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BIOENERGY AND BIOFUELS

Enhanced expression of the codon-optimized *exo*-inulinase gene from the yeast *Meyerozyma guilliermondii* in *Saccharomyces* sp. W0 and bioethanol production from inulin

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Abstract In the present study, after the *exo*-inulinase gene *INU1* from *Meyerozyma guilliermondii* was optimized according to the codon usage bias of *Saccharomyces cerevisiae*, both the optimized gene *INU1Y* and the native gene *INU1* were ligated into the homologous integration expression vector pMIRSC11 and expressed in *Saccharomyces* sp. W0. It was determined that the inulinase activity of the recombinant yeast Y13 with the optimized gene *INU1Y* was 43.84 U/mL, which was obviously higher than that (31.39 U/mL) produced by the recombinant yeast EX3 with the native gene *INU1*. Moreover, it was indicated that the recombinant yeast Y13 could produce 126.30 mg/mL ethanol from 300.0 g/L inulin while the recombinant yeast EX3 and *Saccharomyces* sp. W0 produced 122.75 mg/mL and 114.15 mg/mL ethanol, respectively, under the same conditions. In addition, the ethanol productivity of the recombinant yeast Y13 was 2.25 mg/mL/h within 48 h of the fermentation, which was obviously higher than that of the recombinant yeast EX3 (1.97 mg/mL/h) and *Saccharomyces* sp. W0 (1.77 mg/mL/h) within the same period. The results demonstrated that the recombinant yeast Y13 had higher ethanol production and productivity than the recombinant yeast EX3 and *Saccharomyces* sp. W0. Therefore, it was concluded that the codon optimization of the *exo*-inulinase gene from *M. guilliermondii* effectively enhanced inulinase activity and improved ethanol production from inulin by *Saccharomyces* sp. W0 carrying the optimized inulinase gene.

Keywords *Exo*-inulinase · *Meyerozyma guilliermondii* · *Saccharomyces* sp. W0 · Codon optimization · Ethanol · Inulin

Introduction

Inulin is a polysaccharide composed of linear chains of β-2,1-linked D-fructofuranose molecules terminated by a glucose residue and is present as a reserve carbohydrate in the roots and tubers of plants, such as Jerusalem artichoke, chicory, dahlia, and yacon (Chi et al. 2011). Recently, inulin and inulin-containing materials have received increasing attention as the renewable raw materials for bioethanol, lipid, citric acid, single-cell protein, ultra-high-fructose syrup, fructooligosaccharide, and other production chemicals (Cui et al. 2011; Li et al. 2013; Liu et al. 2010; Risso et al. 2010; Wang et al. 2012). Furthermore, inulin has been recognized as one of the important potential non-grain materials for bioethanol production (Chi et al. 2011). In the current industrial bioethanol production, it is economically advantageous to convert inulin to ethanol in one microorganism by consolidated bioprocessing (CBP) that does not need additional enzymes for inulin hydrolysis. Thus, *Kluyveromyces marxianus*, which can produce both inulinase and ethanol, became the potential CBP strain used for ethanol production from inulin (Yuan et al. 2012). For example, it was reported that 73.6 mg/mL ethanol from Jerusalem artichoke tuber flour (200 g/L) could be achieved by *K. marxianus* PT-1 at 40 °C (Hu et al. 2012). However, it has been well documented that *K. marxianus* cannot tolerate high concentrations of ethanol differently from *Saccharomyces cerevisiae* that is also a widely used industrial microorganism for its high ethanol production (Lane and Morrissey 2010; Nevoigt 2008; Ostergaard et al. 2000). Unfortunately, *S. cerevisiae* cannot produce

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high concentrations of ethanol from inulin due to its weak capability of utilizing fructooligosaccharides with a high degree of polymerization (Li et al. 2013; Lim et al. 2011; Wang and Li 2013). Therefore, it has been confirmed that introduction of a heterologous inulinase gene into *S. cerevisiae* could improve its inulin utilization and ethanol production.

Inulinases are fructofuranosyl hydrolases that target on the β-2,1 linkage of inulin and hydrolyze it into fructose and glucose. According to hydrolysis pattern, inulinase can be divided into *exo*-inulinase (E.C.3.8.1.80) and *endo*-inulinase (E.C. 3.2.1.7) (Liu et al. 2013). Recently, many *exo*-inulinase and *endo*-inulinase genes cloned from *Aspergillus niger*, *Aspergillus fumigatus*, *Meyerozyma guilliermondii*, *K. marxianus*, *Candida kutaonensis*, *Cryptococcus aureus*, and *Arthrobacter* sp. have been successfully expressed in *S. cerevisiae* (Liu et al. 2013). In our previous study (Zhang et al. 2010a), the *exo*-inulinase gene cloned from the marine yeast *M. guilliermondii* (*Pichia guilliermondii*) was ligated into the expression plasmid YCPlac33 PGK/CYC1 and expressed in *Saccharomyces* sp. W0. The positive transformant Inu-66 obtained can produce 34.2 U/mL of inulinase activity within 72 h of cultivation and 117.6 mg/mL of ethanol from inulin during the 2-L fermentation. In another study (Wang et al. 2011), in order to obtain genetically stable recombinant yeast cells, a new 18S ribosomal DNA (rDNA) homologous integration expression vector pMIRSC11 was constructed and used for the expression of the *exo*-inulinase gene. The transformant MguINU1-04 obtained could produce 34.8 U/mL of inulinase activity and keep a high activity of inulinase after five sequential batch cultivations. Even so, the inulinase activity of the recombinant yeasts was still lower than that (60 U/mL) produced by the native producer *M. guilliermondii* strain 1 (Gong et al. 2008). In order to decrease the fermentation period and increase ethanol yield, it became necessary to enhance the expression of heterologous inulinase gene in *S. cerevisiae*. Codon optimization which means converting the codons present in a target sequence into the codons more frequently used by the expression host is one of the important and effective techniques to enhance heterologous gene expression (He et al. 2014; Liu et al. 2012). Increasing evidence indicates that the synonymous codon substitution can have a significant impact on gene expression, protein translational efficiency, and protein folding by involving the secondary structure stability of the expressed messenger RNA (mRNA), the abundance of the corresponding transfer RNA (tRNA), and the translation speed and accuracy (Angov 2011; Saunders and Deane 2010). However, so far, few studies on *exo*-inulinase gene optimization have been conducted in order to enhance its expression in *S. cerevisiae*. Therefore, in this study, first, the *exo*-inulinase gene (*INU1*) from *M. guilliermondii* was optimized and the improved *INU1* gene was expressed in *Saccharomyces* sp. W0. Then, the recombinant yeast was used for ethanol production from inulin.

Materials and methods

Strains, media, and plasmids

Escherichia coli DH5α [F^-, φ80*lacZΔM15*, Δ(*lacZYA-argF*), U169, *deoR*, *recA1*, *endA1*, *hsdR17* (rk^-, mk^+), *phoA*, *supE44*, λ^-, *thi-1*, *gyrA96*, *relA1*] (Takara Biotechnology, Dalian, China) was used to amplify the plasmids carrying the optimized *exo*-inulinase gene and its native one. *Saccharomyces* sp. W0 [collection number 2E001020 at the Marine Microorganisms Culture Collection of China (MCCC)] which is a high ethanol-producing yeast (Chi and Liu 1993) was used as a control in this study. The uracil mutant W12d (collection number 2E001021 at MCCC) of *Saccharomyces* sp. W0 obtained in the previous study (Zhang et al. 2010b) was used as an expression host. YPD medium used for yeast growth contained 20.0 g/L glucose, 10.0 g/L yeast extract, and 20.0 g/L peptone. LB medium with 100.0 μg/mL of ampicillin was used for *E. coli* growth and transformation. The inulinase production medium was composed of 20.0 g/L inulin, 10.0 g/L yeast extract, and 20.0 g/L peptone. The medium used for ethanol production from inulin contained 300.0 or 250.0 g/L inulin (Shaanxi Pioneer Biotech Co. Ltd, Xian, China) and 20.0 g/L malt extract. Plasmid pSIMPLE-19 EcoRV/BAP (Takara Biotechnology, Dalian, China) was used for cloning of PCR products. Plasmid pUC57-INUY carrying the optimized gene *INU1Y* (Accession No. KJ939347) was synthesized by GenScript (Nanjing, China). The 18S rDNA homologous integration expression vector pMIRSC11 (Wang et al. 2011) was used for the *exo*-inulinase expression in this study.

Isolation of DNA, restriction digestions, and transformation

Yeast genomic DNA for amplification of the *exo*-inulinase gene in *M. guilliermondii* strain 1 (collection number 2E001026 at MCCC) was isolated by using TIANamp Yeast DNA Kit (TIANGEN, China). Bacterial plasmid DNA was purified by using TIANprep Rapid Mini Plasmid Kit (TIANGEN, Beijing, China). Restriction *endo*nuclease digestions and DNA ligations were performed according to the manufacturer's recommendations. *E. coli* was transformed with plasmid DNA using the method described by Sambrook et al. (1989).

Codon optimization and synthesis of the modified gene

The gene sequence encoding the *exo*-inulinase (*INU1*) was obtained from GenBank (Accession No. EU195799). The

codon usage of the DNA sequence was analyzed using the graphical codon usage analyzer (http://www.genscript.com/cgi-bin/tools/rare_codon_analysis) and optimized by replacing the codons predicted to be less frequently used in *S. cerevisiae* with the frequently used ones using DNAWorks software (http://helixweb.nih.gov/dnaworks/) without altering the encoded amino acid sequence. The modifications of the *INU1* gene were made throughout its sequence, including the addition of two restriction sites, *Bst*I and *Kpn*I, to the forward primer and reverse primer ends (Inuybst and Inuhiskpn, Table 1), respectively. The mRNA structure and free energy of mRNA folding were analyzed using the program RNAstructure 5.2 (http://rna.urmc.rochester.edu/RNAstructure.html). The optimized inulinase gene *INU1Y* (Accession No. KJ939347) was synthesized by GenScript (Nanjing, China) and ligated into pUC57. Therefore, the resulting plasmid was named pUC57-INU1Y.

Construction of the expression plasmids carrying the optimized gene *INU1Y* and the native gene *INU1*

The plasmid pUC57-INU1Y carrying the optimized gene *INU1Y* was digested with *Bst*I and *Kpn*I, gel purified, and ligated into pMIRSC11, yielding the recombinant vector pMIRSC11-INU1Y (Fig. 1). Then, it was verified by DNA sequencing. At the same time, in order to use the native inulinase gene *INU1* from *M. guilliermondii* as the control, the primers Inuybst and Inuhiskpn were designed (Table 1). The genomic DNA of *M. guilliermondii* strain 1 obtained above was used as the template for PCR. The PCR reaction system (50.0 μL) was composed of 10.0 μL of Q5 Reaction Buffer, 4.0 μL of dNTP mixture (2.5 mM each), 1.0 μL of the template DNA, 1.0 μL of the primer Inuybst, 1.0 μL of the primer Inuhiskpn, 0.5 μL of Q5 High-Fidelity DNA Polymerase (New England BioLabs, Ipswich, MA, USA), and 32.5 μL of distilled water. The conditions for the PCR amplification were as follows: initial denaturation of 98 °C for 3 min, followed by 30 cycles of 98 °C for 15 s, 65 °C for 15 s, and 72 °C for 50 s, with a final extension at 72 °C for 5 min. The PCR products were purified, phosphorylated, and ligated into the plasmid pSIMPLE-19 EcoR V/BAP vector to construct plasmid pSIMPLE-INU1. Finally, the *INU1* gene isolated from pSIMPLE-INU1 was ligated into pMIRSC11, forming the expression plasmid pMIRSC11-INU1 (Fig. 1).

Expression of the *exo*-inulinase genes in *Saccharomyces* sp. W0

Linear DNA fragments with *INU1Y* or *INU1* gene were obtained by the digestion of pMIRSC11-INU1Y or pMIRSC11-INU1 with *Not*I, respectively (Fig. 1), and transformed into the uracil mutant W12d of *Saccharomyces* sp. W0 using the LiAc method described by Gietz and Woods (2006). The transformed cells were spread on YNB plates without uracil. Positive transformants carrying the *INU1Y* or *INU1* gene were cultured in the inulinase production medium for 72 h, and the *exo*-inulinase activity produced by the transformants was determined as described below. The native strain *Saccharomyces* sp. W0 without the *exo*-inulinase gene was used as the control. After the determination of *exo*-inulinase activity in the cultures from over 200 transformants, the recombinant yeasts EX3 and Y13 transformed with the linear DNA fragments with *INU1* gene and *INU1Y* gene, respectively, were used in the subsequent investigations.

Determination of inulinase activity

The recombinant yeasts EX3 and Y13 mentioned above and *Saccharomyces* sp. W0 were grown in 50 mL of the inulinase production medium at 28 °C with shaking at 180 rpm for 72 h. The culture was centrifuged at 4 °C, and the supernatant was employed for inulinase activity determination. Inulinase activity produced by the recombinant yeasts EX3 and Y13 and *Saccharomyces* sp. W0 was determined according to Gong et al. (2007). The reaction mixture containing 0.1 mL of the crude recombinant enzyme and 0.9 mL of 20.0 g/L inulin (Sigma-Aldrich, St. Louis, MO, USA) dissolved in phosphate buffer (50.0 mM, pH 6.0) was incubated at 60 °C for 10 min. The reaction was terminated in boiling water for 10 min. The same mixture with 0.1 mL of the crude recombinant enzyme inactivated in the boiling water for 10 min was used as the control. The amount of reducing sugar in the reaction mixture was assayed by using the method described by Spiro (1966). One unit of inulinase activity was defined as the amount of enzyme required to produce 1.0 μmol of reducing sugar per minute under the assay in this study.

Confirmation of integration of the target gene into the genomic DNA of *Saccharomyces* sp. W0

To get the evidence of integration of the target gene into the genomic DNA of *Saccharomyces* sp. W0, the genomic DNAs from the positive transformants EX3 and Y13 and the uracil mutant of *Saccharomyces* sp. W0 were extracted and used as templates for PCR, respectively. The DNA fragments (1732 bp) containing the partial sequence of the promoter (TDH3 PT) and terminator (CYC1 TT) of the expression plasmid and the whole sequence of the *INU1Y* gene were PCR amplified using the primers Pmis and Pmia (Table 1 and Fig. 1). PCR amplification was performed as described earlier.

The fluorescent real-time RT-PCR analysis

The recombinant yeasts EX3 and Y13 mentioned above and *Saccharomyces* sp. W0 were grown at 28 °C and shaking

Table 1 The primers used in this study

Primer	Sequence	Use
Inuybst	5′-*TTCGAA*TAAACACACATAAACAAACAAAATGAGA GCTTTTTTGGCCTTAA-3′, the italic bases encode *Bst*I site	Amplification of *INU1* for plasmid construction
Inuhiskpn	5′-**GGTACC**TTA*ATGATGATGATGATGATG*TGAAGTTG CCCTCAATTTTAACTCC-3′, the bold bases encode *Kpn*I site and the italic bases encode 6× His	
Pmis	5′-CTGTAAATCTATTTCTTAAACTTC-3′	Amplification for confirmation of integration of the target gene
Pmia	5′-AAGCGTGACATAACTAATTACATG-3′	
W026ss	5′-GAGACCGATAGCGAACAAG-3′	Amplification of 26S rDNA for real-time RT-PCR
W026sa	5′-CTACCCACAAGGAGCAGAG-3′	
InuRs	5′-GGCACGAGCCAACTAACCA-3′	Amplification of *INU1* for real-time RT-PCR
InuRa	5′-TGGAGTGCAAGCTCCAGTTCG-3′	
InuYRs	5′-GTTCCTGATGACTCCCAAGC-3′	Amplification of *INU1Y* for real-time RT-PCR
InuYRa	5′-CCAGGCTAAACCTACGACAC-3′	

speed of 180 rpm in the inulinase production medium for 72 h. The cultures were centrifuged at 8000×g and 4 °C for 10 min and the pellets obtained were used as the samples for total RNA isolation. The total RNA was purified using a RNAprep pure Tissue Kit (TIANGEN, Beijing, China). For fluorescent real-time reverse transcription PCR (RT-PCR), complementary DNA (cDNA) was synthesized from the total RNA using the PrimeScript RT reagent Kit (Takara Biotechnology, Dalian, China) according to the manufacturer's protocol. The fluorescent real-time RT-PCR assay was carried out in a 20.0-μL reaction volume per well containing 10.0 μL of DyNAmo ColorFlash Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), 1.0 μL of 1:10 diluted cDNA, and 200.0 nM of each forward and reverse primer. The primers W026ss and W026sa, the primers InuRs and InuRa, and the primers InuYRs and InuYRa (Table 1) were designed

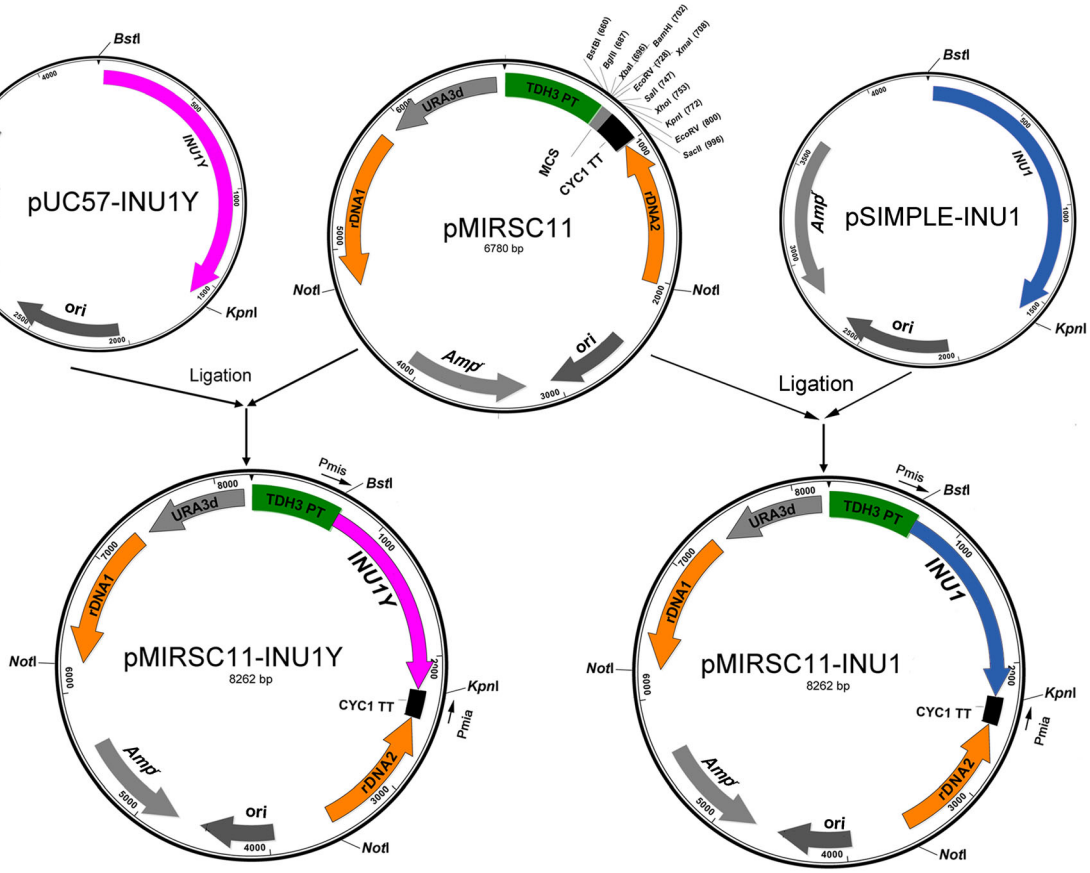

Fig. 1 Construction of the recombinant expression vectors pMIRSC11-INU1 and pMIRSC11-INU1Y

according to the sequences of the 26S ribosomal RNA (rRNA) gene of *S. cerevisiae* (Accession No. AJ508581), the native inulinase gene *INU1* (Accession No. EU195799), and the codon-optimized inulinase gene *INU1Y* (Accession No: KJ939347), respectively. The fluorescent real-time RT-PCR reactions were performed on Rotor-Gene Q 5plex System (QIAGEN, Hilden, Germany). The thermal profile was one initial cycle of 2 min at 95 °C, followed by 40 cycles of 20 s at 95 °C, 20 s at 60 °C, and 35 s at 72 °C.

The fluorescent real-time RT-PCR data obtained were analyzed with Rotor-Gene Q Series Software v2.0.2 (QIAGEN, Hilden, Germany). The comparative computerized tomography (Ct) method (Livak and Schmittgen 2001) was used to analyze the relative expression level of the native gene *INU1* of the recombinant yeast EX3 and *Saccharomyces* sp. W0 and the optimized gene *INU1Y* of the recombinant yeast Y13. The Ct value of 26S rRNA gene was determined as the internal control for each sample. The Ct values for each set of three reactions were averaged for all the subsequent calculations. Each datum represents the average of three experiments. The relative expression quantity was calculated using the formula $RATE=2^{-\Delta\Delta Ct}$. The sample data obtained from the real-time RT-PCR analysis were subjected to one-way analysis of variance (ANOVA) (Wu and Hamada 2000). *P* values were calculated by Student's *t* test ($n=3$). *P* values less than 0.01 were considered statistically significant. Statistical analysis was performed using SPSS 11.5 for Windows (SPSS Inc., Chicago, IL, USA).

Ethanol fermentation

The recombinant yeasts EX3 and Y13 mentioned above and *Saccharomyces* sp. W0 were inoculated into 50.0 mL of the inulinase production medium and incubated at 28 °C with shaking at 180 rpm for 72 h. The seed culture obtained was transferred into 250-mL flasks at an inoculum size of 10 % (*v*/*v*) (the final yeast cell concentration was about 5.0×10^7 cells/mL) with the final volume of 150 mL of the ethanol production medium containing 250.0 or 300.0 g/L inulin (Shaanxi Pioneer Biotech Co. Ltd, Xian, China). The fermentation was performed under the conditions of 30 °C for 120 h without aeration and agitation. The ethanol concentration, residual reducing sugar, and residual total sugar were measured as described below. In order to make the yeast cells produce the maximal ethanol, the ethanol fermentation was kept in the closed fermentation system without aeration and agitation.

Ethanol assay

High performance liquid chromatography (HPLC) was employed to measure ethanol concentration in the fermentation medium. The fermented medium was centrifuged at $5000\times g$ for 5 min. Then, 20.0 μL of the supernatant obtained was analyzed by HPLC (Agilent 1200 series, Agilent Technologies, Santa Clara, CA) equipped with an Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA) and a refractive index detector (Agilent 1260 Infinity RID). The H_2SO_4 solution (5.0 mM) was used as the mobile phase at 55 °C with a flow rate of 0.5 mL per minute (Mohr et al. 2013).

Determination of reducing sugar and total sugar in the fermented media

Reducing sugar in the initial media and fermented media was determined by using the method described by Spiro (1966). Total sugar was assayed by using the same method as reducing sugar after acid hydrolysis of polysaccharides (the supernatant was adjusted to pH=1.0 with sulfuric acid and heated to 100 °C for 30 min) (Li et al. 2013).

Results

Preparation of the codon-optimized inulinase gene and the native inulinase gene

The analysis by using the graphical codon usage analyzer revealed that the native gene *INU1* contained many rare codons, including instances of CGA (Gly) and CGG (Ser), which shared only 15 and 10 % relative adaptiveness in *S. cerevisiae*, respectively (Online Resource Fig. S2a). Therefore, these rare codons of the native *INU1* gene were replaced by the preferred ones which appeared more common in *S. cerevisiae* (Online Resource Fig. S2). In addition, as shown in Table 2, the negative *cis*-acting sites which could reduce the stability of the mRNA secondary structure were eliminated. Consequently, the optimized gene *INU1Y* obtained shared 78.71 % nucleotide sequence identity to the native *INU1* gene (Online Resource Fig. S1). Afterwards, the genes *INU1Y* and *INU1* were inserted into the expression vectors to construct pMIRSC11-INU1Y and pMIRSC11-INU1, respectively (Fig. 1), as described in "Materials and methods."

Table 2 Unstable *cis*-acting elements in the genes *INU1* and *INU1Y*

Cis-acting elements	*INU1*	*INU1Y*
Splice-site (GGTGAT)	2	0
PolyA (AATAAA)	1	0
Destabilizing sequence (ATTTA)	3	0
PolyT (TTTTTT)	1	0

Expression of the *INU1* and *INU1Y* genes in *Saccharomyces* sp. W0

In order to enhance inulinase activity and improve ethanol production, the linear DNA fragments with *INU1Y* or *INU1* obtained from pMIRSC11-INU1Y and pMIRSC11-INU1, respectively, were transformed into the uracil mutant W12d of *Saccharomyces* sp. W0 (Fig. 1). After different positive transformants obtained were cultured in the inulinase production medium at 28 °C for 3 days, the inulinase activity was determined as described in "Materials and methods". The results in Table 3 indicated that the inulinase activity of the recombinant yeast EX3 among all the transformants with the native *exo*-inulinase gene *INU1* was 31.39±0.43 U/mL. However, the inulinase activity of the recombinant yeast Y13 among all the transformants with the optimized *exo*-inulinase gene *INU1Y* was 43.84±0.55 U/mL, which represented a 37.4 % increase compared to that produced by the recombinant yeast EX3 with the native *INU1* gene (Table 3). In addition, the inulinase activity of the wild-type strain *Saccharomyces* sp. W0 was only 2.72±0.17 U/mL (Table 3). All these results demonstrated that the expression of the codon-optimized gene *INU1Y* was indeed greatly enhanced in *Saccharomyces* sp. W0 compared to that of the native gene *INU1* from *M. guilliermondii*.

To prove that the linear DNA fragments carrying the inulinase genes had been integrated into the genomic DNA of *Saccharomyces* sp. W0, the genomic DNAs from the corresponding recombinant yeasts and the uracil mutant of *Saccharomyces* sp. W0 were extracted and used as the templates for PCR checking. The PCR products amplified from the genomic DNAs from the recombinant yeasts Y13 and EX3 were of the expected size, namely 1732 bp (Fig. 2), indicating that the native and optimized inulinase genes had indeed been integrated into the genomic DNA of the recombinant yeasts EX3 and Y13, respectively. However, no such PCR product size was amplified from the genomic DNA of the uracil mutant of *Saccharomyces* sp. W0 without the inulinase gene (Fig. 2).

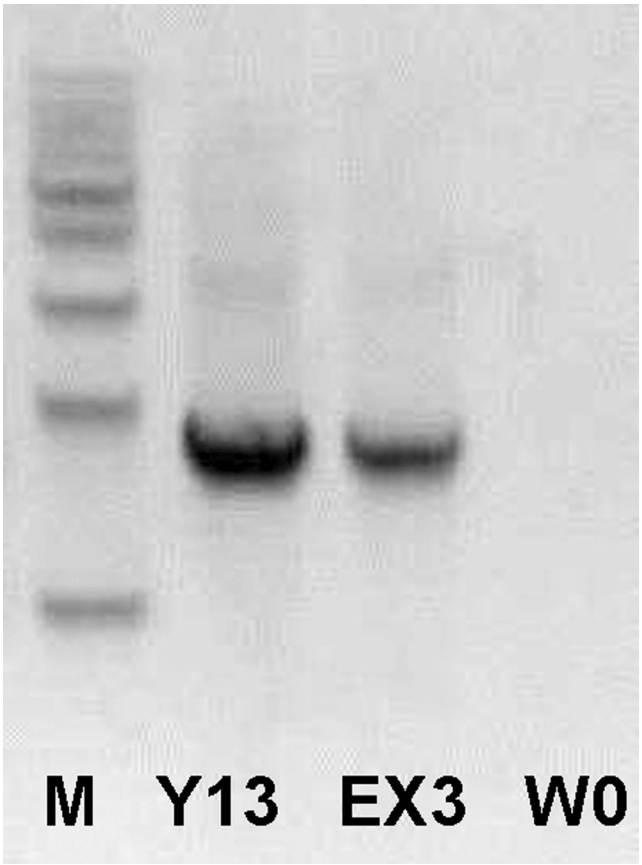

Fig. 2 PCR checking of the transformants and *Saccharomyces* sp. W0. Lane *M*: 1 kb DNA ladder marker which sizes were from 1 to 10 kb; Lane *Y13*, lane *EX3*, and lane *W0*: PCR products from the genomic DNAs of the recombinant yeasts EX3, Y13, and *Saccharomyces* sp. W0, respectively

Table 3 The *exo*-inulinase activities of the different transformants and wild-type strain *Saccharomyces* sp. W0

Strain	*Exo*-inulinase activity (U/mL)
W0	2.72±0.17
EX3 (transformed with the native *INU1* gene)	31.39±0.43**
Y13 (transformed with the optimized *INU1Y* gene)	43.84±0.55**††

Enzyme activities were assayed using inulin as a substrate by determining the concentration of reducing sugars released. The reaction was performed as described in "Materials and methods." Data were given as means±SD, $n=3$.

**($P<0.01$): significant difference compared with the W0 group; ††($P<0.01$): significant difference compared with the EX3 group

Effects of the codon optimization of the *exo*-inulinase gene on its transcription level

It has been confirmed that codon-usage bias is correlated with the abundance of cognate tRNAs available within the cells, therefore with the translation efficiency (Gustafsson et al. 2004; Kanaya et al. 2001). However, whether the codon optimization had an effect on the transcription level of the *exo*-inulinase gene or not was still unknown in this study. Therefore, real-time RT-PCR by using the total RNA of strain EX3, Y13, and W0 was conducted as described above. The result in Fig. 3 showed that, the relative transcription level of the *exo*-inulinase gene was clearly increased from 100 % in the recombinant yeast EX3 with the native gene *INU1* to 212.9 % in the recombinant yeast Y13 with the optimized gene *INU1Y*, while that of the *exo*-inulinase gene in the cells of

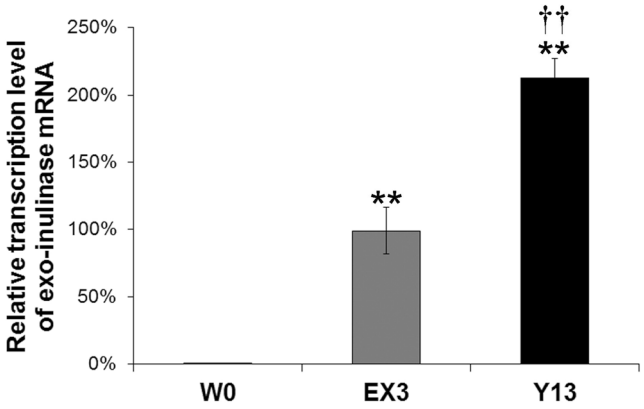

Fig. 3 The relative transcription levels of the *exo*-inulinase mRNA of *Saccharomyces* sp. W0, the recombinant yeast EX3, and Y13. Data were given as means±SD, $n=3$. **($P<0.01$): significant difference compared with the W0 group; ††($P<0.01$): significant difference compared with the EX3 group

Saccharomyces sp. W0 without the inulinase gene was almost 0 %.

Ethanol production from inulin

To test whether the inulin utilization and ethanol production of the recombinant yeast Y13 transformed with the optimized *exo*-inulinase gene *INU1Y* could be more efficient than those of the recombinant yeast EX3 with the native *exo*-inulinase gene *INU1* during the ethanol fermentation, ethanol fermentation from inulin by the yeast strains EX3, Y13, and W0 was conducted as described in "Materials and methods". It can be seen from Table 4 that more ethanol in the medium containing 300.0 g/L inulin was produced by all the strains than that in the medium containing 250.0 g/L inulin. Furthermore, the results in Table 4 indicated that the ethanol concentrations produced by the recombinant yeast Y13 were 114.45 and 126.30 mg/mL in the media containing 250.0 and 300.0 g/L inulin, respectively, which were obviously higher than those produced by the recombinant yeast EX3 (113.15 and 122.75 mg/mL) and *Saccharomyces* sp. W0 (97.45 and 114.15 mg/mL) under the same conditions ($P<0.01$, Table 4). Moreover, it also can be observed from the data in Table 4 that the utilization of total sugar and reducing sugar by the recombinant yeasts Y13 and EX3 were both above 90 % and about 99 %, respectively.

To investigate how the codon optimization of the *exo*-inulinase gene improves ethanol production from inulin, the changes in residual reducing sugar concentration and ethanol concentration within 48 h of the fermentation were analyzed. As shown in Fig. 4a, c, the ethanol concentration caused by the recombinant yeast Y13 was higher than that caused by the recombinant yeast EX3 and *Saccharomyces* sp. W0 within the same period. In addition, the recombinant yeast Y13 showed a remarkable ability in the hydrolysis of inulin and production of reducing sugar because of its higher inulinase activity than any other strains used in this study. It meant that more reducing sugar could be converted to ethanol by the recombinant yeast Y13 than by any other strains used in this study. Furthermore, it also could be observed in Fig. 4b, d that the ethanol productivities for the recombinant yeast Y13 grown in the ethanol production media containing 300.0 g/L inulin and 250.0 g/L inulin were 2.25±0.02 and 2.08±0.06 mg/mL/h within 48 h, while the ethanol productivities for the recombinant yeast EX3 were 1.97±0.01 and 1.95±0.03 mg/mL/h and the ethanol productivities for *Saccharomyces* sp. W0 were 1.77±0.05 and 1.70±0.04 mg/mL/h under the same conditions. Thus, compared to the recombinant yeast EX3, the recombinant yeast Y13 with the optimized *exo*-inulinase gene *INU1Y* showed enhanced ethanol productivity both in the ethanol production media containing 250.0 g/L inulin ($P<0.05$, Fig. 4b) and 300.0 g/L inulin ($P<0.01$, Fig. 4d).

Discussion

Our previous studies indicated that *Saccharomyces* sp. W0 had high ethanol tolerance and produced high concentration of ethanol from glucose and sucrose (Chi and Liu 1993; Wang

Table 4 Ethanol production from different concentrations of inulin in a 150-mL fermentation system

Strain	Inulin concentration (g/L)	Ethanol concentration (mg/mL)	Utilization of total sugar (%)	Utilization of reducing sugar (%)
W0	250.0	97.45±0.12	87.59±3.18	98.76±0.32
EX3	250.0	113.15±0.22**	90.03±2.71	98.80±0.33
Y13	250.0	114.45±0.24**††	92.13±0.99	99.06±0.12
W0	300.0	114.15±0.16	87.41±1.67	98.49±0.20
EX3	300.0	122.75±0.13**	90.69±2.94	99.07±0.29
Y13	300.0	126.30±0.13**††	92.69±0.82**	99.12±0.10**

Data are given as means±SD, $n=3$.

**($P<0.01$): significant difference compared with the W0 group; ††($P<0.01$): significant difference compared with the EX3 group

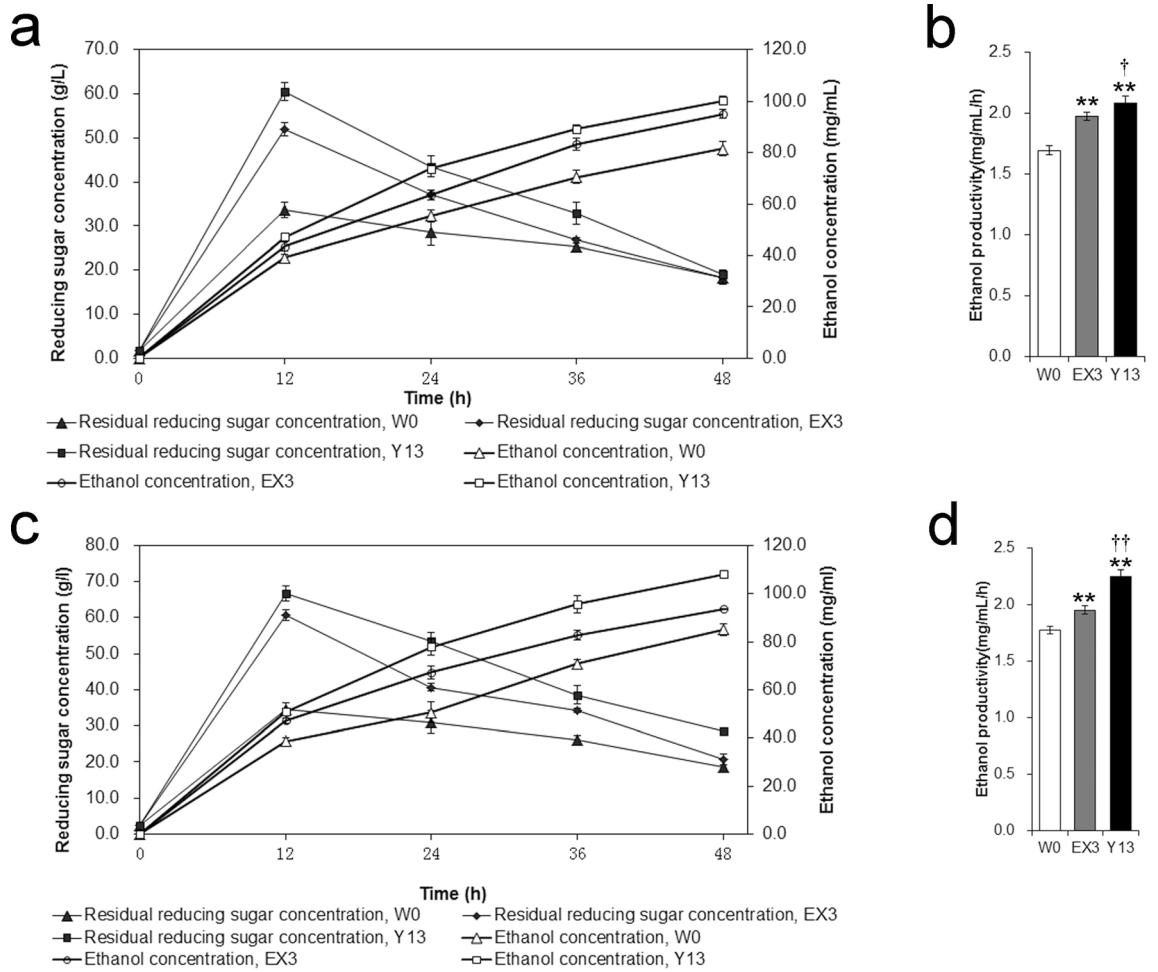

Fig. 4 Time course of ethanol production from inulin by *Saccharomyces* sp. W0 and the recombinant yeasts EX3 and Y13 and their ethanol productivities within 48 h in ethanol production media containing 250.0 g/L (**a**, **b**) and 300.0 g/L (**c**, **d**) inulin, respectively. Changes in reducing sugar concentrations during the fermentation were determined (**a**, **c**). Data were given as means±SD, n=3. **(P<0.01): significant difference compared with the W0 group; ††(P<0.01): significant difference compared with the EX3 group

et al. 2011). However, *Saccharomyces* sp. W0 had weak ability to produce ethanol from inulin as the invertase produced by this high ethanol-producing yeast had low inulinase activity (Li et al. 2013). It has been reported that codon usage bias is evolved relative to regulating gene expression levels, and rare codons could reduce the efficiency of translation or even disengage the translational machinery (Angov 2011). Therefore, in this study, the native inulinase gene *INU1* from the marine yeast *M. guilliermondii* strain 1 was optimized according to the most commonly used codons in *S. cerevisiae* (Figs. 2 and 3, Table 2). After the optimized gene *INU1Y* and native gene *INU1* were ligated into the expression vector in *S. cerevisiae* and overexpressed in *Saccharomyces* sp. W0 (Fig. 1), the inulinase activity of the recombinant yeast Y13 among all the transformants was remarkably higher than that of the recombinant yeast EX3 and their wild-type strain W0 (Table 3). Previously, several successful efforts have been made to express inulinase genes from various hosts including yeasts, molds, and bacteria in *S. cerevisiae* (Liu et al. 2013). For example, the *endo*-inulinase gene *inuA* from *A. niger* was expressed in *S. cerevisiae* strain JZ1C, and the inulinase activity of the recombinant yeast JZ1C-I obtained was 3.1 U/mL (Yuan et al. 2013a, b). Furthermore, the *endo*-inulinase gene *EnIA* from *Arthrobacter* sp. S37 was expressed in *Saccharomyces* sp. W0, the same strain as used in this study, and the inulinase activity of the recombinant yeast D5 reached 8.6 U/mL (Li et al. 2013). It has been found that the engineered strains of *S. cerevisiae* carrying the *exo*-inulinase gene show a higher inulinase activity than those carrying the *endo*-inulinase gene. For example, the *exo*-inulinase gene from *K. marxianus* NCYC2887 was overexpressed by using the GAL10 promoter in a *S. cerevisiae* △*gal80* strain, and the recombinant inulinase activity was 34.6 U/mL (Lim et al. 2010). Moreover, our recent results have indicated that after *INU1* from *M. guilliermondii* strain 1 was actively expressed in *Saccharomyces* sp. W0, the best transformants obtained could produce 34.2 and 34.8 U/mL of extracellular inulinase activities (Wang et al. 2011; Zhang et al. 2010b). So this study

was a resultful attempt to enhance the production of the recombinant *exo*-inulinase in *Saccharomyces* sp. W0 by codon optimization technology since the recombinant yeast Y13 has shown the highest *exo*-inulinase activity (Table 3, 43.84 U/mL) among all the engineered strains of *S. cerevisiae* reported to date.

Although both the optimized and native gene were integrated into the genomic DNAs of the recombinant yeasts Y13 and EX3, respectively (Fig. 2), the inulinase activity produced by the recombinant yeast Y13 was higher than that produced by the recombinant yeast EX3 (Table 3). Furthermore, the transcriptional level of the optimized gene *INU1Y* in the recombinant yeast Y13 was much higher than that of the native gene *INU1* in the recombinant yeast EX3 (Fig. 3). Indeed, the results shown in Fig. 3 strongly demonstrated that the codon optimization and the elimination of unstable *cis*-acting elements (Table 2) in the *exo*-inulinase gene enhanced the stability of mRNA and improved the relative mRNA abundance. The results from Kudla et al. (2009) also indicated that the codon bias played a predominant role in the stability of mRNA and then influenced global expression levels. Consequently, the improvement of translation efficiency (Online Resource Fig. S2) and the increasing of mRNA stability caused by codon optimization both had effects on the enhancement of the *exo*-inulinase gene expression (Fig. 3).

All the data in Table 4 and Fig. 4 represented that the ethanol production and sugar utilization by the recombinant yeast Y13 carrying the optimized gene *INU1Y* were improved compared to those by the recombinant yeast EX3 carrying the native gene *INU1*. So far, many engineered strains carrying heterogenous *exo*-/*endo*-inulinase genes have been constructed to produce ethanol from inulin. For example, after the *exo*-inulinase gene from *K. marxianus* was expressed in *S. cerevisiae*, 72.5 mg/mL ethanol was produced from Jerusalem artichoke tubers (Yuan et al. 2013a, b). After the *endo*-inulinase gene from *A. niger* was expressed in *S. cerevisiae*, the ethanol concentration produced by the recombinant yeast JZ1C-I was 55.3 mg/mL within 48 h (Yuan et al. 2013a, b) while the recombinant yeast D5 with the *endo*-inulinase gene from *Arthrobacter* sp. produced 106.5 mg/mL ethanol from 300.0 g/L inulin within 120 h (Li et al. 2013). In our previous studies (Wang et al. 2011), the recombinant *Saccharomyces* sp. MguINU1-04 with the native *exo*-inulinase gene from *M. guilliermondii* strain 1 could produce 116.0 mg/mL ethanol in an ethanol production medium containing 300.0 g/L inulin within 120 h. Although those recombinant yeasts produced more ethanol from inulin than their wild-type strains (Li et al. 2013; Wang et al. 2011; Yuan et al. 2013a, b; Yuan et al. 2013a, b), the ethanol concentrations produced by them were still lower than those produced by the recombinant yeast Y13 obtained in this study owe to its enhanced inulinase expression level (Fig. 3) and inulinase activity (Table 3). The results in Fig. 4 also demonstrated that the improvement in inulin hydrolysis by the inulinase produced by the recombinant yeast Y13 could cause rapid increase in reducing sugar within 48 h of the fermentation, thus improving the ethanol productivity from inulin. In addition, when the concentration of inulin in the ethanol production media increased from 250 to 300 g/L, the recombinant yeast Y13 showed an obvious advantage over the recombinant yeast EX3 in the ethanol production (Table 4) and productivity (Figs. 4b, d). This meant that a high inulinase activity could be essential to help the engineered *Saccharomyces* sp. W0 to reach its full potential for the ethanol production from inulin. Consequently, the recombinant yeast Y13 obtained in this study and the inulinase produced by it have highly potential applications in biofuel industry.

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