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Expression of the inulinase gene from the marine-derived *Pichia guilliermondii* in *Saccharomyces* sp. W0 and ethanol production from inulin

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Summary

It has been confirmed that *Saccharomyces* sp. W0 can produce high concentration of ethanol. In this study, the *INU1* gene cloned from the marine-derived *Pichia guilliermondii* was transformed into uracil mutant of *Saccharomyces* sp. W0. The positive transformant Inu-66 obtained could produce 34.2 U ml⁻¹ of extracellular inulinase within 72 h of cultivation. It was found that 15.2 U of inulinase activity per one gram of inulin was suitable for inulin hydrolysis and ethanol production by the transformant Inu-66. During the small-scale fermentation, 13.7 ml of ethanol in 100 ml of medium was produced and 99.1% of the added inulin was utilized by the transformant. During the 2 l fermentation, 14.9% (v/v) of ethanol was produced from inulin and 99.5% of the added inulin was converted into ethanol, CO₂ and cell mass.

Introduction

Ethanol is the most used liquid biofuel either as a fuel or as a gasoline enhancer (Sanchez and Cardona, 2008). It has been reported that ethanol has greater octane booster properties, is not toxic, and does not contaminate water sources. It is also an excellent raw material for synthetic chemicals. Currently, *Saccharomyces cerevisiae* is the main producer of bioethanol as it can produce high concentration of ethanol from glucose and sucrose and has high ethanol tolerance (Hirasawa *et al.*, 2007; Abe *et al.*, 2009). The price of the sugar source is a very

important process parameter in determining the overall economy of ethanol production, starch is a good substrate for ethanol production and other fermentation products on a large scale due to its low price and easily available raw material in most regions of the world (Chi *et al.*, 2003; Gupta *et al.*, 2003). In the course of conventional enzymatic saccharification by amylases, starch must be gelatinized at high temperature and pressure. The gelatinized starch is then liquefied with high temperature α -amylase, followed by the saccharification with glucoamylase at a much lower temperature of 50–60°C. In the case of ethanol fermentation by using *S. cerevisiae*, temperature of the saccharified slurry is further decreased to around 30°C. Therefore, the conventional enzymatic saccharification by amylases has many disadvantages and the process is very complicated. Inulin is present as a reserve carbohydrate in the roots and tubers of plants such as Jerusalem artichoke, chicory, dahlia and yacon. The yields of the roots and tubers are very high. The dried materials of the tubers contain over 50% inulin (Pandey *et al.*, 1999). Inulin consists of linear chains of β -2, 1-linked D-fructofuranose molecules terminated by a glucose residue through a sucrose-type linkage at the reducing end. Such inulin sources have recently received increasing attention as a renewable raw material for fructose syrup production, ethanol fermentation, and inulooligosaccharide (IOS) production (Chi *et al.*, 2009). The exoinulinases catalyse removal of the terminal fructose residues from the non-reducing end of the inulin molecule in one step, producing fructose as main products and glucose as minor products, which can be easily converted into ethanol by *S. cerevisiae*. Furthermore, inulin is easy to be dissolved in water and the solution with high concentration of inulin has low viscosity. Therefore, it is a better material for ethanol production than starch. The extract from Jerusalem artichoke tuber was hydrolyzed and the hydrolyzed Jerusalem artichoke was used as the substrate for ethanol production by *S. cerevisiae* strain (Remize *et al.*, 1998). Ethanol was also produced from Jerusalem artichoke mashed tubers using *Klyuveromyces fragilis*, a yeast with an active inulinase, together with either a commercial distillery yeast, *S. cerevisiae* or the bacterium *Zymomonas mobilis* (Szambelan *et al.*, 2004). The exoinulinases and endoinulinases produced by

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Aspergillus niger mutant 817 were used in the simultaneous saccharification and fermentation of pure inulin and Jerusalem artichoke tuber to ethanol in combination with an ethanol tolerant strain *S. cerevisiae* 1200 (Nakamura *et al.*, 1996). In our previous studies (Gong *et al.*, 2007), we found that over 61.5 ± 0.4 U ml⁻¹ of inulinase activity is produced by the marine-derived *Pichia guilliermondii* strain 1. The inulinase has been purified (Gong *et al.*, 2008) and characterized, and the gene encoding the inulinase (*INU1* gene) has been cloned, characterized and overexpressed (Zhang *et al.*, 2009). *Saccharomyces* sp. W0 can produce high concentration of ethanol from glucose and sucrose and has high ethanol tolerance (Chi and Liu, 1993; Chi *et al.*, 1999). Therefore, the main aims of this present study were to express the *INU1* gene in *Saccharomyces* sp. W0, and the inulinase-producing transformant obtained was used to produce ethanol from inulin directly in one-step fermentation.

Results

Construction of the expression vector carrying the inulinase gene and transformation

The *INU1* gene encoding the extracellular inulinase was amplified from the genomic DNA of *P. guilliermondii* strain 1 by PCR and inserted into the expression vector YCplac33 PGK/CYC1. The resulting plasmid was named YCplac33-*INU1*. The construction of the expression vector carrying the inulinase gene is shown in Fig. 1. From this figure, it can be seen that the recombinant plasmid contains the constitutive promoter PGK and the effective terminator CYC1. Therefore, the cloned inulinase gene can be expressed constitutively in *S. cerevisiae* and the gene expression can be effectively terminated. The expression vector also contains CEN4 sequence so that the vector in the host can be distributed in each daughter cell during the vegetative growth. The recombinant plasmid was transformed into the uracil

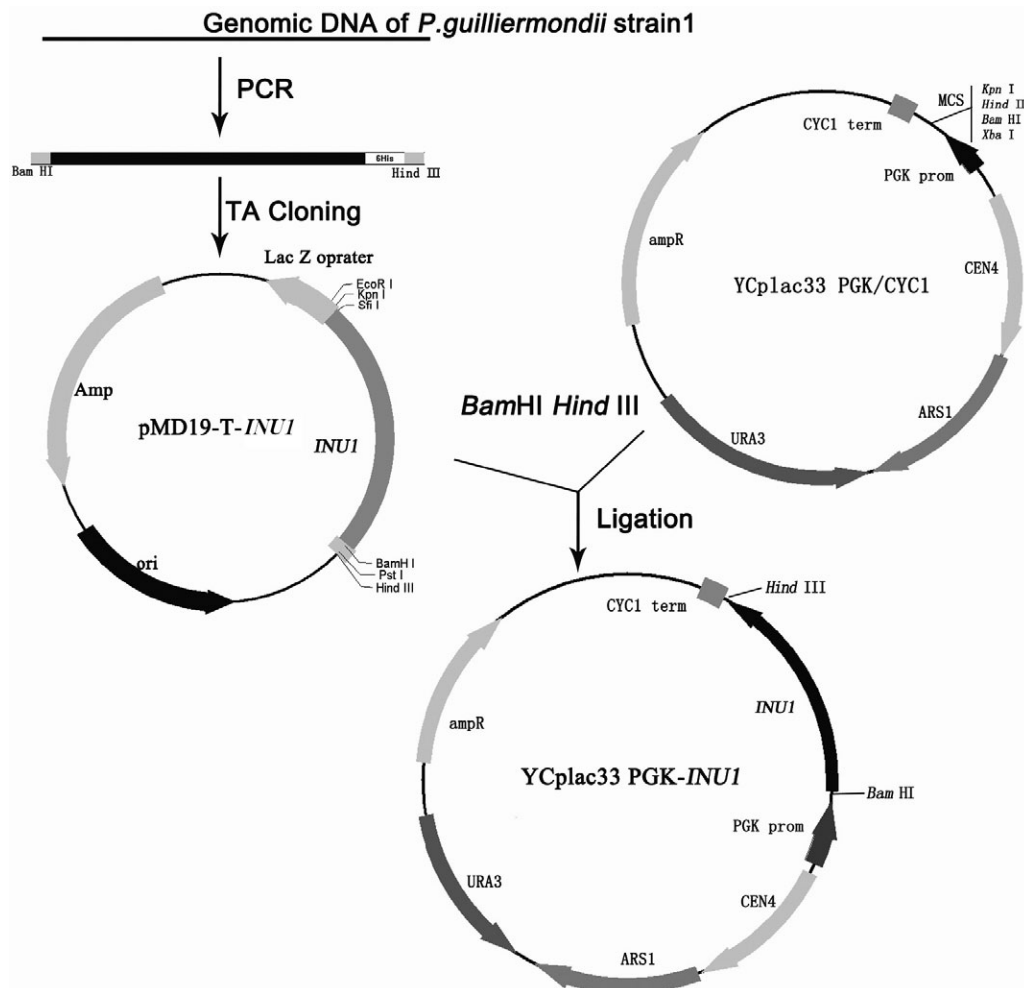


Fig. 1. Construction of the recombinant plasmid YCplac33 PGK-*INU1*.

Table 1. The inulinase activity of the different transformants.

Transformants	Inulinase activity (U ml ⁻¹)	Transformants	Inulinase activity (U ml ⁻¹)
Inu-2	19.6 ± 0.3	Inu-31	20.8 ± 0.3
Inu-6	24.0 ± 0.4	Inu-33	16.6 ± 0.4
Inu-7	28.9 ± 0.3	Inu-36	20.8 ± 0.3
Inu-8	21.8 ± 0.2	Inu-39	29.2 ± 0.3
Inu-9	31.6 ± 0.5	Inu-40	17.11 ± 0.43
Inu-10	23.67 ± 0.19	Inu-47	21.22 ± 0.21
Inu-11	26.78 ± 0.32	Inu-48	30.78 ± 0.53
Inu-16	27.89 ± 0.47	Inu-52	20.44 ± 0.26
Inu-17	20.22 ± 0.55	Inu-63	18.66 ± 0.35
Inu-18	17.78 ± 0.36	Inu-66	33.67 ± 0.42
Inu-22	21.89 ± 0.41	Inu-72	28.56 ± 0.28
Inu-25	17.00 ± 0.31	Inu-95	30.44 ± 0.39
Inu-26	31.22 ± 0.35	Inu-97	19.78 ± 0.45
Inu-27	26.55 ± 0.33	Inu-102	26.56 ± 0.33
<i>Saccharomyces</i> sp. W0	0.0		

Data are the mean standard derivatives ($n = 3$).

mutant *Saccharomyces* sp. W101. After determination of extracellular inulinase activity from over 100 positive transformants, the results in Table 1 indicate that the inulinase activity was in the range of 17 and 33 U ml⁻¹, while no inulinase activity of *Saccharomyces* sp. W0 was detected. The results in Table 1 also show that the inulinase activity produced by the transformant Inu-66 was the highest (33.67 ± 0.42 U ml⁻¹). Therefore, it was used in the subsequent investigation. The results in Fig. 2 confirm that the cloned *INU1* gene was amplified from the genomic DNA of the transformant Inu-66 by PCR and no such PCR products were obtained from the genomic DNA of *Saccharomyces* sp. W0. This means that the transformant Inu-66 indeed carried the cloned *INU1* gene.

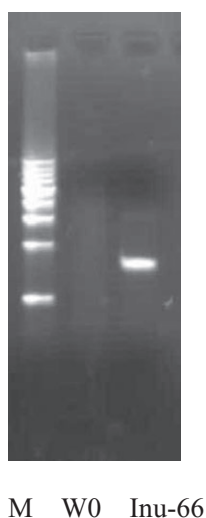


Fig. 2. PCR products of *Saccharomyces* sp. W0 and the transformant Inu-66. M, DNA markers; W0, PCR products from genomic DNA of *Saccharomyces* sp. W0; Inu-66, PCR products from genomic DNA of the transformant Inu-66.

Time-course of cell growth and inulinase production by the transformant Inu-66

During cell growth in the inulinase production medium, inulinase activity in the supernatant and cell mass was measured. The results in Fig. 3 show that the highest inulinase activity (34.2 U ml⁻¹) of the transformant Inu-66 was reached within 72 h of the cell cultivation when cell growth reached the end of log phase.

Effects of different inoculation size on CO₂ liberation during the fermentation

During ethanol fermentation by *Saccharomyces* spp., 1 mol of glucose is converted to 2 mol of CO₂, and 2 mol of CH₃-CH₂OH and CO₂ will be released from the fermentation system. The molecular weight (44) of CO₂ is almost the same as that (46) of CH₃-CH₂OH. Therefore, the lost weight (CO₂) was used to estimate the weight of ethanol

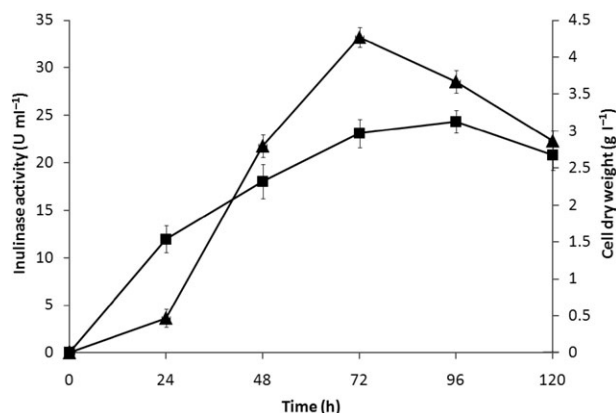


Fig. 3. Time-course of cell growth (■) and inulinase production (▲) by the transformant Inu-66. Data are the mean standard derivatives ($n = 3$).

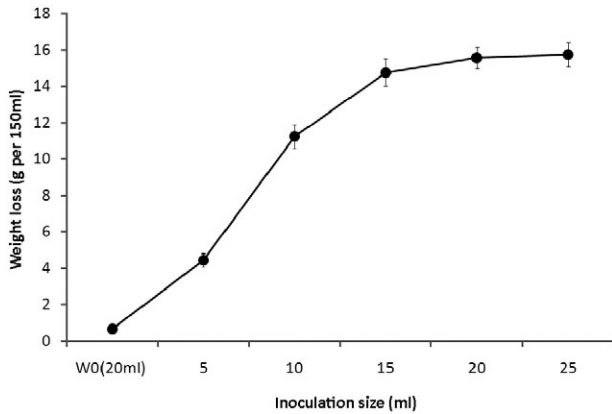


Fig. 4. Effects of different inoculation size on CO₂ liberation during the fermentation. Data are the mean standard derivatives ($n = 3$).

produced in the closed fermentation system. This was the basis of carbon dioxide quantification in this study and CO₂ evolved was monitored by the decrease in weight of the whole culture. Different volumes of the culture of the transformant Inu-66 grown in the inulinase production medium for 72 h were inoculated into 150 ml of the ethanol fermentation medium and CO₂ liberation (weight loss) during the fermentation was monitored. The results in Fig. 4 reveal that when the inoculation size was increased from 5 ml to 20 ml, CO₂ liberation was also continuously increased. However, when the inoculation size was increased from 20 to 25 ml, CO₂ liberation was not further increased. This means that the optimal inoculation size was 20 ml of the culture per 150 ml of the ethanol fermentation medium. This also indicates that 15.2 U of inulinase activity per one gram of inulin was suitable for inulin hydrolysis and ethanol production by the transformant Inu-66 as 20 ml of the culture contained 684 U of inulinase activity and the total grams of inulin in 150 ml of the fermentation medium were 45. However, growth of *Saccharomyces* sp. W0 in the ethanol fermentation medium only caused a small amount of weight loss because it had no inulinase activity (Table 1 and Figs 3 and 4).

Analysis of the fermented media during the small-scale fermentation

When the small-scale fermentation was finished, residual total sugar, residual reducing sugar and ethanol

Table 2. Analysis of the media before and after the fermentation.

Yeast strains	Ethanol concentration (% v/v)	Initial inulin concentration (% w/v)	Initial concentration of reducing sugar (% w/v)	Concentration of residual reducing sugar (% w/v)	The concentration of residual total sugar (% w/v)
<i>Saccharomyces</i> sp. W0	0.6 ± 0.1	30	1.1 ± 0.3	0.04 ± 0.01	28.7 ± 0.2
Transformant Inu-66	13.7 ± 0.5	30	1.0 ± 0.4	0.06 ± 0.01	0.3 ± 0.1

Data are the mean standard derivatives ($n = 3$).

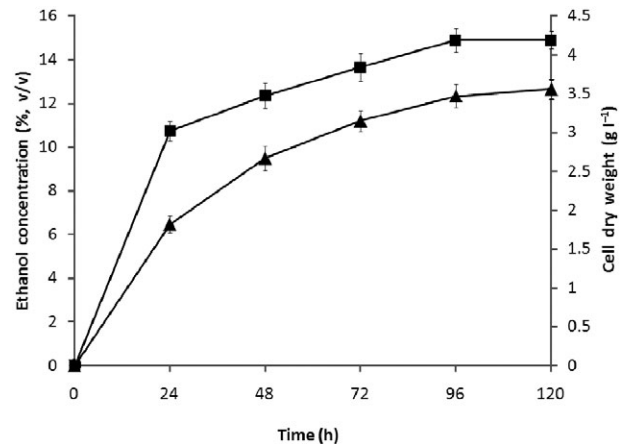


Fig. 5. Time-course of ethanol production (■) and cell growth (▲) during the 2 l fermentation. Data are the mean standard derivatives ($n = 3$).

concentration were analysed as described in *Experimental procedures*. It can be seen from the data in Table 2 that the transformant Inu-66 could produce 13.7 ml of ethanol in 100 ml of medium and 99.1% of the added total sugar was utilized by the transformant while *Saccharomyces* sp. W0 only produced 0.57 ml of ethanol in 100 ml of the medium and 4.3% of the added total sugar was utilized by the wild-type strain. This suggests that most of the added sugar (inulin) can be used for ethanol production and cell growth by the transformant Inu-66 while only a small amount of the added sugar was used by *Saccharomyces* sp. W0 because it had no inulinase activity (Table 1). This is the first report that the inulinase gene cloned from the marine-derived yeast strain was well expressed in *Saccharomyces* sp. W0 and the recombinant yeast was used to produce ethanol from inulin directly.

Ethanol production during 2 l fermentation

Subsequently, ethanol production and cell growth by the transformant Inu-6 were carried out in 2 l fermentor, the results in Fig. 5 show that the fermentation period was 96 h, and the final ethanol concentration in the fermented medium was 14.9% (v/v) when the cell growth reached the end of log phase. This indicates that more ethanol was produced during the 2 l fermentation than during the

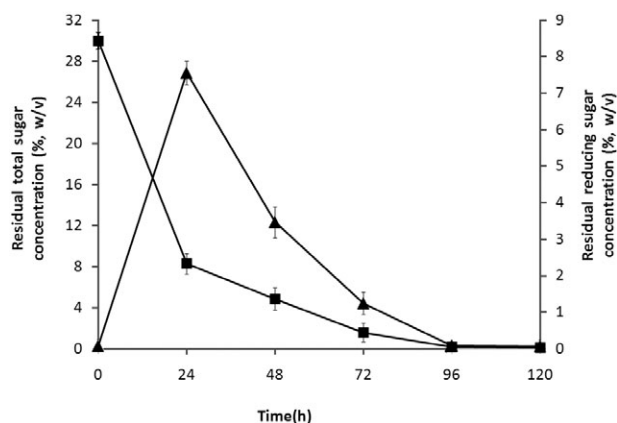


Fig. 6. The time-course of residual total sugar change (■) and residual reducing sugar change (▲) during the 2 l fermentation. Data are the mean standard derivatives ($n = 3$).

small-scale fermentation. At the end of the fermentation, 0.09% (w/v) of reducing sugar and 0.15% (w/v) of total sugar were left in the fermented media, indicating that 99.5% of added inulin was converted into ethanol, CO₂ and cell mass (Fig. 6). This means that the engineered *Saccharomyces* sp. W0 carrying the *INU1* gene cloned from the marine-derived yeast has high potential application in ethanol production from inulin extracted from the non-food material.

Discussion

In recent years, due to the rising grain prices, many countries have been concerned that further promoting ethanol using food grains as feedstocks will contribute to persistent high prices of food (Li and Chan-Halbrendt, 2009). Therefore, the feedstocks that are non-food grain that could sustainability grow on marginal and abandon lands are being used for ethanol production. Inulin and inulin-containing materials such as Jerusalem artichoke tubers are ones of such materials. However, *S. cerevisiae* cannot synthesize and secrete inulinase that hydrolyze inulin into fructose and glucose (Chi *et al.*, 2009). Therefore, in this study, the inulinase gene cloned from the marine-derived yeast was transformed into the cells of *Saccharomyces* sp. W0, which has been confirmed to be able to produce high concentration of ethanol (Chi and Liu, 1993; Chi *et al.*, 1999) (Figs 1 and 2). The cloned gene could be expressed well in the cells and the expressed inulinase could be secreted into the medium (Table 1 and Fig. 3). When the cell cultures containing high activity of inulinase were used to both hydrolyze inulin and produce ethanol from the hydrolysate, 14.9% (v/v) ethanol was produced and 99.5% of added inulin was utilized by the cells (Figs 5 and 6) during the 2 l fermentation. In our previous studies (Chi and Liu, 1993),

it was found that the tetraploid fusant strain between *Saccharomyces* sp. W0 and *S. cerevisiae* 1200, *Saccharomyces* sp. W0 and *S. cerevisiae* 1200 could produce 18.8% (v/v), 17.7% (v/v) and 18.7% (v/v) ethanol from hydrolysate of raw corn starch, respectively. It was also found that *Saccharomyces* sp. W0 could yield 16.3% (v/v) ethanol from sucrose by the fed-batch fermentation (Chi *et al.*, 1999). This suggests that *Saccharomyces* sp. W0 could produce more ethanol from hydrolysate of corn starch and sucrose than the engineered yeast from inulin. However, as mentioned above, corn starch is not suitable for ethanol production in developing country like China and corn starch must be hydrolyzed into glucose that then is converted into ethanol by *S. cerevisiae* using many amylases (Li and Chan-Halbrendt, 2009). *Aspergillus niger* 12 had been shown to produce extracellular exo-inulinases and endo-inulinases constitutively. The *A. niger* mutant 817 from *A. niger* 12 was found to have fourfold higher inulinase activity than the wild-type strain in the submerged culture. Therefore, *A. niger* 817 was used in the simultaneous saccharification and fermentation of pure inulin to ethanol in combination with an ethanol tolerant strain *S. cerevisiae* 1200. This single-step process permits the production of high concentrations of ethanol (20–21%, v/v) from chicory and dahlia inulins as model substrates in 3 days (Nakamura *et al.*, 1996). This suggests that the one step ethanol production process from inulin developed in this study has still potential for further improvement.

Experimental procedures

Gene, plasmids and yeast strains

The *INU1* gene was amplified from the genomic DNA of *Pichia guilliermondii* strain 1 according to the methods described by Zhang and colleagues (2009). The expression vector YCPlac33 PGK/CYC1 in *S. cerevisiae* was kindly supplied by Dr Jean-Luc Parrou at INSA-Toulouse, France. *Saccharomyces* sp. W0, which is a high ethanol-producing yeast (Chi and Liu, 1993), was used as the producer of ethanol in this study. *P. guilliermondii* strain 1 was isolated from the surface of a marine alga (Gong *et al.*, 2007). The two-yeast strains were preserved at -80°C in this laboratory. The uracil mutant *Saccharomyces* sp. W101 was isolated from *Saccharomyces* sp. W0 as described below.

Media

Growth medium was yeast-peptone-dextrose medium containing 2.0% (w/v) glucose, 2.0% (w/v) peptone, 1.0% (w/v) yeast extract. The inulinase production medium contained 2.0% (w/v) inulin, 1.0% (w/v) yeast extract and 2.0% (w/v) polypeptone. The ethanol fermentation medium was composed of 30.0% inulin (w/v) and 4.0% (w/v) of the hydrolysate of soybean cake.

Preparation of the hydrolysate of soybean meal

The hydrolysate of soybean meal was prepared as described by Chi and colleagues (2003).

Isolation of DNA, restriction digestions and transformation

DNA manipulations were carried out using the standard methods (Sambrook *et al.*, 1989). Bacterial plasmid DNA was purified using Perfectprep plasmid minikits (Eppendorf). Yeast genomic DNA for amplification of the extracellular inulinase gene in *P. guilliermondii* strain 1 was isolated as described by Sambrook and colleagues (1989). Restriction endonuclease digestions and DNA ligations were performed according to the manufacturer's recommendations. *Escherichia coli* was transformed with plasmid DNA according to Sambrook and colleagues (1989). The *E. coli* transformants were plated out onto LB medium containing ampicillin (100 µg ml⁻¹). *Saccharomyces* sp. W101 was transformed with the YCPlac33 PGK/CYC1 carrying the *INU1* gene encoding inulinase.

Isolation of uracil mutants

The uracil mutant of *Saccharomyces* sp. W0 was isolated as described by Wang and colleagues (2009). One of the mutants was named *Saccharomyces* sp. W101.

Construction of the expression vector carrying the *INU1* gene and transformation

To obtain the *INU1* gene encoding the extracellular inulinase, the genomic DNA of *P. guilliermondii* strain 1 was extracted and the *INU1* gene was amplified using the genomic DNA as template and the primers (the forward primer Epu: 5'-GGATCCATGAGAGCTTTTCTTGCCTTAATT-3', underlined bases encode *Bam*HI; the reverse primer Epd: 5-**AAGCTTTCAGTGATGGTGATGGTGATGCTATGAAGTTGCCCTCAA**-3', underlined and bold bases encode *Hind*III and underlined and italic bases encode 6 his tag), which were designed according to the cloned inulinase gene (the Accession number: EU195799) and multi-cloning sites of the expression vector YCPlac33 PGK/CYC1. The reaction system (50 µl) was composed of 5.0 µl of 10× buffer, 1.0 µl (10 mM) of dNTPs, 5.0 µl (50 mM) of Epu, 5.0 µl (50 mM) of Epd, 0.5 µl of Taq DNA polymerase, 2.0 µl (10.0 ng ml⁻¹) of the template DNA and 28.5 µl of H₂O. The conditions for the PCR amplification were as follows: initial denaturation at 94°C for 8 min, denaturation at 94°C for 1 min, annealing temperature at 48–51°C for 1 min, extension at 72°C for 1 min, final extension at 72°C for 8 min. PCR was run for 32 cycles and the PCR cycler was GeneAmp PCR System 2400 (PerkinElmer, Waltham, MA, USA). The PCR products amplified were ligated into pMD19-T with restriction sites of *Bam*HI and *Hind*III to generate construct pMD19-T-*INU1*. The recombinant plasmid was transformed into *E. coli* DH5a. The recombinant plasmids extracted from the positive transformants were digested with the restriction enzymes *Bam*HI and *Hind*III, and the digests carrying the *INU1* gene were ligated

into YCPlac33 PGK/CYC1 digested with the same restriction enzymes. The resulting plasmid was designated as YCPlac33 PGK/CYC1-*INU1* (Fig. 1). The recombinant plasmid was transformed into *E. coli* DH5a. The recombinant plasmids extracted from the positive transformants were used to transform the competent cells of the uracil mutant *Saccharomyces* sp. W101, and different positive transformants were selected from YNB medium with 2.0% glucose and 1.0% ammonium sulfate.

Preparation of the crude recombinant inulinase

The different transformants with the cloned *INU1* gene were cultivated in 100 ml of the inulinase production medium (pH 5.0) by shaking at 250–300 r.p.m. and 28°C for 72 h. The cultures were centrifuged at 5000 g and 4°C for 10 min and the inulinase activity in the supernatant was determined as described below. After determination of inulinase activity from over 100 transformants, it was found that the transformant Inu-66 could produce the highest level of the extracellular inulinase (Table 1). Therefore, it was used in the subsequent investigation.

Determination of the recombinant inulinase activity

The recombinant inulinase activity in the supernatant obtained above was determined according to Gong and colleagues (2007). The amount of reducing sugar in the reaction mixture was assayed by the method of Nelson–Somogyi (Spiro, 1966). One inulinase unit (U) was defined as the amount of enzyme that produces 1 µmol of reducing sugar per minute under the assay conditions used in this study.

Ethanol fermentation

Saccharomyces sp. W0 and the transformant Inu-66 mentioned above were grown at 28°C and shaking speed of 250 r.p.m. in the inulinase production medium (pH 5.0) for 72 h. The different volumes of the culture (1 × 10⁸ cells ml⁻¹) were transferred to a 250 ml bottle with the final volume of 150 ml of the ethanol production medium (pH 5.0) containing 30.0% (w/v) inulin and 4.0% (w/v) of hydrolysate of soybean meal. The bottles were fitted with rubber bungs perforated by a needle and incubated statically at 28°C. The loss of weight by CO₂ liberation during the fermentation was monitored daily until fermentation ceased. The final ethanol concentration, residual reducing sugar and residual total sugar in the fermented media were determined as described below.

Two-litre fermentation was carried out in a Biostat B2 2 l fermentor (B. Braun, Germany) equipped with baffles, a stirrer, alkali pump, heating element, oxygen sensor and temperature sensor. Two hundred and fifty millilitres of the seed culture grown in the inulinase production medium (pH 5.0) at 28°C and at shaking speed of 250 r.p.m. for 72 h was transferred into 1500 ml of the hydrolysate of soybean cake containing 495 g of inulin. The fermentation was performed under the conditions of 28°C for 120 h without aeration and agitation.

Ethanol assay

One hundred millilitres of the fermented medium and 100 ml of tap water were mixed thoroughly. The mixture was distilled

at 100°C and 100 ml of the distillate was collected. The distillate was diluted to 1000-fold. The ethanol concentration in the dilute was determined by using Gas Chromatography (HP5890II, Hewlett-Packard, USA); The chromatography column was a fused silica AC-20 capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) and column temperature was 60°C; detector: FID; detector temperature was 150°C; injector temperature: 150°C; carrier gas: helium, 1.0 ml min⁻¹; injection volume: 1.0 μl; the concentration of standard ethanol was 1.0 μl ml⁻¹.

Determination of reducing sugar and total sugar in the fermented media

Reducing sugar in the initial media and fermented media was determined by the Nelson-Somogyi method (Spiro, 1966). Total sugar was measured as reduction of sugar after hydrolysis of the initial and fermented media (Chi *et al.*, 2003).

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