A81P* and *ScMTH1-ΔT*. pZB022 was constructed for the expression of the *KmRPE1* and *KmRKI1*. The pZJ056 vector was constructed for the disruption of pyruvate decarboxylase (*KmPDC1*).

The plasmid construction procedures are described in detail below. *KmMTH1* was amplified from the genomic DNA of *K. marxianus* NBRC1777 with primers KMMTH1-ECORI-F and KMMTH1-NOTI-R (Table 2), and the resulting fragment was digested with *Eco*RI and *Not*I and then ligated into plasmid pZJ042 (Zhang et al., 2015b) at the *Eco*RI and *Not*I sites to form the pZB009 plasmid (Table 1). pZB012 or pZB013 which contain *KmMTH1-A66P* or *KmMTH1-ΔT* mutant genes, respectively, were constructed by site-directed mutagenesis with the primer pairs KMMTH1-A66P-F/KMMTH1-A66P-R and KMMTH1-ΔT-F /KMMTH1-ΔT-R (Table 2). After amplification with pZB009 as a template, the product was digested with *Dpn*I to remove the template and was then transfected into *E. coli*. The mutated plasmid was then isolated and confirmed by sequencing. *ScMTH1* was amplified from the genomic DNA of *S. cerevisiae* W303-1A (ATCC 208352) with primers SCMTH1-NOTI-F and SCMTH1-NOTI-R (Table 2), and the resulting fragment was digested with *Not*I and then ligated into the pZJ042 plasmid (Zhang et al., 2015b) at the *Not*I site to form plasmid pZB014 (Table 1). pZB015 and pZB016, which carry mutant genes *ScMTH1-A81P* and *ScMTH1-ΔT*, respectively, were constructed via site-directed mutagenesis from pZB014 with the primer pairs SCMTH1-A66P-F/SCMTH1-A81P-R and SCMTH1-ΔT-F/SCMTH1-ΔT-R. The procedure was the same as that used for pZB012 and pZB013 construction. *KmPDC1* was amplified from the genomic DNA of *K. marxianus* NBRC1777 with the primer pair KMPDC1-F and KMPDC1-R (Table 2). "A" was added to the resulting fragments by Taq DNA polymerase, and the resulting DNA fragment was inserted into pMD18T (Takara, Dalian, China) to form plasmid pMD18T-*KmPDC1* (Table 1). The expression cassette of *ScURA3* was amplified from plasmid YEUGAP (Zhang et al., 2014) with the primers SCURA3-SMAI-F and SCURA3-SMAI-R (Table 2), and the amplicon was digested with *Sma*I. The plasmid frame and a part of the *KmPDC1* fragment was amplified with the primer pair KMPDC1-MF and KMPDC1-MR with pMD18T-*KmPDC* as a template. *ScURA3* and pMD18T-*KmPDC2* fragments were ligated to form pZJ056 (Table 1). In pZJ056, *ScURA3* was inserted into the ORF of *KmPDC1* and used for the disruption cassettes. pZB022, which contained the *KmRKI1* and *KmRPE1* expression cassettes, was constructed by amplifying fragment *ScGAPDHp-KmRPE1-ScGAPDHt* with the primer pair SCGAP-XBAI-F and TER-XBAI-R (Table 1) from pZJ022 (Zhang et al., 2015b), and the amplicon was inserted into pZJ027 (Zhang et al., 2015b) at the *Xba*I site.

A strain (YZJ051) with a modified xylose assimilation pathway (Zhang et al., 2015b) served as the starting strain in this study. The *URA3* gene in YZJ051 was disrupted to recover the selection marker, and thus we obtained strain named YZJ057 (Table 3) (Zhang et al., 2015b). The above plasmids were linearized and transfected into *K. marxianus* by the lithium acetate method (Abdel-Banat et al., 2010). First, the *KmPDC1* gene (GenBank: P33149.1) in strain YZJ057 (Zhang et al., 2015b) was disrupted.

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