Direct production of bioethanol from Jerusalem artichoke inulin by gene-engineering Saccharomyces cerevisiae 6525 with exoinulinase gene

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Direct production of bioethanol from Jerusalem artichoke inulin by gene-engineering Saccharomyces cerevisiae 6525 with exoinulinase gene

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Abstract
Jerusalem artichoke (Helianthus tuberosus L.), an important crop, containing over 50% inulin in its tubers on a dry weight basis is an agricultural and industrial crop with a great potential for production of ethanol and industrial products. Inulin is a good substrate for bioethanol production. Saccharomyces cerevisiae 6525 can produce high concentrations of ethanol, but it cannot synthesize inulinase. In this study, a new integration vector carrying inuA1 gene encoding exoinulinase was constructed and transformed into 18SrDNA site of industrial strain S. cerevisiae 6525. The obtained transformant, BR8, produced 1.1 U mL\(^{-1}\) inulinase activity within 72 h and the dry cell weight reached 12.3 g L\(^{-1}\) within 48 h. In a small-scale fermentation, BR8 produced 9.5% (v/v) ethanol, with a productivity rate of 0.385 g ethanol per gram inulin, while wild-type S. cerevisiae 6525 produced only 3.3% (v/v) ethanol in the same conditions. In a 5-L fermentation, BR8 produced 14.0% (v/v) ethanol in fermentation medium containing inulin and 1% (w/v) (NH\(_4\))_2SO\(_4\). The engineered S. cerevisiae 6525 carrying inuA1 converted pure nonhydrolyzed inulin directly into high concentrations of ethanol.

Keywords: Bioethanol, Jerusalem artichoke, inulinase, Saccharomyces cerevisiae, gene engineering

Introduction
Bioethanol represents an alternative to petroleum, and it is receiving increasing attention because of oil price hikes, greenhouse gas emissions from fossil fuels, and gradual depletion of crude oil resources. At present, the main raw materials for bioethanol production include sugarcane, cassava starch, sweet potato, and grain-based feedstocks (Li & Chan-Halberndt 2009; Kabir & El-Shehawy 2012). This drives significant increases in grain prices, and consequently, there is much debate about food security and affordability for the world’s poorest populations (Manchanda & Garg 2011; Kalavrouziotis & Koukoulakis 2012; Zhang et al. 2012; Radhakrishnan & Kumari 2013). Therefore, non-food grain feedstocks are being sought for bioethanol production, especially in developing countries such as China.

Inulin and inulin-containing plants have received increasing attention as a renewable raw material for ethanol production (Chi et al. 2009; Liu et al. 2011; Mahesh & Jayachandran 2011). Inulin consists of linear chains of \(\beta-2\rightarrow1\)-fructosfuranose terminated by a glucose residue linked to fructose by an \(\alpha(1\rightarrow2)\) bond (Martellos 2012). Exo-inulinase catalyzes the one-step removal of the terminal fructose residue from the nonreducing end of the inulin molecule, producing fructose as the main product which can be easily converted into ethanol by S. cerevisiae. Furthermore, inulin dissolves readily in water, and solutions containing high concentrations of inulin have low viscosity. Inulin is naturally present in a wide variety of plants. For example, Jerusalem artichoke (Helianthus tuberosus L.) containing over 50% inulin in its tubers on a dry weight basis is an agricultural and industrial crop with a great potential for production of ethanol and
industrial products (Zhang et al. 2010; Yuan et al. 2012).

*S. cerevisiae* is traditionally used for production of bioethanol because of its ability to tolerate low pH and high concentrations of ethanol and inhibitors, and its ability to grow anaerobically (Hirasawa et al. 2007; Nevoigt 2008; Stanley et al. 2010). However, *S. cerevisiae* cannot synthesize and secrete inulinase, the enzyme that hydrolyzes inulin into fructose. Therefore, it would be useful to clone and actively express the inulinase gene in this yeast. In previous studies, the exoinulinase gene *inuA1* (GenBank ID: JF961344.1) was cloned from *Penicillium janthinellum* strain B01 and expressed at high levels in *Pichia pastoris*. In this study, to produce a strain of *S. cerevisiae* capable of direct production of ethanol from inulin, we constructed an integration expression vector carrying the exoinulinase gene (*inuA1*), and transformed it into *S. cerevisiae* 6525, an industrial ethanol production strain. We selected the transformant with the highest inulinase activity and used it to produce ethanol from inulin in a one-step process (Ow & Sim 2012).

### Materials and methods

#### Gene, plasmids, and yeast strains

The *inuA1* gene encoding exo-inulinase was amplified from *Penicillium janthinellum* strain B01 (Wang et al. 2011; Sandionigi et al. 2012). We used *Saccharomyces cerevisiae* 6525 (kindly supplied by Dr. Bai, Dalian, China), a strain with good fermentation ability, as the host for harboring the inulinase gene. The plasmid pUG6 was used to construct the integration expression vector.

#### Media

The yeast growth medium was yeast peptone dilution (YPD) medium containing 2.0% (w/v) glucose, 2.0% (w/v) peptone, and 1.0% (w/v) yeast extract. The yeast transformants were selected on YPD medium with 200 mg mL$^{-1}$ G418 (Genview, Carlsbad, CA, USA). The inulinase production medium (fermentation medium) contained 2.0% (w/v) inulin, 1.0% (w/v) yeast extract, and 2.0% (w/v) peptone. The medium used for small-scale ethanol production in flask cultures consisted of 20% (w/v) pure nonhydrolyzed inulin and 1.0% (w/v) (NH$_4$)$_2$SO$_4$. The pH of the suspension (5.5–6.0) was not adjusted. We used 30% (w/v) inulin for the 5-L fermentation study and the pH of the suspension was adjusted to 5.0. The suspension was autoclaved for 15 min and used for fermentation without any addition of other nutrients except for 1% (w/v) (NH$_4$)$_2$SO$_4$.

#### Construction of the 18SrDNA integration vector

DNA was manipulated according to standard procedures (Skornik et al. 2010). First, the PGK1 promoter (approx. 780 bp) was polymerase chain reaction (PCR) amplified from genomic DNA of *S. cerevisiae* 6525, using the primers PGK1-F and PGK1-R (Table I), which were designed according to the PGK1 promoter sequence (GenBank ID: X59720.2). The PCR product was extracted and digested with *SacII* and *SpeI*, and then inserted into pUG6 that had been pre-linearized with the same enzymes, yielding pUG-PGK.

Then, the CYC1 terminator (approx. 250 bp) was PCR-amplified from DNA of the pYES2.0 plasmid, using the following primers: CYC1-F, which shared the same sequence as the *inuA1*-specific primer *inuA1*-R used in subsequent PCR amplifications, and CYC1-R, which contained an *EcoRV* site. The *inuA1* gene fragment was PCR-amplified using primers *inuA1*-F with a *SpeI* site and *inuA1*-R. The PCR products from the two PCR amplifications were mixed, denatured, allowed to anneal using the shared sequence of the primers, and subjected to a fusion PCR, using primers *inuA1*-F and CYC1-R as described earlier. A fusion fragment including the *inuA1* gene and the CYC1 terminator was generated by overlap extension PCR, then digested by *EcoRV* and *SpeI*, and inserted into pUG-PGK digested with same enzymes, generating pUG-PIC. The PCR reaction and conditions for the

### Table I. Primers used to construct the recombinant vector.

<table>
<thead>
<tr>
<th>Primers name</th>
<th>Primer sequence 5′–3′</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGK1-F</td>
<td>TCCCCCGGGGAGTTTCAACTCAAGACGCAACAG (SacII site italicized)</td>
</tr>
<tr>
<td>PGK1-R</td>
<td>CGGACTAGTGGTTTTATATTGGTGTGTA (SpeI site italicized)</td>
</tr>
<tr>
<td><em>inuA1</em>-F</td>
<td>GGAATCTAGATGTTGACGCTTTTCAAACCCCTT (SpeI site italicized)</td>
</tr>
<tr>
<td><em>inuA2</em>-R</td>
<td>GTGACATACTAATTACATGATCTAATCTCCACGGTCAAA (shared bases shown in bold type)</td>
</tr>
<tr>
<td>CYC1-F</td>
<td>TTGGACACTAGATGTTTATATTGGTGTGTA (SpeI site italicized)</td>
</tr>
<tr>
<td>CYC2-R</td>
<td>CCGGATATCGCAAAATTTAAGGGTCACGGTCAAA (EcoRV site italicized)</td>
</tr>
<tr>
<td>18SrDNA-F</td>
<td>GCCTAGATGAGACGGTACCCCATC (XbaI site italicized)</td>
</tr>
<tr>
<td>18SrDNA-R</td>
<td>GGTCACCTCTGGATGAGAGTCACGGTCAAA (BstEI site italicized)</td>
</tr>
</tbody>
</table>
PCR amplification were as recommended in the manual of the PrimeSTAR® HS DNA polymerase.

To integrate the *inuA1* gene into the chromosomes of *S. cerevisiae* 6525, a partial 18SrDNA fragment of approximately 840 bp was PCR-amplified from the genomic DNA of strain 6525, using the primers 18SrDNA-F and 18SrDNA-R, which were designed according to the 18S rDNA gene sequence (GenBank ID: AB628065.1). The partial 18S rDNA fragment was extracted, digested with *Xba*I and *EcoRI*, and then inserted into pUG-PIC which was digested with same enzymes, resulting in recombinant PUG-PICS which contained the PGK1 promoter, the inuA1 open-reading frame, the CYC1 terminator, and 18S rDNA. The recombinant pUG-PICS was linearized with *Rsr*II, and then transformed into *S. cerevisiae* 6525 by electroporation. A 200 μL aliquot of cells was plated on YPD medium containing 200 mg mL⁻¹ G418 and grown at 30°C until the transformants became visible. The different positive transformants were grown in inulinase production medium at 30°C for 3 days. Inulinase activity was determined in the supernatant of each sample by the method that will be described further. The transformant with the highest inulinase activity in the culture supernatant was used for subsequent investigations.

To confirm that the linear DNA fragments carrying the inulinase gene had been integrated into the chromosomes of *S. cerevisiae* 6525, we carried out PCR amplifications using genomic DNAs from the transformant and the wild-type *S. cerevisiae* 6525 as templates. The forward primer was inuA1-F and the reverse primer was CYC1-R (see Table I for sequences). The sizes of the PCR products were as expected, approximately 2400 bp.

**Determination of recombinant inulinase activity**

The recombinant inulinase activity in the supernatants of the transformants obtained earlier was determined according to Wang et al. (2011). We determined the concentration of reducing sugars in the mixture using the 3,5-dinitrosalicylic (DNS) acid assay with fructose as the standard (Miller 1959). The amount of enzyme that produces 1 mmol reducing sugars per minute under the assay conditions used in this study.

**Ethanol fermentation**

For flask cultures, seed cultures were prepared by inoculating *S. cerevisiae* 6525 and the transformant BR8 grown on YPD overnight was transferred into a 250-mL Erlenmeyer flask containing 50 mL yeastpectone liquid medium. The cultures were then incubated at 30°C for 3 days with shaking at 200 rpm. Then, 10 mL of the seed culture was transferred into a 250-mL flask containing 100 mL ethanol production liquid medium. The fermentation was carried out at 30°C for 120 h. The final concentrations of ethanol, residual reducing sugars, and residual total sugars in the fermented media were determined as will be described further.

The 5-L fermentation was carried out in a GUCS-5 bioreactor (DongFang BioTech Zheng Jiang Co., Ltd, Hangzhou, China). A 300 mL aliquot of the seed culture obtained earlier was transferred into 3000 mL medium containing 30% inulin with the initial pH adjusted to 5.0. The fermentation was carried at 30°C for 192 h without aeration and agitation.

**Analytical methods**

After the system reached equilibrium, samples were taken every 24 h. The cell dry weight was measured according to the method of Chi et al. (2001) and Verma et al. (2012). Reducing sugars were assayed by the DNS method (Wang et al. 2012) and are expressed as fructose equivalents. Total reducing sugars were estimated by the same method after acid hydrolysis (pH adjusted to 2 with H₂SO₄; 60 min at 100°C). The ethanol concentration was determined by gas chromatography (Agilent Company, Chicago, USA). Chromatography column: HP-FFAP (30 m × 0.25 mm i.d., 0.25 m film thickness); column temperature: 80°C; injector temperature 160°C; detector temperature: 230°C and isopropanol were used as the internal standard. The ethanol concentration was expressed as a percentage (v/v), the unit used by distillers; 1% (v/v) ethanol corresponds to 7.92 g ethanol per liter.

**Results and discussion**

**Construction of the 18SrDNA integration vector**

To create a strain of *S. cerevisiae* that could directly ferment inulin into ethanol, we constructed the expression vector pUG-PICS (Figure 1) to integrate *inuA1* into the genome of *S. cerevisiae* 6525 using 18SrDNA as the target site for homologous recombination. Previously, it was reported that the inulinase activity of a yeast transformant with an inulinase gene integrated into its genome remained very stable during five sequential batch cultivations in a nonselective medium. In contrast, the inulinase activity of a transformant carrying the same gene in a plasmid (Ycplacc33) decreased gradually (Zhao et al. 2010; Wang et al. 2011). The integration strategy used in the present study not only facilitates stable recombination of the heterologous gene, but also increases the possibility of integrating multiple copies.
of the gene into the yeast genome. Since there are approximately 200 copies of rDNA present as tandemly repeated sequences in the yeast genome, up to 100 copies of the heterologous gene could be integrated. The recombinant plasmid (Figure 1) contained the constitutive promoter PGK1 (782 bp) and the effective terminator CYC1 (249 bp). Therefore, the cloned inulinase gene could be expressed constitutively in S. cerevisiae and gene expression could be effectively terminated.

The recombinant plasmid was linearized and transformed into S. cerevisiae 6525. We measured the inulinase activity in the supernatant of cultures of the transformants, and selected the one with highest activity, BR8, for further experiments. The wild-type S. cerevisiae 6525 had no inulinase activity (data not shown). As shown in Figure 2, the PCR products obtained from the transformant were of the expected size, 2381 bp. However, no such PCR products were amplified from the genomic DNA of the wild-type S. cerevisiae 6525. This indicated that the linear DNA fragments containing the inulinase gene were indeed integrated into the genome of S. cerevisiae 6525.

**Time-course of cell growth and inulinase production by the transformant BR8**

We measured inulinase activity in the supernatant and cell mass during cell growth in the inulinase production medium. As shown in Figure 3, the transformant BR8 showed the highest inulinase activity (10.9 U mL⁻¹) within 72 h of culture, when cell growth reached the stable phase. The maximum dry cell weight was 12.3 g L⁻¹ at 48 h of culture. In another study, the INU2 gene encoding an endoinulinase of Aspergillus ficuum was expressed under the control of the Kluyveromyces marxianus INU1 promoter in a SUC2-deleted strain of S. cerevisiae, and the endo-inulinase activity of the recombinant was 4.04 U mL⁻¹. Optimization of the culture conditions could increase the inulinase activity of the transformant BR8. Further experiments are required to study the expression and secretion of inulinase in yeast in greater detail. Such experiments, which are currently in progress, will include replacement of the P. jathinellum signal sequence by homologous signal sequences (e.g. α-factors) as well as more detailed studies on the cellular localization of the inulinase protein in yeast. Surprisingly, although the expression level of inulinase in S. cerevisiae was less than that in Picha pastoris reported earlier (Wang et al. 2011), it greatly enhanced the production of ethanol from inulin, as described further in the text.

**Ethanol fermentation**

To determine whether the transformant BR8 constructed earlier could directly convert inulin into ethanol, we carried out fermentation experiments using BR8 and the wild-type S. cerevisiae 6525 in flask. As shown in Table II, the transformant BR8 produced 9.5% (v/v) ethanol and the ethanol productivity rate was 0.385 g ethanol per gram inulin. Most of the added sugar (inulin) was used for ethanol production and cell growth by the transformant BR8, with only 2.7% (w/v) total sugars remaining in the medium at the end of the fermentation. In the same conditions, the wild-type S. cerevisiae 6525 produced only 3.3% (v/v) ethanol, and used only some of the inulin with 8.0% (w/v) residual total sugars remaining in the culture medium at the end of the fermentation. It was reported that the wild-type S. cerevisiae cannot secret inulinase to directly convert inulin to ethanol (Zhang et al. 2010). In our study, however, the wild-type S. cerevisiae 6525 was able to use some of the inulin because it contained the invertase
gene encoding invertase (EC.3.2.1.26), which catalyzes the hydrolysis of sucrose. *S. cerevisiae* KCCM50549 strain produced 4.6% (v/v) concentrations of ethanol at 34 h in a 5-L jar fermentor without addition of inulinase. Our results indicated that direct production of ethanol from inulin was significantly increased in the *S. cerevisiae* 6525 transformant harboring the inulinase gene.

Next, we conducted an experiment in which the transformant BR8 was grown and its ethanol production assessed in a 5-L fermentor over a 192-h period. The ethanol concentration reached 12.1% (v/v) after 120 h fermentation and increased to 14.0% (v/v) at 192h (Figure 4). The ethanol productivity rate was 0.483 g ethanol per gram total consumed sugars. The conversion efficiency of inulin-type sugars to ethanol was 94.8% of the theoretical ethanol yield. The cell growth reached the stationary phase after 120 h cultivation and the maximum dry cell weight was 12.1 g L\(^{-1}\). Yeast cells can be recycled and utilized to produce single-cell protein. At the end of the fermentation period, 1.8% (w/v) reducing sugars and 2.5% (w/v) total sugars remained in the fermented medium (Figure 4), indicating that most of the added inulin was
converted into ethanol. Our study indicated that the engineered *S. cerevisiae* 6525 carrying the *inuA1* gene cloned from *P. janthinellum* could directly convert inulin extracted from a nonfood material into ethanol. Such consolidated bioprocessing integrating inulinase production, saccharification of inulin and ethanol production from sugars released from inulin by the enzyme is a potential breakthrough for low-cost production of ethanol from inulin biomass. Ethanol fermentations from Jerusalem artichoke tubers were carried out with two trains from the inulinase-producing species *K. marxianus* Y179 and ATCC8554, and the experimental results indicated that *K. marxianus* Y179 with improved inulinase production is more efficient than *K. marxianus* ATCC8554 (Yuan et al. 2012). Therefore, improving inulinase activity facilitates the direct conversion of inulin into ethanol (Bogani et al. 2012). At present, we are conducting experiments to increase the amount and activity of the secreted inulinase, which could increase the rate of ethanol production and reduce the duration of the fermentation period (Kabir & El-Shehawy, 2012).

**Conclusion**

In summary, we have successfully constructed an integrated expression vector and transformed it into the chromosomes of *S. cerevisiae*. The inulin derived from Jerusalem artichoke could be converted efficiently to ethanol by transformant BR8 without acidic or enzymatic hydrolysis prior to fermentation. The transformant BR8 produced approximately three times more ethanol compared with the wild-type *S. cerevisiae* 6525. Direct fermentation of inulin at 30% (w/v) yielded 14.0% (v/v) of ethanol in a 5-L fermentor, thus it is a very promising strain for industrial applications.

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