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Recombinant Production of an Inulinase in a *Saccharomyces cerevisiae* gal80 Strain

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The inulinase gene (INU1) from *Kluyveromyces marxianus* NCYC2887 was overexpressed by using the GAL10 promotor in a Δgal80 strain of *Saccharomyces cerevisiae*. The inulinase gene lacking the original signal sequence was fused in-frame to a mating factor α signal sequence for secretory expression. Use of the Δgal80 strain allowed for the galactose-free induction of inulinase expression using a glucose-only medium. Shake-flask cultivation in YPD medium produced 34.6 U/ml of the recombinant inulinase, which was approximately 13-fold higher than that produced by *K. marxianus* NCYC2887. It was found that the use of the Δgal80 strain improved the expression of inulinase in the recombinant *S. cerevisiae* in both aerobic and anaerobic conditions by about 2.9- and 1.7-fold, respectively. A 5-l fed-batch fermentation using YPD medium was performed under aerobic condition with glucose feeding, which resulted in the inulinase production of 31.7 U/ml at the OD_{600} of 67. Ethanol fermentation of dried powder of Jerusalem artichoke, an inulin-rich biomass, was also performed using the recombinant *S. cerevisiae* expressing INU1 and *K. marxianus* NCYC2887. Fermentation in a 5-l scale fermentor was carried out at an aeration rate of 0.2 vvm, an agitation rate of 300 rpm, and with the pH controlled at 5.0. The temperature was maintained at 30°C and 37°C, respectively, for the recombinant *S. cerevisiae* and *K. marxianus*. The maximum productivities of ethanol were 59.0 and 53.5 g/l, respectively.

Keywords: Inulinase, recombinant, *Saccharomyces cerevisiae*, Δgal80, Jerusalem artichoke

Microbial inulinases are an important class of industrial enzymes, which are widely used in the food and pharmaceutical industries [10]. Inulin is a fructan oligosaccharide composed of α-D-glucopyranosyl-[β-(2,1)-D-fructofuranosyl-D-fructofuranosides]. Inulin consists of a linear β-2,1-linked polyfructose chain, terminated by a glucose residue through a sucrose-type linkage at the reducing end, and it is the major storage carbohydrate in the roots and tubers of plants such as Jerusalem artichoke, chicory, dahlia, and yacon [2, 13]. Inulinase can be divided into exoinulinase and endoinulinase. Exoinulinases (β-D-fructan fructohydrolase; E.C. 3.2.1.80) consecutively cut off terminal fructose units from the nonreducing end of inulin. Endoinulinases (2,1-β-D-fructan fructanohydrolase; E.C. 3.2.1.7) hydrolyze the internal β-2,1-fructofuranosidic linkages and produce such main products as inulotriose, inulotetraose, and inulopentaose [1]. Inulinase can be produced by different microorganisms, including fungi, yeasts, and bacteria. Among them, *Kluyveromyces* sp. (*Kluyveromyces marxianus* and *Kluyveromyces fragilis*) are often used for commercial applications [2, 13].

Recently, an exoinulinase gene (INU1) of *K. marxianus* was cloned and expressed in *Saccharomyces cerevisiae* [7], and the inulinase of *K. marxianus* has been purified and characterized [5]. The INU1 gene encodes a 555 amino acids polypeptide and consists of a signal peptide (23 amino acids). When the inulinase gene of *K. marxianus* was expressed in *S. cerevisiae*, the majority of the inulinase activity was found in the extracellular medium [9]. However, detailed studies for the recombinant production of inulinase in *S. cerevisiae* have been rarely described, especially for the practical aspects of industrial production. In this study, the INU1 gene isolated from *K. marxianus* NCYC2887 was cloned into *S. cerevisiae* Δgal80 that enabled the glucose-only fermentation of the transformant for the expression under a GAL10 promoter. The effect of Δgal80 mutation on the inulinase productivity under aerobic and anaerobic fermentation conditions was investigated. The recombinant *S. cerevisiae* strain expressing inulinase was also employed for ethanol fermentation using Jerusalem artichoke.

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Materials and Methods

Strains and Plasmids

The *Kluyveromyces marxianus* NCYC2887 strain was purchased from the National Collection of Yeast Cultures (Norwich, UK). The expression vector pYEGα and the host strain *Saccharomyces cerevisiae* Y2805 (*MATα pep::HIS3 prb-Δ1.6R can1 his3-20 ura3-52*) and its Δgal80 mutant strain were as previously described [3, 14, 15].

Inulinase Gene Cloning and Expression Vector Construction

Genomic DNA from *K. marxianus* NCYC2887 was isolated and used as a template [11]. The inulinase gene of *K. marxianus* NCYC2887 was amplified using PCR primers, which were designed based on the other *K. marxianus* inulinase gene sequences from GenBank. The forward primer, pYEG Inu1F (5'-AAAATCTAGATAAGAGAGATGGTGACAGCAAGGC-3') incorporating an *Xba*I restriction site (underlined), and the reverse primer pYEG Inu1R (5'-AAAAGTCGACGCGGCCGCTCAAACGTTAAATTGGGTA-3') having a *Sal*I restriction site (underlined), were used for PCR. The PCR reaction was performed using an I-MAX II 2× PCR Master mix Solution kit (Intronbio, Korea). The PCR reaction mixture (50 µl) contained 25 µl of 2× PCR Master mix solution, 1.0 µl DNA template, 1.5 µl pYEG Inu1F (10 pmol), 1.5 µl pYEG Inu1R (10 pmol), and 21 µl of distilled water. The conditions for the PCR amplification were initial denaturation at 94°C for 1 min, denaturation at 94°C for 30 s, annealing at 55°C for 30 min, extension at 72°C for 1 min 45 s, and a final extension at 72°C for 10 min. The PCR was run for 25 cycles using a TaKaRa PCR Thermal Cycler DICE TP600 (Takara, Japan). The PCR products were cloned into the T&A cloning vector (RBC Bioscience, Taiwan) and the nucleotide sequences of the inserts were determined.

The PCR fragment of the INU1 gene was designed to exclude the original signal sequence of the inulinase. The PCR product was digested with *Xba*I and *Sal*I and subcloned into the *Xba*I/*Sal*I site to include the secretion sequence of the expression vector (MFα signal sequence of *S. cerevisiae*) [3], which was designated as pYEGα-Inu1. The plasmid was transformed into the *S. cerevisiae* Y2805 and Δgal80 strain using the LiAc/TE method [12]. The cells were spread onto UD agar medium (0.67% yeast nitrogen base without amino acid, 0.077% Ura$^-$ DO supplements, 2% glucose, 2% bacto-agar, pH 5.6 ~6.0) for selection of transformants.

Enzyme Assay

Enzyme activities were assayed by measuring the concentration of reducing sugars released from inulin. The reaction mixture containing 50 µl of enzyme solution and 500 µl of 1% (w/v) inulin (from chicory) (Sigma, USA) in 0.2 M sodium acetate buffer (pH 5.2) was incubated at 50°C for 10 min. The enzyme was inactivated by boiling the reaction mixture at 100°C for 5 min. The increase in reducing sugar was estimated by the 3,5-dinitrosalicylic acid method [8]. The optical densities of the solutions at 520 nm were used to construct the standard curve. One inulinase unit was defined as the amount of enzyme that produced one micromole of fructose equivalent from inulin per minute under standard assay conditions.

Analytical Methods

SDS−PAGE was performed using 10% polyacrylamide gel containing 0.1% SDS, according to the method of Laemmli [6]. After electrophoresis, the gels were stained with Coomassie Blue R-350 and a protein marker (SeeBlue plus2 pre-stained standard; Invitrogen, USA) was used as the molecular mass marker. Activity staining of the inulinase on the gel was done as previously described [4]. After electrophoresis, the gel was treated with 1% Triton X-100 (Sigma, USA) for 1 h to remove SDS, followed by soaking in 1% inulin dissolved in 0.2 M sodium phosphate buffer (pH 5.2) for 30 min at 50°C. After reaction, the gel was heated in 0.1% 2,3,5-triphenyl tetrazolium chloride (TTC) in 0.5 M NaOH. The band showing inulinase activity appeared red.

Oligosaccharide chains from both recombinant and wild-type inulinases were removed by endoglycosidase H (Endo-H) (New England Biolabs, USA). Inulinase samples were mixed with glycoprotein denaturing buffer and heated at 100°C for 10 min. Endo-H and 0.5 M sodium citrate buffer (pH 5.5) were added to the inulinases, and the solutions were incubated at 37°C for 2 h.

The ethanol and sugar concentrations in the supernatant were measured by high-pressure liquid chromatography (Gilson, Middleton, WI, USA) using an HPX87H column (Bio-Rad, Hercules, CA, USA) and an ERC-7515A RI detector (ERC, Tokyo, Japan).

Aerobic Fed-Batch Fermentation Using Δgal80 Strain

For the 5-l fed-batch fermentation, a seed culture was grown in 200 ml of YPD broth (10 g/l yeast-extract, 20 g/l Bacto-peptone, and 20 g/l glucose) overnight at 30°C and was inoculated into a 5-l jar fermentor (KoBiotech, Seoul, Korea) containing 1.8 l of medium containing (per liter): 100 g glucose, 40 g yeast extract, and 10 g Bacto-peptone. The pH and temperature were adjusted to 6.0 and 30°C, respectively. When the initially added glucose was completely consumed, a feeding medium containing 600 g/l glucose was supplied. The hourly feeding rate was regulated according to the glucose concentration in the culture medium. To determine the residual glucose concentrations, 1 ml of culture broth was centrifuged and the glucose concentration in the supernatant was measured using a glucose and lactate analyzer (YSI Model 2000; Yellow Springs, OH, USA).

Ethanol Fermentation from Jerusalem Artichoke Flour

The *K. marxianus* NCYC2887 strain and the recombinant *S. cerevisiae* strains were grown in 500-ml flasks containing 50 ml of YPD medium and incubated at 30°C for 16 h with shaking at 180 rpm. The precultured cells were then inoculated into a 5-l jar fermentor with a working volume of 2 l containing 180 g/l of Jerusalem artichoke flour. The fermentation was carried out at an aeration rate of 0.2 vvm, an agitation rate of 300 rpm, and the pH controlled at 5.0. The temperature conditions for the wild type and the recombinant *S. cerevisiae* strain were controlled at 37°C and 30°C, respectively.

Results and Discussion

Cloning of the Inulinase Gene from *K. marxianus* NCYC2887 and Its Expression in *S. cerevisiae*

The inulinase gene (INU1) without signal sequence obtained by PCR from the genomic DNA of *K. marxianus* NCYC2887 was found to consist of 1,602 nucleotides encoding a protein of 534 amino acids. The INU1 gene showed 99.4% and 98.5% nucleotide identities to the inulinase genes (GenBank Accession Nos. X68479 and

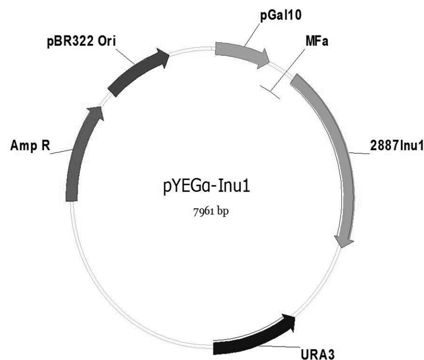

Fig. 1. Construction of the inulinase expression vector, pYEGα-Inu1.
pGal10, GAL10 promoter; MFa, MFα signal sequence; 2887Inu1, INU1 gene from *K. marxianus* NCYC2887; URA3, the gene coding for orotidine-5'-phosphate decarboxylase; Amp R, Ampicillin resistance marker.

X57202) from the other two *K. marxianus* strains, CBS6556 and ATCC12424, respectively. The amino acid identities were 100.0% and 98.9%, respectively.

The INU1 gene was overexpressed under the control of the GAL10 promotor and, for the secretory expression, the INU1 gene without its original signal sequence was fused to the mating factor α signal sequence of *S. cerevisiae* to yield the expression plasmid, pYEGα-Inu1 (Fig. 1). pYEGα-Inu1 was transformed into *S. cerevisiae* Y2805.

The inulinase expression in the recombinant *S. cerevisiae* Y2805 strain was analyzed by SDS−PAGE (Fig. 2). The inulinase obtained from the culture supernatant of *K. marxianus* NCYC2887 was relatively homogeneous with the molecular mass of about 100 kDa. The supernatant of the recombinant *S. cerevisiae* strain showed polydisperse protein bands of higher molecular masses ranging from 55 to 100 kDa, indicating the possible glycosylation of the inulinase in *S. cerevisiae*. When the inulinase of recombinant *S. cerevisiae* was analyzed by SDS−PAGE after treatment with Endo-H, the inulinase migrated as a single discrete band with a size of 59 kDa, which explicitly showed that the recombinant inulinase contained N-linked high-mannose-type oligosaccharides. Activity staining of the gel showed that the band near the 98 kDa marker exhibited inulinase activity (Fig. 2B).

Effect of Δgal80 Mutation on Inulinase Expression Under Aerobic and Anaerobic Conditions

For recombinant production of inulinase in *S. cerevisiae*, the Y2805 strain and its Δgal80 mutant strain were used as hosts for expression of the INU1 gene. These two host cells were cultivated in both the aerobic and anaerobic conditions. Table 1 shows the comparison of the expression levels of inulinase under these conditions.

The secretory expression of the *K. marxianus* inulinase in *S. cerevisiae* Y2805 under the aerobic condition resulted in the inulinase level of 17.0 U/ml in the culture supernatant. The *K. marxianus* NCYC2887 strain produced a considerably low level of inulinase of 2.7 U/ml in the culture supernatant. Thus, the recombinant production of INU1 in *S. cerevisiae* Y2805 yielded an about 6-fold increased amount of inulinase compared with that produced in the original *K. marxianus* NCYC2887 strain.

Since the expression under the GAL10 promoter in *S. cerevisiae* Y2805 requires the addition of galactose as inducer, which is prohibitably expensive for use in large scale fermentation, the use of the Δgal80 mutant strain of *S. cerevisiae* Y2805 was tested for the proper expression of inulinase under both the aerobic and anaerobic conditions.

The use of the Δgal80 mutant strain enabled the glucose-only fermentation in both the aerobic and anaerobic conditions. In the aerobic condition, the Δgal80 mutant strain produced about 2- and 2.9-fold increases in the amount of inulinase in the culture supernatant and in the inulinase amount per cell mass, respectively, compared with the Y2805 wild-type strain. In the anaerobic

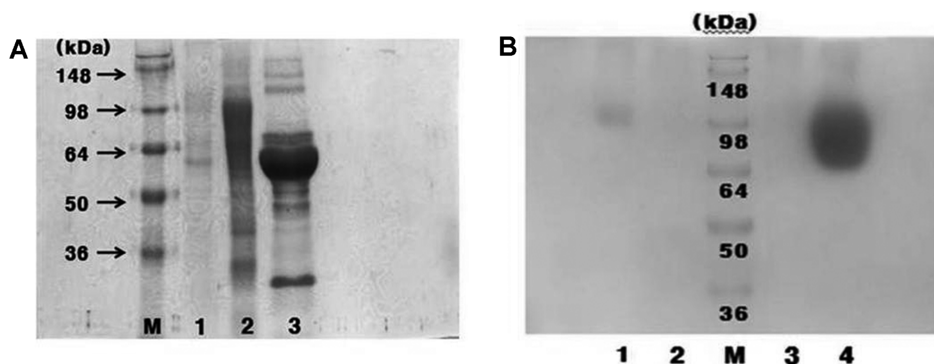

Fig. 2. SDS−PAGE analysis of the inulinase with Coomassie staining (**A**) and activity staining (**B**).
A. The extracellular inulinases from *K.marxianus* NCYC2887 (lane 1) and recombinant *S. cerevisiae* (lane 2) were denatured by boiling. Lane 3, Endo-H treated recombinant *S. cerevisiae*. **B**. The inulinases from *K.marxianus* NCYC2887 (lane 1) and recombinant *S. cerevisiae* (lane 4) were activity-stained after renaturation. *S. cerevisiae* Y2805 without plasmid (lane 2) and *S. cerevisiae* Y2805 containing mock plasmid pYEGα (lane 3) were used as control strains. M denotes the protein molecular weight markers (Invitrogen, USA).

Table 1. Inulinase expression in different yeast strains under aerobic and anaerobic cultivation conditions.

Strain/plasmid	Condition	Medium[a]	OD	Act (U/ml)	Act/OD (U/ml/OD)
	Aerobic				
K. marxianus NCYC2887		YPD	30.2	2.7	0.09
S. cerevisiae Y2805/pYEGα- Inu1		$YPD_{1\%}G_{1\%}$	28.8	17.0	0.59
S. cerevisiae Y2805 Δgal80/pYEG α-Inu1		YPD	20.4	34.6	1.69
	Anaerobic				
S. cerevisiae Y2805/pYEGα- Inu1		$YPD_{1\%}G_{1\%}$	16.1	9.7	0.60
S. cerevisiae Y2805 Δgal80/pYEG α-Inu1		YPD	9.8	9.8	1.00

[a]YPD medium contained 10 g/l yeast extract, 20 g/l Bacto-peptone, and 20 g/l glucose.
$YPD_{1\%}G_{1\%}$ medium contained 10 g/l yeast extract, 20 g/l Bacto-peptone, 20 g/l glucose, and 10 g/l galactose.

condition, the inulinase expression level was 2−3.5-fold lower than in the aerobic cultivation. The Δgal80 strain did not show any difference in the amount of inulinase obtained, but the level of inulinase per cell OD was 1.7-fold higher with the Δgal80 strain. Therefore, we selected *S. cerevisiae* Δgal80 as the host for inulinase expression.

Aerobic Fermentation of *S. cerevisiae* Δgal80 for Inulinase Production

The inulinase expression in the Δgal80 strain was performed using a 5-l jar fermentor. Fed-batch cultivation was performed with a medium containing only glucose as the carbon source for the Δgal80 strain. Fig. 3 shows the result of a 5-l fed-batch fermentation with the Δgal80 strain. The cell mass of the Δgal80 strain reached an OD_{600} of 67 and inulinase activity of about 31.7 U/ml. Inulinase production occurred when the initially added glucose was consumed. This glucose-only mode of expression of the GAL10 promotor in the Δgal80 strain should be a valuable yeast gene expression system that does not demand the addition of an inducer, galactose. The fermentation was essentially completed within 48 h (Fig. 3).

Ethanol Production from Jerusalem Artichoke

The ethanol fermentation from inulin contained in Jerusalem artichoke requires the acid or enzymatic hydrolysis of inulin prior to ethanol fermentation with *S. cerevisiae*, whereas *K. marxianus* does not require pretreatment owing to the endogenous inulinase. The ethanol fermentations with Jerusalem artichoke by *K. marxianus* NCYC2887 and the recombinant *S. cerevisiae* Y2805 Δgal80 strain expressing inulinase were compared. Fig. 4 shows the result of 5-l fermentor experiments with *K. marxianus* NCYC2887 (Fig. 4A) and the recombinant *S. cerevisiae* strain (Fig. 4B). Fermentation with *K. marxianus* NCYC2887 proceeded very rapidly (Fig. 4A). The sugars were rapidly consumed within 12 h, and ethanol increased correspondingly. There was no significant change in pH during fermentation and the cell growth decreased gradually from 24 h. The fermentation was completed within 24 h, and the maximum ethanol production of 53.5 g/l was obtained. In the case of the recombinant *S. cerevisiae* strain (Fig. 4B), fructose was released up to 6 h of fermentation and then rapidly consumed within 24 h. The maximum ethanol production of 59.0 g/l was obtained in 24 h.

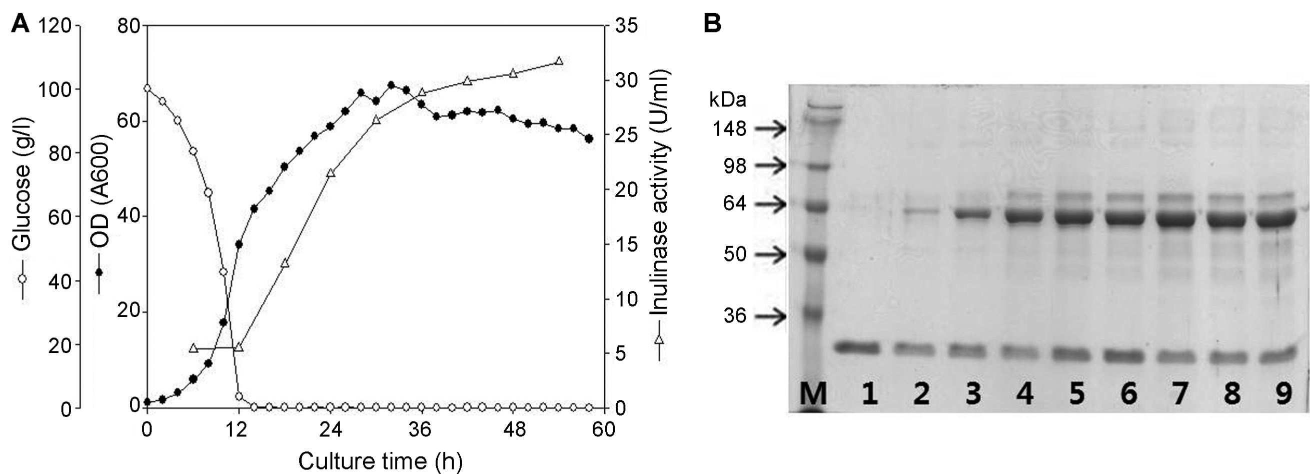

Fig. 3. Fed-batch culture (5 l) of *S. cerevisiae* Y2805 Δgal80/pYEGα-Inul (**A**) and SDS−PAGE of Endo-H-treated culture medium (**B**). Lane 1, 6 h; lane 2, 12 h; lane 3, 18 h; lane 4, 24 h; lane 5, 30 h; lane 6, 36 h; lane 7, 42 h; lane 8, 48 h; lane 9, 54 h.

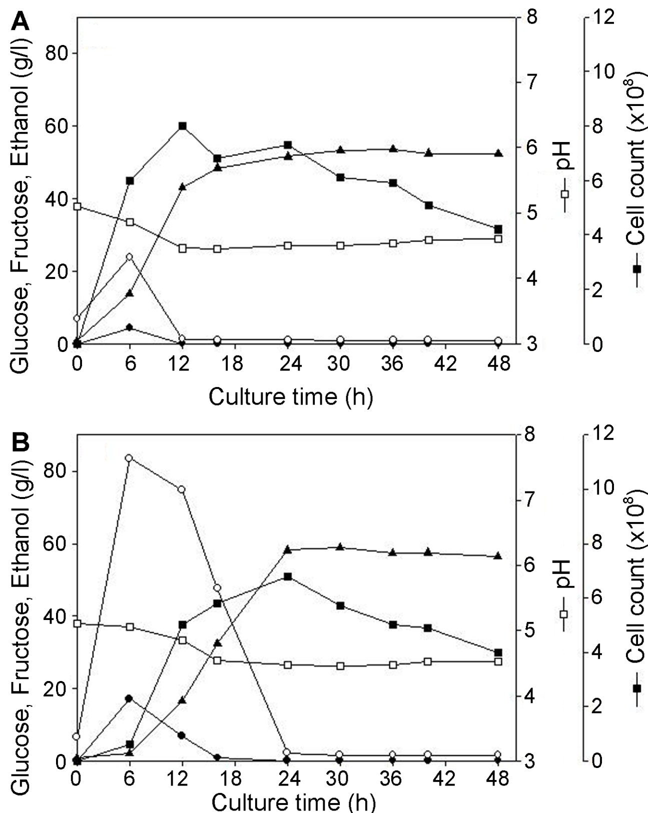

Fig. 4. Ethanol production from Jerusalem artichoke.
Fermentations with *K. marxianus* NCYC2887 (**A**) and the recombinant *S. cerevisiae* strain expressing the INU1 gene (**B**) were carried out at 37°C and 30°C, respectively. Symbols: ●, glucose; ○, fructose; ▲, ethanol; □, pH; ■, cell count.

In conclusion, the efficient recombinant production of inulinase in *S. cerevisiae* was achieved by using the Δgal80 strain. Use of the Δgal80 strain enabled the glucose-only fermentation of the recombinant strain for inulinase expression under the GAL10 promoter without requiring galactose that is otherwise required as an inducer. In addition, the Δgal80 strain further improved the productivity of inulinase production in both the aerobic and anaerobic culture conditions by 2.9- and 1.7-fold, respectively, compared with the wild-type strain. Aerobic fermentation with the *S. cerevisiae* Δgal80 strain produced 31.7 U/ml of inulinase in a 48-h fed-batch fermentation with glucose feeding. Ethanol fermentation with the *S. cerevisiae* Δgal80 strain expressing the inulinase gene yielded 59.0 g/l ethanol from 180 g/l of Jerusalem artichoke flour within 30 h.

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