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ORIGINAL PAPER

Optimizing promoters and secretory signal sequences for producing ethanol from inulin by recombinant *Saccharomyces cerevisiae* carrying *Kluyveromyces marxianus* inulinase

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Abstract Inulin is a polyfructan that is abundant in plants such as Jerusalem artichoke, chicory and dahlia. Inulinase can easily hydrolyze inulin to fructose, which is consumed by microorganisms. Generally, *Saccharomyces cerevisiae*, an industrial workhorse strain for bioethanol production, is known for not having inulinase activity. The inulinase gene from *Kluyveromyces marxianus* (KmINU), with the ability of converting inulin to fructose, was introduced into *S. cerevisiae* D452-2. The inulinase gene was fused to three different types of promoter (GPD, PGK1, truncated HXT7) and secretory signal sequence (KmINU, MFα1, SUC2) to generate nine expression cassettes. The inulin fermentation performance of the nine transformants containing different promoter and signal sequence combinations for inulinase production were compared to select an optimized expression system for efficient inulin fermentation. Among the nine inulinase-producing transformants, the *S. cerevisiae* carrying the PGK1 promoter and MFα1 signal sequence (*S. cerevisiae* D452-2/p426PM) showed not only the highest specific KmINU activity, but also the best inulin fermentation capability. Finally, a batch fermentation of the selected *S. cerevisiae* D452-2/p426PM in a bioreactor with 188.2 g/L inulin was performed to produce 80.2 g/L ethanol with 0.43 g ethanol/g inulin of ethanol yield and 1.22 g/L h of ethanol productivity.

Keywords Ethanol · Inulin · Inulinase · *Saccharomyces cerevisiae* · Consolidated bioprocessing

Introduction

Recently, bioethanol is considered one of the most promising biofuels due to greater octane booster properties compared to others [1]. It is also virtually non-toxic and unable to contaminate water sources. Bioethanol is a form of renewable energy that can be produced from agricultural feedstocks. Energy crops for bioethanol production include maize, wheat, sorghum, sugar cane and so on. A certain food prices increased globally because of production of bioethanol from food crops. Therefore, the feedstock Jerusalem artichoke (*Helianthus tuberosus* L.), not competing with grain crops for arable land, is regarded as a promising bioenergy crop for production of bioethanol. Unlike most starch-based plants, one of the major constituents of Jerusalem artichoke is inulin. The dry tubers of Jerusalem artichoke contain about 60–80 % (w/w) inulin [2]. More important, Jerusalem artichoke can be used directly as a fermentable carbon source after a relatively simple pretreatment process, compared to lignocellulosic biomass.

Inulin, a non-digestible carbohydrate, is a polyfructan, consisting of linear β-2,1-linked polyfructose chains terminated by a glucose residue. The roots and tubers of plants such as Jerusalem artichoke, chicory, dahlia and yacon contain inulin as a reserve carbohydrate [3]. Inulin

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shows high solubility in water and also exhibits low viscosity in solution [1]. Inulin can be hydrolyzed by a chemical method such as acid hydrolysis. However, fructose is easily degraded at low pH, and the acid hydrolysis leads to coloring of the inulin hydrolysate and by-product formation of difructose anhydrides [4]. Moreover, fructose is liable under acidic conditions to form 5-hydroxymethylfurfural, a known inhibitor of microbial cell growth [5].

Production of fructose from complete hydrolysis of inulin is a single-step reaction catalyzed by inulinase, which yields up to 95 % of fructose [6]. Inulinases produced by microbes are often exo-acting as well as inducible [2]. The microbial inulinases hydrolyze inulin by cleaving terminal fructosyl units to form fructose [2]. Inulin can be utilized for producing ethanol by enzymatic hydrolysis, but enzymatic hydrolysis may increase the cost of production [7].

Consolidated bioprocessing (CBP) integrating inulinase production, inulin hydrolysis and fermentation in one vessel can be used for ethanol fermentation from inulin. The CBP strategy is not only cost-effective, but also competitive for producing ethanol from inulin as no sugar and toxic materials accumulate during ethanol fermentation and contamination can be prevented effectively [8]. There were reports about applications of CBP to direct production of ethanol from inulin [8–10] by using naturally inulin-utilizing *Kluyveromyces* species. In this study, *Saccharomyces cerevisiae* was employed to produce ethanol directly from inulin. *S. cerevisiae* is an industrial workhorse for bioethanol production, which has various characteristics such as high osmo- and ethanol-tolerance and availability of numerous genetic tools. However, *S. cerevisiae* strains are generally unable to ferment inulin. Therefore, we introduced the inulinase gene from the yeast *K. marxianus* (KmINU) into *S. cerevisiae* with three different signal sequences to secrete inulinase into the fermentation medium. The inulinase of *K. marxianus* (KmINUp) showed high thermostability and catalytic activity among the reported inulinases [8, 11, 12]. To optimize yeast strains for expressing KmINU for efficient production of fructose from inulin, three different promoters and three different signal sequences were fused to the KmINU gene in different combinations. We compared the specific inulinase activity, inulin consumption rate, ethanol yield and productivity among the nine different inulinase-producing recombinant *S. cerevisiae* strains.

Materials and methods

Reagents

Glucose, agarose, ampicillin, ethidium bromide, yeast synthetic drop-out supplement, yeast nitrogen base (YNB, w/o amino acid), bovine serum albumin and EDTA were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Synthetic complete (SC) supplement mixture was purchased from Sunrise Science Products (San Diego, CA, USA). Inulin powder was purchased from NOW FOOD (Bloomingdale, IL, USA). Bacto-peptone, bacto-tryptone, yeast extract and bacto-agar were obtained from Difco (Detroit, MI, USA); glycine from Junsei (Tokyo, Japan); ethanol from Merck (Darmstadt, Germany); HPLC-grade water from J.T. Baker (Phillipsburg, NJ, USA); 1 N NaOH, 1 N HCl, NaCl, H_2SO_4 and potassium phosphate from Duksan (Ansan, Korea).

Strains and plasmids

Escherichia coli TOP10 (Invitrogen, Carlsbad, CA, USA) was employed for the propagation and preparation of plasmid DNA. *S. cerevisiae* D452-2 [Matα, leu2 his3 ura3 can1] was used as a host strain to ferment inulin to ethanol because of its versatility as a biofuel producer [13–15]. *Kluyveromyces* genus is well known for its ability to grow on inulin [11] and has been widely used for production of inulinase which is secreted into medium efficiently [16–20]. Therefore, the inulinase gene from *K. marxianus* (KmINU) was selected to introduce into *S. cerevisiae* D452-2. The *K. marxianus* inulinase gene (KmINU) was subcloned into plasmids p426GPD, p426PGK1, and p426HXT7. The KmINU signal sequence and KmINU gene were isolated from the chromosomal DNA of *K. marxianus* by the polymerase chain reaction (PCR) using primers designed from the KmINU sequences reported in NCBI. The MFα1 and SUC2 signal sequences were isolated from the chromosomal DNA of *S. cerevisiae* by PCR using primers listed in Table 1. Signal sequences of KmINU, MFα1, and SUC2 were assigned into plasmids p426GPD, p426PGK1, and p426HXT7, resulting in total nine plasmids (Table 2). All vector constructs had the myc-tag at the C-terminal end of the KmINU gene.

DNA manipulation and transformation

Transformation of *E. coli* Top10 using the heat shock method was carried out as described previously [21]. An appropriate volume of the transformed cells was spread on LB agar plates with an ampicillin selection marker. AxyPrep™ Plasmid Miniprep Kit from Axygen Biosciences Co. (San Francisco, CA, USA) was employed for Mini-scale preparation of plasmid DNA. Introduction of expression vectors into *S. cerevisiae* D452-2 was performed using the yeast EZ-Transformation kit (BIO 101, Vista, CA, USA).

Table 1 List of primers used in this study

Name	Sequence	Relevant work
F-SpeI_KmINU	gactagtATGAAGTTCG CATACTCCCTCTTGCTT	Cloning KmINU cassette into p426GPD, p426PGK1
R-HindIII_KmINU_myc	cccaagcttTCACAGATCCTCTTCTGAGATGAGTTT TTGTTCAACGTTAAATTGGGTAACGTTAAA	Cloning KmINU cassette into p426GPD
F-BamHI_KmINU	cgggatccATGAAGTTCGCATACTCCCTCTTGC	Cloning KmINU cassette into p426HXT7
RXhoI_KmINU_myc	ccgctcgagTCACAGATCCTCTTCTGAGATGAGTTT TTGTTCAACGTTAAATTGGGTAACGTTAAATGAGTTAATGGT	Cloning KmINU cassette into p426PGK1, p426HXT7
FBamHI_ovMF	cgggatccATGAGATTTCCTTCAATTTTTACTGCTG	Overlapping KmINU cassette and *MFα1* signal sequence, cloning into p426GPD, p426PGK1, p426HXT7
R-ovMFKmINU	GGCCTTGCTGTCACCATCAGCTTCAGCCTCTCTTTTCTCG	Overlapping KmINU cassette and *MFα1* signal sequence
F-ovKmINU	GATGGTGACAGCAAGGCCATCA	Overlapping KmINU cassette and signal sequence
R-SalI_ovKmINU_myc	acgcgtcgacTCACAGATCCTCTTCTGAGATGAGTT TTTGTTCAACGTTAAATTGGGTAACGTTAAATGAGTTAATGG	Overlapping KmINU cassette and signal sequence, cloning into p426GPD, p426PGK1, p426HXT7
F-BamHI_ovSUC2	cgggatccATGCTTTTGCAAGCTTTCCTTTTCCTT	Overlapping KmINU cassette and *SUC2* signal sequence, cloning into p426GPD, p426PGK1, p426HXT7
R-ovSUC2KmINU	GGCCTTGCTGTCACCATCTGATGCAGATATTTTGGCTGCAAAAC	Overlapping KmINU cassette and *SUC2* signal sequence

The lowercase sequences correspond to restriction enzymes site

Media and culture conditions

LB medium (1 % tryptone, 0.5 % yeast extract, 1 % NaCl) was used for *E. coli* cultivation. YEPD medium (1 % yeast extract, 2 % bacto-peptone, 2 % glucose) and YNB medium which lacked appropriate amino acids were used for selection of yeast strains. YNB synthetic complete medium (6.7 g/L YNB, 2.0 g/L synthetic complete supplement mixture) that contains a mixture of amino acids was used for cultivation of yeast strains. All cultivations of recombinant *S. cerevisiae* were carried out at 30 °C. Cell stock was transferred to a test tube containing 5 mL of appropriate medium and incubated overnight at 250 rpm. In flask culture, the cells were transferred into a 250-mL glass flask containing 50 mL YEPD medium and cultivated at the shaking speed of 80 rpm. For bioreactor cultivation, 100 mL of the pre-culture grown in YNBD medium at 30 °C and shaking speed of 250 rpm for 60 h was transferred into a 500-mL bioreactor vessel. Batch fermentation was performed in 500 mL YEP medium supplemented with 20 % (w/v) inulin or fructose at 30 °C and pH 5.0 (adjustment by 1 N HCl and 1 N NaOH) using a bench-top bioreactor (KoBioTech, Seoul, Korea). The 20 % (w/v) of inulin was soluble in medium. For oxygen-limited conditions, an agitation speed of 200 rpm with no aeration was used.

Immunoblot analysis

To determine inulinase secreted into the medium from *S. cerevisiae* D452-2/p426PM during fermentations, cell-free culture medium was taken at 0, 24, 48 and 72 h. A 50 mL of the culture broth (YNB + 2 % glucose) from the flask was taken and the sample was immediately centrifuged at 5,000×*g*, 4 °C for 10 min for separation of cells and culture medium. A Vivaspin 30,000 MWCO (GE Healthcare Life science, Piscataway, NJ, USA) was employed to concentrate the cell-free culture medium. A 50 mL of cell-free culture medium was concentrated to 50 μL and applied onto a 12 % sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and subjected to Western blot analysis.

To determine the glycosylation state of the KmINUp, samples were treated by Endo-H that cleaves the linkage between the innermost GlcNAc and asparagine residues of high mannose, hybrid, and complex oligosaccharides from N-linked glycoproteins [22]. Briefly, the KmINUp sample was combined with 1 μL of 10× glycoprotein denaturing buffer and H_2O to make a 10 μL total reaction volume. Then the sample was denatured by heating at 100 °C for 10 min. After heating, this solution was added to 2 μL of 10× G5 Reaction Buffer, 1–5 μL Endo-H and H_2O to

Table 2 Nine recombinant *S. cerevisiae* D452-2 strains containing a different KmINU construct

Strain	Promoter	Signal sequence
S. cerevisiae D452-2/p426GI	*GPD*	*KmINU*
S. cerevisiae D452-2/p426PI	*PGK1*	*KmINU*
S. cerevisiae D452-2/p426HI	Truncated *HXT7*	*KmINU*
S. cerevisiae D452-2/p426GM	*GPD*	*MFα1*
S. cerevisiae D452-2/p426PM	*PGK1*	*MFα1*
S. cerevisiae D452-2/p426HM	Truncated *HXT7*	*MFα1*
S. cerevisiae D452-2/p426GS	*GPD*	*SUC2*
S. cerevisiae D452-2/p426PS	*PGK1*	*SUC2*
S. cerevisiae D452-2/p426HS	Truncated *HXT7*	*SUC2*

make a total reaction volume of 20 μL. Finally, the reaction mixture was incubated at 37 °C for 1 h.

Following SDS-PAGE, the protein gels were immune-blotted onto 0.45 μm polyvinylidene difluoride (PVDF) membranes (PALL, Port Washington, NY, USA). Blots were rinsed three times in 1× phosphate-buffered saline (PBS) buffer and the blots were sequentially blocked with 5 % non-fat skim milk powder (Bio-Rad, Hercules, CA, USA) in PBS for 1.5 h at room temperature. After blocking, the blots were washed three times in PBS and probed with anti-myc mouse monoclonal antibody (IG Therapy Co., Kangwon, Korea) at a 1/1,000 dilution in PBS for 1 h at room temperature. The blots were further washed and the membranes reprobed with anti-mouse lgG (Fc specific)–peroxidase, antibody produced in goat (A2554, Sigma, St. Louis, MO, USA) at a 1/5,000 dilution in PBS for 40 min at room temperature. Washing was repeated with an additional 2 × 5 min washes in PBS only. The blots were incubated in the detection reagents, WEST-ZOL (Intron biotechnology, Seongnam, Korea) for 2 min in dark room and immediately exposed to films in cassette for 30 s to 10 min. The films were incubated in developer buffer for several minutes, and then the reaction was stopped by stop buffer and dipped in fixer buffer sequentially.

Analytical methods

Dry cell weight concentration was estimated by measuring absorbance at 600 nm by a spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan). Optical density was converted into dry cell mass by using the following conversion equation

$$\text{Dry cell weight (g/L)} = 0.30 \times \text{OD}_{600}.$$

Concentrations of inulin, fructose, glycerol, acetic acid and ethanol were measured by high performance liquid chromatography (HPLC) (Agilent 1100LC, Palo Alto, CA, USA) equipped with the organic acid column (Phenomenex, Torrance, CA, USA). The carbohydrate analysis ion exclusion column heated at 60 °C was applied to analyze 20 μL of diluted culture broth. Detection was made with a refractive index detector at 35 °C. HPLC operation conditions were set as described previously [23]. A 5 mM H_2SO_4 solution was used as mobile phase at a flow rate of 0.6 mL/min.

Measurement of inulinase activity

The cultures were harvested by centrifugation at 5,000×*g* at 4 °C for 10 min to separate the cells from the media. The supernatant was designated as the extracellular fraction. The cells were lysed by treatment of Y-PER (Thermo scientific, IL, USA) and vortexed. After centrifugation at 5,000×*g* for 10 min, the supernatant was designated as the intracellular fraction and used for the measurement of cellular inulinase activity. To measure the inulinase produced by the recombinant strains carrying the KmINU, MFα1, and SUC2 signal sequences, periplasmic fractions were prepared. After the culture was centrifuged at 5,000×*g*, at 4 °C for 10 min to separate the cells from the media the first time, the cells were treated with lyticase from *Arthrobacter luteus* (L4025, Sigma, St. Louis, MO, USA). The supernatant was designated as the periplasmic fraction.

The extracellular, intracellular and periplasmic fractions were incubated with 2 % inulin in 0.1 M phosphate buffer (pH 6.0) at 60 °C for 10 min. The reaction was inactivated by keeping the reaction mixture at 100 °C for 10 min. The amount of reducing sugars released was determined by the 3,5-dinitrosalicylic acid (DNS) method. One unit of inulinase was defined as the amount of enzyme liberating 1 μmol of fructose equivalent from inulin per minute at 60 °C and the total specific activity is the sum of extracellular, periplasmic and intracellular activity per mg dry cell weight (U/mg DCW).

Results and discussion

Selection of an optimized expression cassette for inulinase production

To introduce a fermentation capability for inulin in *S. cerevisiae* D452-2, the expression system of KmINU has to be optimized. Three types of signal sequences (KmINU, MFα1 and SUC2 signal sequence) were fused to the N-terminal of the KmINU gene and the C-terminal myc tag was fused to the C-terminal of the KmINU gene to construct KmINU expression cassettes (Fig. 1). Consequently, the amplified inulinase expression cassettes were inserted

into the expression vectors p426GPD, p426PGK1, p426HXT7 containing the GPD, PGK1 and truncated HXT7 promoter, respectively. The resulting plasmids, each with the KmINU signal sequences were named p426GI (carrying the GPD promoter), p426PI (carrying the PGK1 promoter) and p426HI (carrying the truncated HXT7 promoter). Among the well-known yeast signal sequences (MFα1 signal sequence and SUC2 signal sequence) and the native KmINU signal sequence, one signal sequence was selected to introduce into each construct, resulting in nine recombinant yeast strains carrying a different KmINU expression cassette. A recombinant *S. cerevisiae* D452-2 strain containing a constructed vector was screened using an appropriate selective marker (Table 2). The nine recombinant yeast strains could grow on inulin as a carbon source unlike the wild-type *S. cerevisiae* D452-2 which could not grow in this condition.

To evaluate the enzyme activity of inulinase in the nine different recombinant *S. cerevisiae* D452-2 strains, crude extract enzyme assays were performed. Figure 2 shows the total specific activity of KmINUp in the nine recombinant *S. cerevisiae* strains. The activities of KmINUp were in the range of 0.09 ± 0.01 to 1.34 ± 0.06 U/mg DCW. The *S. cerevisiae* D452-2/p426PM strain carrying the inulinase fused to the combination of the PGK1 promoter and MFα1 signal sequence expressed the highest inulinase activity of 1.34 ± 0.06 U/mg of DCW among the nine recombinant *S. cerevisiae* strains tested. The GPDp, PGK1p and truncated HXT7p are commonly used promoters in *S. cerevisiae* [24–28]. These constitutive promoters were employed to express the KmINU gene. The truncated promoter of HXT7 that abolishes glucose repression leads to higher expression of the gene on glucose media than other promoters of genes involved in the glycolytic pathway [29]. Interestingly, the recombinant yeast strains carrying inulinase with the PGK1 promoter in this study tended to show higher total specific activity of inulinase than that with the truncated HXT7 and GPD promoters. This result suggests that the strength of the promoter may depend on the target gene fused to the promoter. The *SUC2* and *MFα1* signal sequences have been applied to secretion of enzymes in *S. cerevisiae* [30–32]. The yeast strains carrying inulinase with the SUC2 signal sequence showed lower inulinase activity than that with the MFα1 or native KmINU signal sequence. Furthermore, the specific activities of inulinase of the recombinant yeast strains carrying MFα1 were higher than that with the native KmINU signal sequence except for the GPD promoter. This indicates that the assigned signal sequence has impact on total specific activity of the inulinase. In addition, a combination of promoter and signal sequence might be important in the enhancing total specific activity of inulinase in *S. cerevisiae*.

Flask fermentations (250 mL) were performed to test the inulin fermentation capability of the nine recombinant *S. cerevisiae* strains and summarized in Table 3. The representative fermentation profiles of D452-2/p426GM and D452-2/p426GS were displayed in Fig. 3. The overall ethanol productivity was calculated by dividing the maximum ethanol concentration by the fermentation time. Fermentation capabilities of the recombinant *S. cerevisiae* D452-2 strains were in correlation with the total specific activity of inulinase of the recombinant yeast strains (Fig. 4) as expected. Strain D452-2/p426PM which expressed KmINU by the PGK1 promoter and MFα1 signal sequence showed the highest total specific activity of inulinase, inulin consumption rate, maximum ethanol concentration, ethanol yield and productivity among the nine recombinant strains. The recombinant strains displaying lower specific activities of inulinase, however, tended to show higher final dry cell mass (Fig. 4b). Consistent with the result of total specific activity of inulinase, the *S. cerevisiae* D452-2/p426PM harboring the PGK1 promoter and MFα1 signal sequence showed the best inulin fermentation capability among the recombinant yeast strains and was chosen for further analyses.

Expression of inulinase gene in recombinant *S. cerevisiae* D452-2/P426PM

To monitor the expression pattern of KmINU by *S. cerevisiae* D452-2/p426PM, an immunoblot analysis was employed. The cell-free culture broth of *S. cerevisiae* D452-2/p426PM was taken at indicated times for immunoblot analysis (Fig. 5a). The total specific activity of inulinase increased with cultivation time and reached a maximum value (1.34 U/mg DCW) at 60 h after cell cultivation (Fig. 6), indicating that KmINUp produced from *S. cerevisiae* D452-2/p426PM accumulated in the fermentation broth with increasing culture time, and appeared not to be degraded even after the yeast strain finished the inulin fermentation (Fig. 5a). Additionally, an immunoblot analysis of the culture supernatant revealed that a protein band of KmINUp was around 90 kDa. The size of the band is about 30 kDa larger than the estimated molecular weight of the mature KmINUp (~60 kDa) (Fig. 5a). When the sample was treated with endoglycosidase H (Endo-H) to determine the glycosylation of KmINUp, the band, of which size is corresponding to about 60 kDa, appeared (Fig. 5b). These results suggested that KmINUp was glycosylated in the recombinant *S. cerevisiae* D452-2/p426PM. As glycosylation is involved in the stability of the secreted protein, the recombinant *S. cerevisiae* D452-2/p426PM may stabilize KmINUp by glycosylation [33, 34]. Time profiles of total specific activity of KmINUp and cell mass of the *S. cerevisiae* D452-2/p426PM strain were shown in Fig. 6.

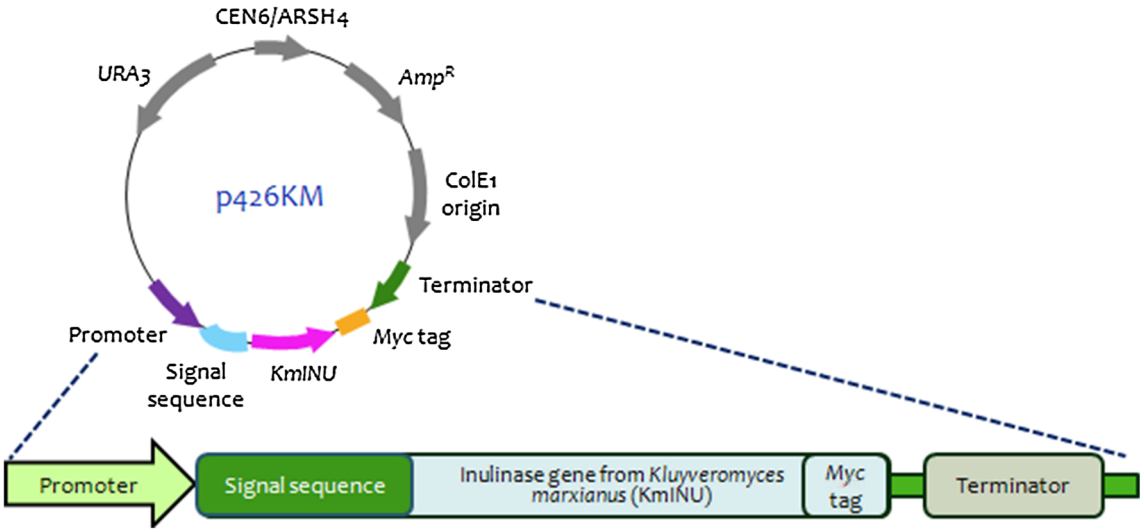

Fig. 1 KmINU expression cassette

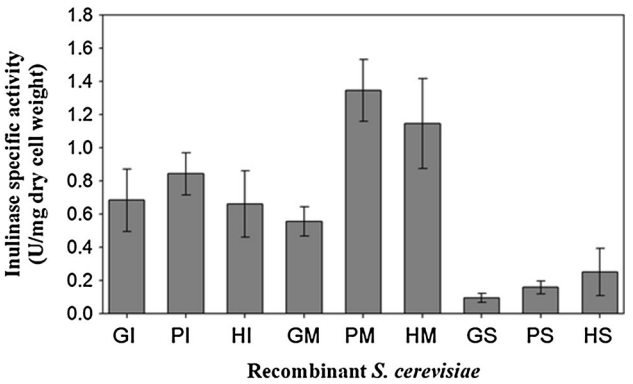

Fig. 2 Total specific activity of KmINUps obtained by overexpression in *S. cerevisiae* using different promoter and signal sequence combinations. Specific activities were measured from recombinant *S. cerevisiae* D452-2 strains shown in Table 2. *GI* D452-2/p426GI, *PI* D452-2/p426PI, *HI* D452-2/p426HI, *GM* D452-2/p426GM, *PM* D452-2/p426PM, *HM* D452-2/p426HM, *GS* D452-2/p426GS, *PS* D452-2/p426PS, *HS* D452-2/p426HS. The values represent the averages ± standard deviations (*error bars*)

Comparison of fermentation capability for inulin and fructose

It is necessary to compare the fermentation properties of *S. cerevisiae* D452-2/p426PM for inulin as a carbon source with those of the wild-type *S. cerevisiae* for fructose which is a fully hydrolyzed form of inulin. Batch fermentations using the wild-type *S. cerevisiae* D452-2 with fructose and recombinant *S. cerevisiae* D452-2/p426PM with inulin were performed. The wild-type *S. cerevisiae* D452-2 strain consumed the fructose added in 60 h to produce 80.4 g/L of ethanol. On the other hand, simultaneous hydrolysis of inulin to fructose and conversion of fructose to ethanol occurred for the *S. cerevisiae* D452-2/p426PM strain. Inulin was completely hydrolyzed in 36 h and 80.2 g/L of ethanol was produced in 66 h (Fig. 7). It appeared that the fructose hydrolyzed from inulin accumulated in the medium because the hydrolysis rate of inulin was higher than the consumption rate of fructose. Recently, two fructose-specific transporter genes were identified and characterized [35]. More experiments are on-going to facilitate the consumption rate of fructose by introducing the fructose-specific transporter genes to *S. cerevisiae* D452-2. The wild-type *S. cerevisiae* D452-2 resulted in 0.43 g ethanol/g fructose of ethanol yield and 1.34 g/L h of ethanol productivity, while the *S. cerevisiae* D452-2/p426PM strain resulted in 0.43 g ethanol/g inulin of ethanol yield and 1.22 g/L h of ethanol productivity. Although the ethanol productivity of inulin fermentations using the recombinant strain was slight lower than that of fructose fermentations by the wild-type strain, the consolidated bioprocessing of inulin with *S. cerevisiae* D452-2/p426PM was comparable to the fructose fermentation. The ethanol yield of *S. cerevisiae* D452-2/p426PM (0.43 g ethanol/g inulin) was similar when using fructose as a carbon source (0.43 g ethanol/g fructose). This result indicates that ethanol fermentation with a high concentration of inulin is feasible using the *S. cerevisiae* D452-2/p426PM strain. There were previous reports on direct fermentation of inulin from Jerusalem artichoke by *K. marxianus*. The ethanol yield and productivity of *S. cerevisiae* D452-2/p426PM (0.43 g ethanol/g inulin and 1.22 g/L h) were comparable to the corresponding values of native inulin-consuming *K. marxianus* strains using inulin as a carbon source [9, 10, 12]. Ethanol fermentation was also carried out by inulin-fermenting *S. cerevisiae* KCCM50549 using inulin-containing carbohydrates in Jerusalem artichoke as a carbon source [36]. The

Table 3 Fermentation parameters of nine recombinant *S. cerevisiae* D452-2 strains containing a different KmINU construct

Strain	Total specific activity (U/mg dry cell weight)	Final dry cell weight (g/L)	Inulin consumption rate (g/L h)	Max. ethanol concentration (g/L)	Ethanol yield (g/g inulin)	Ethanol productivity (g/L h)
D452-2/p426GI	0.65	13.5	0.7	5.9	0.29	0.29
D452-2/p426PI	0.86	14.6	1.0	7.0	0.35	0.35
D452-2/p426HI	0.66	16.1	0.8	6.0	0.32	0.18
D452-2/p426GM	0.73	11.3	1.0	7.3	0.36	0.36
D452-2/p426PM	1.34	12.0	1.7	8.0	0.40	0.67
D452-2/p426HM	1.14	13.6	1.0	6.8	0.34	0.27
D452-2/p426GS	0.09	16.2	0.4	4.3	0.21	0.14
D452-2/p426PS	0.16	15.3	0.4	4.1	0.21	0.14
D452-2/p426HS	0.25	16.8	0.4	4.5	0.22	0.18

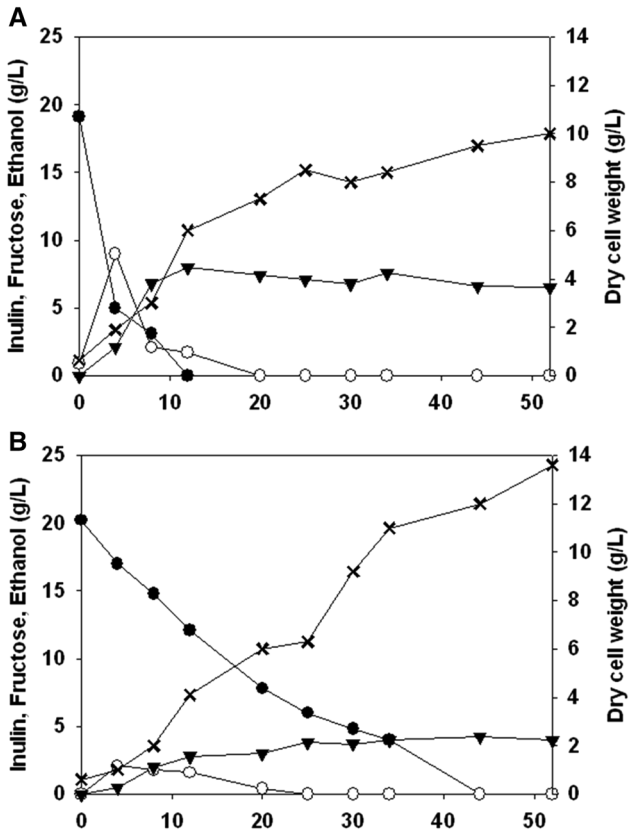

Fig. 3 Flask fermentation profiles of **a** D452-2/p426PM and **b** D452-2/p426GS (*filled circle* inulin, *unfilled circle* fructose, *inverted filled triangle* ethanol, *cross symbol* dry cell weight)

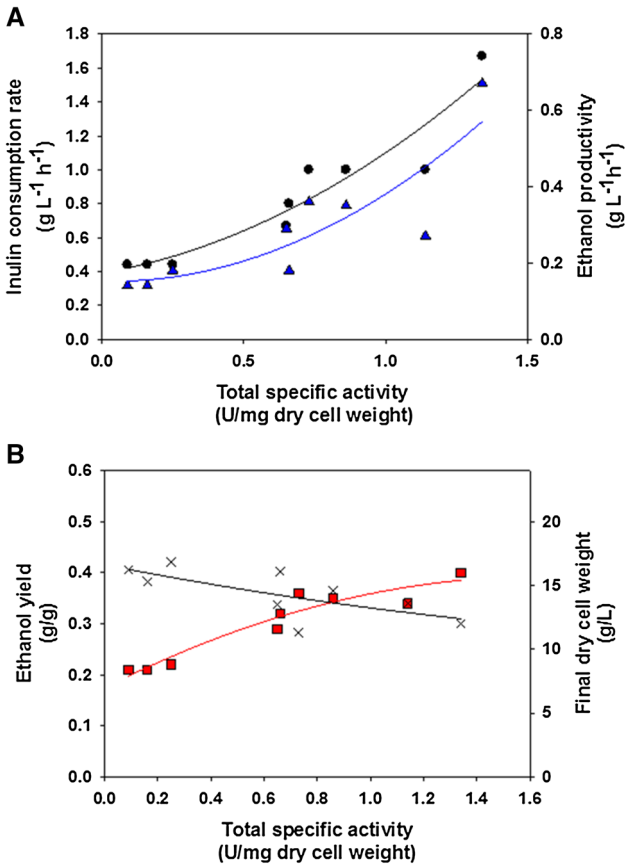

Fig. 4 Correlation between the total specific activity of inulinase (U/mg DCW) and **a** inulin consumption rate (*filled circle*) or ethanol productivity (*triangle*), **b** ethanol yield (*square*) or final dry cell weight (*cross symbol*) for the recombinant strains carrying inulinases in flask cultivation

ethanol productivity (1.22 g/L h) of *S. cerevisiae* D452-2/p426PM was higher than that of inulin-fermenting *S. cerevisiae* (1.06 g/L h). Additionally, the maximum ethanol concentration (80.2 g/L) and ethanol yield (0.43 g ethanol/g inulin) were much higher than the previous report (36.2 g/L and 0.36 g ethanol/g inulin) [36].

Conclusions

Bioethanol is considered one of the most competitive biofuels due to its greater octane booster properties and

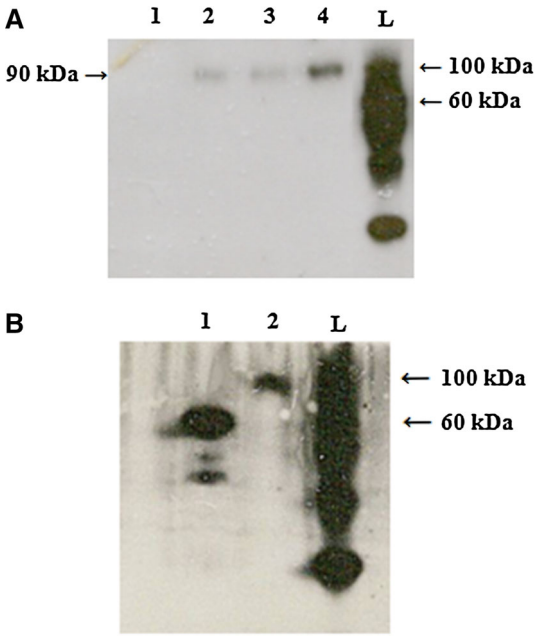

Fig. 5 Immunoblot analysis of KmINUp from *S. cerevisiae* D452-2/p426PM. **a** Expression of KmINUp in the culture medium of *S. cerevisiae* D452-2/p426PM with cultivation time. *Lanes 1*, *2*, *3* and *4* refer to samples after 0, 24, 48, 72 h, respectively. *L* refers to the protein marker. **b** Native and carbohydrate-depleted KmINUp. *Lane 1* Endo-H treated KmINUp. The major band is the targeted protein. *Lane 2* native KmINUp. *L* refers to the protein marker

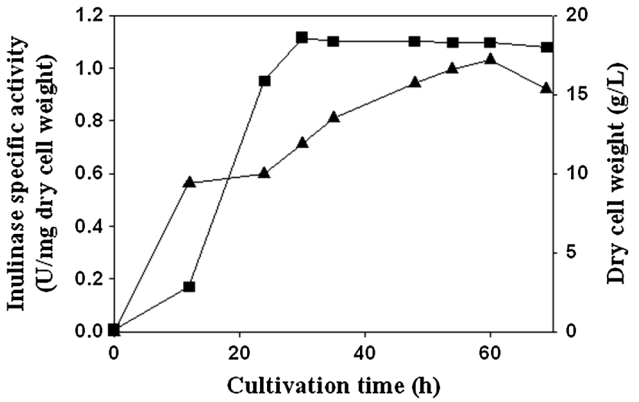

Fig. 6 Total specific activity change of KmINUp from *S. cerevisiae* D452-2/p426PM with cultivation time (*filled triangle* total specific activity, *filled square* dry cell weight)

environmentally friendly nature. Among potential feedstocks for bioethanol production, inulin is very promising since energy crops such as Jerusalem artichoke can be cultivated in non-arable area. Our strategy was the introduction of the *K. marxianus* inulinase gene, into an industrial workhorse, *S. cerevisiae*, for bioethanol production. Optimization of the promoter and signal sequence for expression of the *K. marxianus* inulinase gene revealed that the inulinase activity of *S. cerevisiae* carrying the PGK1 promoter and the MFα1 signal sequence was the highest. In correlation with the enzymatic activity result, *S. cerevisiae* carrying the PGK1 promoter and the MFα1 signal sequence showed the best inulin fermentation capability in CBP using inulin as a carbon source. Inulin consumption rate and ethanol productivity of the selected yeast strain were dramatically improved as compared to other recombinant yeast strains (Fig. 8). This indicates that the optimized combination of the promoter and secretory signal sequence for the expression of the *K. marxianus* inulinase gene enhanced the production of ethanol through hydrolysis of inulin using CBP. Finally, batch fermentation of the recombinant *S. cerevisiae* in a bioreactor resulted in 0.43 g ethanol/g inulin of ethanol yield and 1.22 g/L h of ethanol productivity. More research is in progress to accelerate the

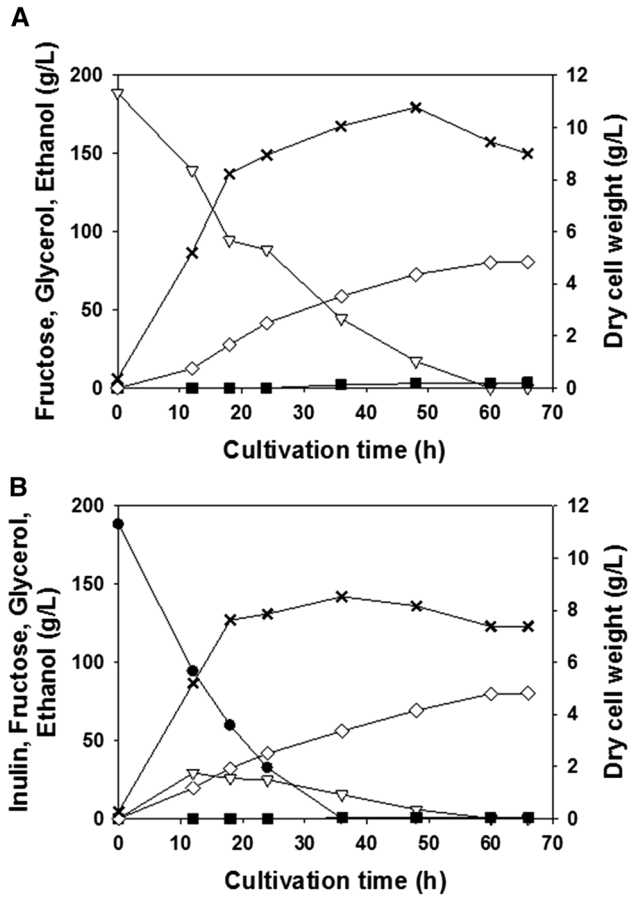

Fig. 7 Bioreactor fermentation profiles of **a** wild-type *S. cerevisiae* D452-2 with 180 g/L fructose and **b** *S. cerevisiae* D452-2/p426PM with 180 g/L inulin (*filled circle* inulin, *inverted unfilled triangle* fructose, *filled square* glycerol, *unfilled diamond* ethanol, *cross symbol* dry cell weight)

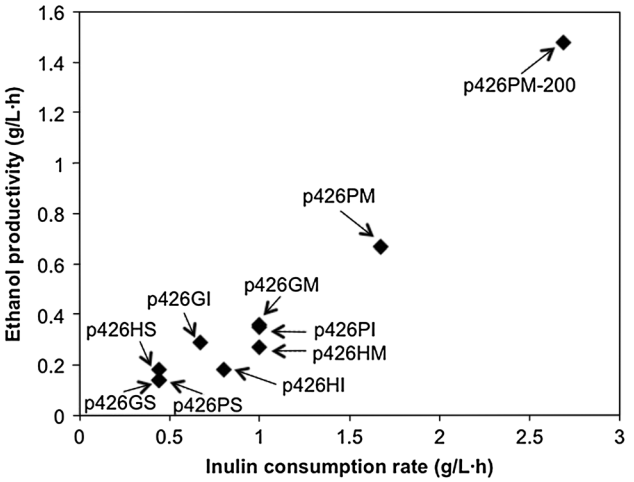

Fig. 8 Correlation between ethanol productivity (g/L h) and inulin consumption rate (g/L h) for the nine recombinant yeast strains. Each fermentation data point represents a yeast strain carrying a depicted plasmid. The data point of 426PM-200 represents a fermentation result of D452-2/p426PM with 200 g/L inulin

fructose uptake rate by introducing the fructose-specific transporter genes to *S. cerevisiae*.

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