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ORIGINAL RESEARCH PAPER

Expression of exoinulinase genes in *Saccharomyces cerevisiae* to improve ethanol production from inulin sources

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Abstract To improve inulin utilization and ethanol fermentation, exoinulinase genes from the yeast *Kluyveromyces marxianus* and the recently identified yeast, *Candida kutaonensis,* were expressed in *Saccharomyces cerevisiae*. *S. cerevisiae* harboring the exoinulinase gene from *C. kutaonensis* gave higher ethanol yield and productivity from both inulin (0.38 vs. 0.34 g/g and 1.35 vs. 1.22 g l^{-1} h^{-1}) and Jerusalem artichoke tuber flour (0.47 vs. 0.46 g/g and 1.62 vs. 1.54 g l^{-1} h^{-1}) compared with the strain expressing the exoinulinase gene from *K. marxianus*. Thus, the exoinulinase gene from *C. kutaonensis* is advantageous for engineering *S. cerevisiae* to improve ethanol fermentation from inulin sources.

Keywords Biofuel · Ethanol · Inulinase · Jerusalem Artichoke · Yeast

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Introduction

Jerusalem artichoke is a herbaceous perennial plant that grows well on non-farm land and resists regular plant diseases. The tubers of Jerusalem artichoke are rich in inulin, a dominant reserve carbohydrate that can be converted into ethanol by microorganisms. It is low-cost to convert inulin to ethanol by consolidated microbial bioprocessing that does not need additional enzymes for inulin hydrolysis (Lynd et al. 2005; Lim et al. 2011; Hu et al. 2012). *Saccharomyces cerevisiae* is widely-used for large-scale ethanol fermentations. However, it cannot convert inulin to ethanol without pretreatment of inulin because it has no genes encoding for inulinase (Lim et al. 2010; Hu et al. 2012; Wang and Li 2013).

Exoinulinase (EC 3.2.1.80) hydrolyses inulin from the non-reducing end, releasing fructose as the main product. Expression of heterologous exoinulinase genes in *S. cerevisiae* is expected to improve ethanol production from inulin sources. The exoinulinase gene in *Kluyveromyces marxianus* is a preferred choice for heterologous exoinulinase expression because of its

advantage in specific enzyme activity (Lim et al. 2010; Li et al. 2011). Recently, we identified an exoinulinase that displayed a high affinity for inulin from a new yeast species, *Candida kutaonensis* sp. nov (Yuan et al. 2012). This study is to evaluate the potential application of the exoinulinase gene from *C. kutaonensis* to improve ethanol production from inulin sources by engineered *S. cerevisiae* strains. The exoinulinase gene from *K. marxianus* was compared with that from *C. kutaonensis* after expressing in *S. cerevisiae*.

Materials and methods

Strains

Saccharomyces cerevisiae strain JZ1C (CGMCC AS 2.3878) used in this study was described previously (Hu et al. 2012). *Escherichia coli* strain DH5α was used for plasmids construction and propagation. *Kluyveromyces marxianus* PT-1 (CGMCC AS2.4515) and *Candida kutaonensis* (CGMCC AS 2.4027) were used for cloning of exoinulinase genes.

Plasmid construction and yeast transformation

Plasmids used for expression of heterologous exoinulinase genes were constructed as follows. The constitutive promoter *PGK1p* and the terminator *PGK1t* were amplified from *S. cerevisiae* JZ1C using primers as described in Supplementary Table 1. The fragments of *PGK1p* and *PGK1t* were digested by restriction enzymes and ligated successively into the plasmid pYC230 to produce the plasmid pYC230-*PGK1pt*. The exoinulinase gene *inuKM* in *K. marxianus* PT-1 together with the nucleotide sequence of its inherent signal peptide were amplified and ligated into the plasmid pYC230-*PGK1pt* to generate the plasmid pYC230-*PGK1pt- inuKM* after digestion by restriction enzymes (Supplementary Table 1 and Fig. 1). The exoinulinase gene *inuCK* in *C. kutaonensis* was amplified and fused to the nucleotide sequence of the signal peptide in *K. marxianus* by overlap extension PCR (OE-PCR) (Supplementary Table 1). The fused fragment was ligated into the plasmid pYC230-*PGK1pt* to generate the plasmid pYC230-*PGK1pt-inuCK* (Fig. 1). All plasmids were validated by restriction enzyme digestions and sequencing. Yeast transformation was performed according to the lithium acetate method.

Ethanol fermentation

Ethanol fermentation from inulin (chicory) or Jerusalem artichoke tuber flour was performed as follows. The seed medium consisted of 50 g inulin/l, 10 g peptone/l, 6 g yeast extract/l, 2 g $(NH_4)_2SO_4$/l, 1 g KH_2PO_4/l, 1.5 g $MgSO_4.7H_2O$/ml, and 0.55 g $CaCl_2$/l. Yeast cells were inoculated into 50 ml fermentation seed medium and incubated at 30 °C with shaking at 200 rpm for 16 h. The seed culture was transformed into 250 ml flasks containing 100 ml fermentation medium with an inoculum of 10 % (v/v) for ethanol fermentation at 30 °C without shaking. Fermentation media contained 200 g inulin/l and equal concentrations of other components as in the seed medium or 200 g Jerusalem artichoke tuber flour/l without any other nutrient supplements (Hu et al. 2012). Jerusalem artichoke tubers were collected from Dongying, Shandong, China. The total sugar content in the flour (71 %) was estimated after acid hydrolysis. The fermentation process was repeated twice and three samples were assayed at each time during the fermentation.

Analytical methods

Enzyme activities were assayed using chicory inulin (Bio Basic Inc., Canada) as substrates by determining the concentration of reducing sugars released. The reaction was performed as described previously (Hu et al. 2012). Diluted crude extract (0.1 ml) with 0.9 ml of 20 g inulin/l prepared in 100 mM acetate buffer (pH 5.0) was incubated at 50 °C for 30 min. The reaction was terminated by boiling, and the

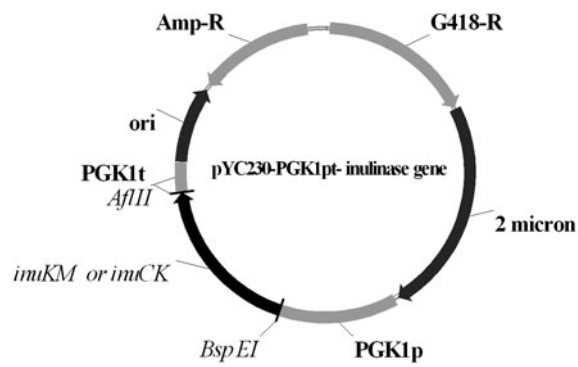

Fig. 1 Structure of the plasmids pYC230-*PGK1pt-inuKM* and pYC230-*PGK1pt- inuCK*

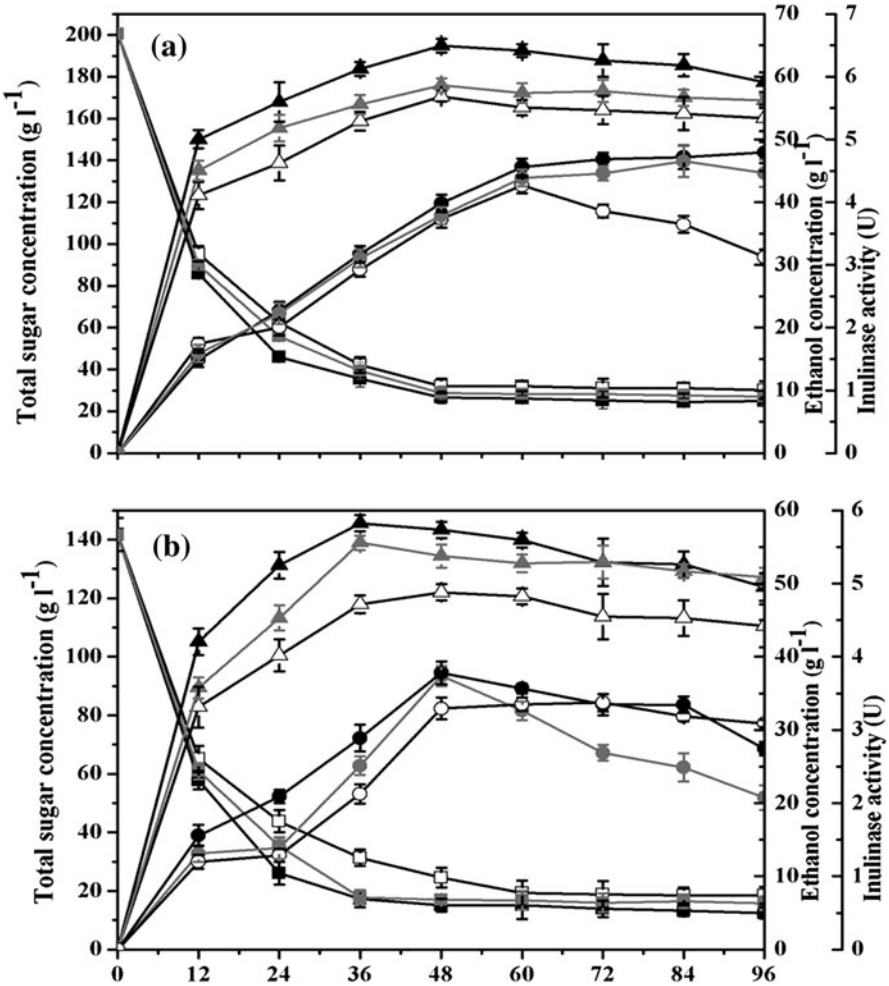

Fig. 2 Ethanol fermentation from inulin (**a**) and Jerusalem artichoke tuber flour (**b**) by JZ1C, JZ1C-*inuKM* and JZ1C-*inuCK*. Ethanol concentration: JZ1C (*white triangle*), JZ1C-*inuKM* (*gray triangle*), and JZ1C-*inuCK* (*black triangle*); Reducing sugar concentration: JZ1C (*white square*), JZ1C-*inuKM* (*gray square*), and JZ1C-*inuCK* (*black square*); Inulinase activity: JZ1C (*white circle*), JZ1C-*inuKM* (*gray circle*), JZ1C-*inuCK* (*black circle*)

reducing sugar was assayed by the 3,5-dinitrosalicylic acid method. One inulinase (U) unit was defined as the amount of enzyme which yields 1 μmol fructose per min under the assay conditions used in this study. Total sugar and reducing sugar were assayed by phenol–sulfuric acid method (Masuko et al. 2005). The ethanol concentration was assayed by Biosensor SBA 40C (Biology Institute of Shandong Academy of Sciences, Jinan, China).

Results and discussion

Extracellular inulinase activity

Two transformants, *S. cerevisiae* JZ1C-*inuKM* and JZ1C-*inuCK*, were constructed by transforming plasmids pYC230-*PGK1pt-inuKM* and pYC230-*PGK1pt-inuCK* into the strain JZ1C, respectively. The exoinulinase genes *inuKM* and *inuCK* were cloned from *K. marxianus* and *C. kutaonensis*, respectively. The two exoinulinase genes were placed under the control of an identical constitutive promoter *PGK1p* and an identical signal peptide sequence of the exoinulinase gene from *K. marxianus* (Fig. 1).

To evaluate the expression of the heterologous exoinulinase genes, extracellular inulinase activity was measured in the transformants JZ1C-*inuKM* and JZ1C-*inuCK* together with the host strain JZ1C during growing in YPI medium. The highest extracellular inulinase activity for JZ1C, JZ1C-*inuKM* and JZ1C-*inuCK* was 1.08 U ml^{-1}, 2.73 U ml^{-1}, and 3.46 U ml^{-1}, respectively, indicating the expression of both heterologous exoinulinase genes in *S. cerevisiae*.

Ethanol fermentation from inulin

Ethanol fermentation from 200 g inulin/l was performed. The highest ethanol yield achieved by *S. cerevisiae* JZ1C, JZ1C-*inuKM* and JZ1C-*inuCK* was 0.34 g/g, 0.34 g/g, and 0.38 g/g at 48 h, respectively (Fig. 2a). The highest ethanol yield of strain JZ1C-*inuCK* was increased by ~11 % compared with the host strain JZ1C, while that of the strain JZ1C-*inuKM* was increased by 0.9 %. As depicted in Fig. 2a, the ethanol productivity was also improved after expression of heterologous exoinulinase genes in *S. cerevisiae* JZ1C, which was 1.19 g l^{-1} h^{-1} for JZ1C, 1.22 g l^{-1} h^{-1} for JZ1C-*inuKM*, and 1.35 g l^{-1} h^{-1} for JZ1C-*inuCK* at 48 h (Fig. 2a).

Ethanol fermentation from Jerusalem artichoke tuber flour

Ethanol fermentation from 200 g Jerusalem artichoke tuber flour/l was conducted without any other nutrient supplements. The highest ethanol yield achieved by strains JZ1C, JZ1C-*inuKM*, and JZ1C-*inuCK* was 0.43 g/g, 0.46 g/g and 0.47 g/g at 36 h, respectively (Fig. 2b). The total sugar remained was 24.9 g l^{-1} for JZ1C, 18.8 g l^{-1} for JZ1C-*inuKM*, and 16.9 g l^{-1} for JZ1C-*inuCK* when ethanol yield reached to the highest value (Fig. 2b). The ethanol productivity was 1.02 g l^{-1} h^{-1} for JZ1C, 1.54 g l^{-1} h^{-1} for JZ1C-*inuKM*, and 1.62 g l^{-1} h^{-1} for JZ1C-*inuCK*, respectively (Fig. 2b). These data indicated that both JZ1C-*inuCK* and JZ1C-*inuKM* improved ethanol production from inulin sources due to expression of heterologous exoinulinase genes. Comparison between JZ1C-*inuCK* and JZ1C-*inuKM* displayed the advantage of JZ1C-*inuCK* on ethanol fermentation from inulin sources. The yeast *K. marxianus* has drawn much attention as an exoinulinase producer and an ethanol fermentation microorganism from inulin sources. The ethanol yield and productivity could reach up to 0.47 g/g and 1.53 g l^{-1} h^{-1}in fermentation from Jerusalem artichoke tuber flour by *K. marxianus* (Yuan et al. 2011; Hu et al. 2012). In this study, the engineered *S. cerevisiae* strain JZ1C-*inuCK* presented an ethanol yield of 0.47 g/g and an ethanol productivity of 1.62 g l^{-1} h^{-1} in fermentation of Jerusalem artichoke tuber flour.

In summary, expression of heterologous exoinulinase genes in *S. cerevisiae* improved ethanol production from inulin sources. Compared with native *K. marxianus* strains and strain JZ1C-*inuKM*, the engineered *S. cerevisiae* strain JZ1C-*inuCK* exhibited advantageous ethanol production from inulin sources, indicating the predominance of the exoinulinase gene from *C. kutaonensis* in engineering *S. cerevisiae*.

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