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ORIGINAL ARTICLE

Inulinase hyperproduction by *Kluyveromyces marxianus* through codon optimization, selection of the promoter, and high-cell-density fermentation for efficient inulin hydrolysis

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Abstract

This study aimed to overexpress an inulinase gene in *Kluyveromyces marxianus* to achieve the inulinase overproduction and preparation of ultra-high-fructose syrup. First, the inulinase gene (*INU1* gene) was overexpressed through codon optimization and selection of a suitable promoter. Then, the inulinase was overproduced by high-cell-density fermentation. Finally, ultra-high-fructose syrup was prepared. It was found that optimization of the codons of the native *INU1* gene encoding inulinase from *Kluyveromyces marxianus* KM-0 made a recombinant strain KM-N70 carrying the optimized *INU1N* gene produce 251.4 U/mL of the inulinase activity. Furthermore, inulinase activity produced by a recombinant KM-KN16 strain carrying the optimized *INU1N* gene directed by the native *TPS1* promoter from *K. marxianus* KM-0 reached 338.5 U/mL and expression level of the optimized *INU1N* gene in the recombinant KM-KN16 strain was also greatly enhanced. During a 10-L fermentation, the recombinant KM-KN16 strain could produce 374.3 U/mL of inulinase activity within 24 h, while during a high-cell-density fed-batch fermentation, the recombinant KM-KN16 strain could produce 896.1 U/mL of inulinase activity and OD_{600nm} value of its culture reached 108. The crude inulinase preparation obtained in this study had an inulinase activity of 18,699.8 ± 736.4 U/g of the crude preparation. It was found that 90.3% of 332.4 g/L of inulin was hydrolyzed to produce 41.0 g/L of glucose and 256.0 g/L of fructose and 91.1% of 328.2 g/L of inulin in the extract of the tubers of Jerusalem artichoke was hydrolyzed to produce 48.3 g/L of glucose and 250.5 g/L of fructose by the crude inulinase preparation (75 U/g of the substrate) within 8 h. The hydrolysates contained major monosaccharides and a trace amount of trisaccharides and the monosaccharides were composed of around 85% fructose and 15% glucose. So far, any other yeasts available have produced only up to 120 U/mL of inulinase activity. Together, this made the recombinant KM-KN16 strain be the best inulinase producer at this moment. The inulinase activity of 18,699.8 ± 736.4 U/g of the crude preparation and the ultra-high-fructose syrup with 41.0 g/L of glucose and 256.0 g/L of fructose were obtained. The inulinase activity obtained in this study was the highest among all the inulinase activities produced by yeast, fungal, and bacterial strains obtained so far.

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Keywords Inulinase hyperproduction · *Kluyveromyces marxianus* · Codon optimization · High-cell-density fermentation · Inulin hydrolysis

Introduction

Inulinases are the enzymes produced by several microorganisms, including some bacteria, fungi, and yeasts, to hydrolyze the β-2,1-glycosidic linkages present in inulin, sucrose, and levan to produce fructose and glucose. These enzymes can be used to hydrolyze inulin to produce ultra-high-fructose syrup and inulooligosaccharides. The produced fructose and glucose can be biotransformed into ethanol, lipids, pullulan, citric acid, single-cell protein, and other chemicals (Chi et al. 2011; Cui et al. 2011; Liu et al. 2013; Ma et al. 2015; Wang et al. 2014; Zhang et al. 2015). Inulin-containing materials can be obtained from the tubers of many plants, such as Jerusalem artichoke, chicory, dahlia, and yacon which are regarded as the non-food grains (Singh et al. 2016; Singh and Shukla 2012; Singh et al. 2017). Therefore, the inulin-containing materials have many advantages over the starchy materials from food grains such as corn, wheat, and rice because of the shortage of food grain, high price of food, and grain insecurity in this world (Chi et al. 2011). So inulinase with high hydrolytic activity against inulin will become more and more important in the biotechnology industries. However, the inulinase activities available are still too low to be used in the biotechnology sections (Liu et al. 2013). In this case, it is very crucial how to enhance inulinase production by metabolic engineering. So far, it has been well documented that some yeast strains such as different strains of *Kluyveromyces marxianus* have much higher potential for producing commercially acceptable yields of the inulinase than fungal, bacterial, and any other yeast strains (Liu et al. 2013; Zhang et al. 2015). In our previous studies (Zhou et al. 2014), disruption of the *MIG1* gene and overexpression of the native *INU1* gene rendered a recombinant strain KM-526 to yield 133.5 U/mL of inulinase activity within a very short time (24 h). Expression of an inulinase gene from the yeast *Kluyveromyces marxianus* CBS 6556 in a methylotrophic host *Pichia pastoris* also made a transformant greatly enhance secretory production of the recombinant inulinase (rKmINU) via methanol induction (Zhang et al. 2012).

Recently, it has been shown that codon optimization of the native and heterologous genes is one of the important and effective techniques to enhance their gene expression (He et al. 2014; Liu et al. 2012) because the synonymous codon substitution can have a significant impact on gene expression, protein translational efficiency, and protein folding (Angov 2011; Saunders and Deane 2010). For example, expression of an optimized inulinase gene *INU1Y* from *Meyerozyma guilliermondii* in *Saccharomyces* sp. W0 greatly enhanced inulinase activity of a recombinant yeast Y13 (Liu et al. 2014).

Trehalose has many physiological functions in adaption of the yeast and fungal cells to extreme environments. Usually, in yeast cells, first, trehalose-6-phosphate is formed from uridine diphosphate glucose (UDPG) and α-glucose-6-phosphate under the catalysis of trehalose-6-phosphate synthase (Tps1). Then, this compound is dephosphorylated to trehalose under the catalysis of trehalose-6-phosphate phosphatase (Tps2) (Chi et al. 2009a, b). It has been known that the promoters of the *TPS1* and *TPS2* genes involved in the trehalose biosynthesis were used to express the target protein because the promoters were reported to be very strong (Chi et al. 2009a, b; Li et al. 2012). For example, the flocculin production and ethanol-induced yeast flocculation can be directed by the promoter of the *TPS1* gene from *Saccharomyces cerevisiae* (Li et al. 2012). However, it is still unknown if the promoter of the *TPS1* gene from different yeasts can affect the expression of the *INU1* gene in *K. marxianus.*

It has been well known that D-fructose is a sugar with the highest sweetness and has also become important owing to their functional properties in addition to its sweetness. The demand for fructose is increasing because of its beneficial role for diabetics, increased iron absorption in children, and increased ethanol removal from the blood of highly intoxicated individuals, in addition to its use as a low-calorie sweetener. In addition, fructose syrup is useful in pastry and confectionary production, as it prevents desiccation and sugar crystallization (Chi et al. 2011). Fructose can be produced from glucose by glucose isomerase. However, this process has many disadvantages. An easier, direct, cheap, and quicker alternative for production of ultra-high-fructose syrup (fructose> 80%) could be enzymatic hydrolysis of inulin using an exo-inulinase with high activity (Chi et al. 2011).

In this study, the *INU1* gene from *K. marxianus* KM-0 was optimized and effects of different *TPS1* promoters on expression of the *INU1* gene in this yeast were examined. Finally, hyperproduction of inulinase by the genetically modified yeast was fulfilled by a high-cell-density fermentation and the produced inulinase was used for preparation of an ultra-high-fructose syrup from inulin.

Materials and methods

Strains and media

The wild-type strain *K. marxianus* KM-0, *K. marxianus* KM-69 which was glucose-derepressed mutant by deletion of the *MIG1* gene, and *K. marxianus* KM-526 overexpressing its own *INU1* gene (Zhou et al. 2014) were used in this study.

Saccharomyces sp. W0 was a high-ethanol-producing yeast for industrial ethanol production (Zhang et al. 2015) and *Guehomyces pullulans* 17-1 was a psychrotolerant yeast isolated from sea sediment in Antarctica (Zhang et al. 2013). The medium composition for cultivation of the seed cultures was the YPD medium. The inulinase production medium contained (g/L) the following: inulin 30.0, yeast extract 15.0, KH_2PO_4 10.0, $(NH_4)_2SO_4$ 10.0, $FeSO_4{\cdot}7H_2O$ 0.5, $CaCl_2{\cdot}2H_2O$ 0.3, $MgSO_4{\cdot}7H_2O$ 0.3, pH 4.5. The *Escherichia coli* strain used for amplification of the plasmids in this study was DH5α [*F*¯ *endA1 hsdR17* (*rK*¯/*mK*$^+$ *110*) *supE44 thi*$^-$ $^{\lambda\kappa-}$ *recA1 gyrlacU* (*80*¯*lacZM15)*] and was grown in 5.0 mL of a Luria broth (LB) at 37 °C overnight (Zhou et al. 2014).

Plasmids

The pMD19-T simple for cloning of PCR products was purchased from TaKaRa Biotechnology Co., Ltd. (Dalian, China). The *E. coli* plasmid pFA6a-GFPS65T-kanMX6 containing the geneticin (G418) resistance gene and the expression vector pMD-rDNA-G418-INU1 were constructed in this laboratory (Zhou et al. 2014).

Isolation of genomic DNA, restriction digestions, and transformation

Yeast genomic DNAs for amplification of the different genes were isolated with TIANamp Yeast Genomic DNA Kits (Tiangen Biotech (Beijing) Co., Ltd.). Restriction endonuclease digestions and DNA ligations were conducted according to the manufacturer's recommendations. *Escherichia coli* was transformed with a plasmid DNA according to Sambrook et al. (1989). *Escherichia coli* transformants were plated onto the LB medium containing 100 μg/mL of ampicillin.

Codon optimization of the native *INU1* gene from *K. marxianus*

A gene (*INU1*) sequence encoding exo-inulinase of *K. marxianus* KM-0 mentioned above was obtained from GenBank (GenBank accession no. X57202.1). The codon usage of the DNA sequence from *K. marxianus* was analyzed using a graphical codon usage analyzer (http://www.gcua.schoedl.de/) and optimized by replacing the codons predicted to be less frequently used in *K. marxianus* with the frequently used ones according to a codon usage database (http://www.kazusa.or.jp/codon/) without altering the encoded amino acid sequence of the inulinase through PCR techniques. The modifications of the *INU1* gene were made throughout its sequence by using the primers (1F, 1R, 2F, 2R, 3F, 3R, 4F, and 4R), including the addition of two restriction sites, *Sal*I and *Pst*I, to the forward primer and reverse primer ends (1F and 4R, Supplementary 1), respectively. The PCR products from the four PCR amplifications were mixed, denatured, allowed to anneal based on the shared bases, and subjected to a fusion PCR using the primers of 1F and 4R as described above. At the same time, the promoter of the native *INU1* gene was PCR amplified from the genomic DNA of *K. marxianus* KM-0 using the primers PF which was added a 5′-*Sac*I site and PR which was added a 5′-*Sal*I site and the terminator of the native *INU1* gene was PCR amplified from the genomic DNA of *K. marxianus* KM-0 using the primers TF which was added a 5′-*Pst*I site and TR which was added a 5′-*Spe*I site (Supplementary file 1). After digestion of all the PCR products with the enzymes *Sal*I, *Sal*I, *Pst*I, and *Spe*I, the digested PCR products were ligated into the plasmid pMD-KM-rDNA-G418 digested with the enzymes *Sac*I and *Spe*I. Finally, the resulting plasmid was named pMD-KM-rDNA-G418-INU1N (Supplementary file 2).

Selection of a suitable promoter for efficient expression of the optimized *INU1N* gene

The promoter of the *GPTPS1* gene was PCR amplified from the genomic DNA of the psychrotolerant yeast *G. pullulans* 17-1 using the primers GP-TPS1prom-F which added a 5′-*Sal*I site and GP-TPS1prom-R which added a 5′-*Sal*I site (Supplementary file 1). The primers GP-TPS1prom-F and GP-TPS1prom-R were designed according to the *GPTPS1* gene (accession number: JX046041.1). The promoter of the *W0TPS1* gene was PCR amplified from the genomic DNA of the high-ethanol-producing yeast *Saccharomyces* sp. W0 using the primers W0-TPS1prom-F which added a 5′-*Sac*I site and W0-TPS1prom-R which added a 5′-*Sal*I site. The primers W0-TPS1prom-F and W0-TPS1prom-R were designed according to the *W0TPS1* gene (accession number: FJ536256.1). The promoter of the *KMTPS1* gene was PCR amplified from the genomic DNA of *K. marxianus* KM-0 using the primers KM-TPS1prom-F which added a 5′-*Sac*I site and KM-TPS1prom-R which added a 5′-*Sal*I site. The primers KM-TPS1prom-F and KM-TPS1prom-R were designed according to the *KMTPS1* gene (accession number: KC900085.1). The native *INU1* promoter in the plasmid pMD-rDNA-G418-INU1 (Zhou et al. 2014) was replaced by each promoter obtained above, respectively. Finally, the resulting plasmids carrying the different promoters and different inulinase genes were obtained (Fig. 1). The *INU1* promoter in the plasmid pMD-KM-rDNA-G418-INU1N (Supplementary File 2) was also replaced by the promoter of the *KMTPS1* gene from *K. marxianus* KM-0 (D in Fig. 1).

Transformation and selection

The plasmid pMD-KM-rDNA-G418-INU1N (Supplementary file 2) and the plasmids carrying the different promoters and different inulinase genes (Fig. 1) obtained above were

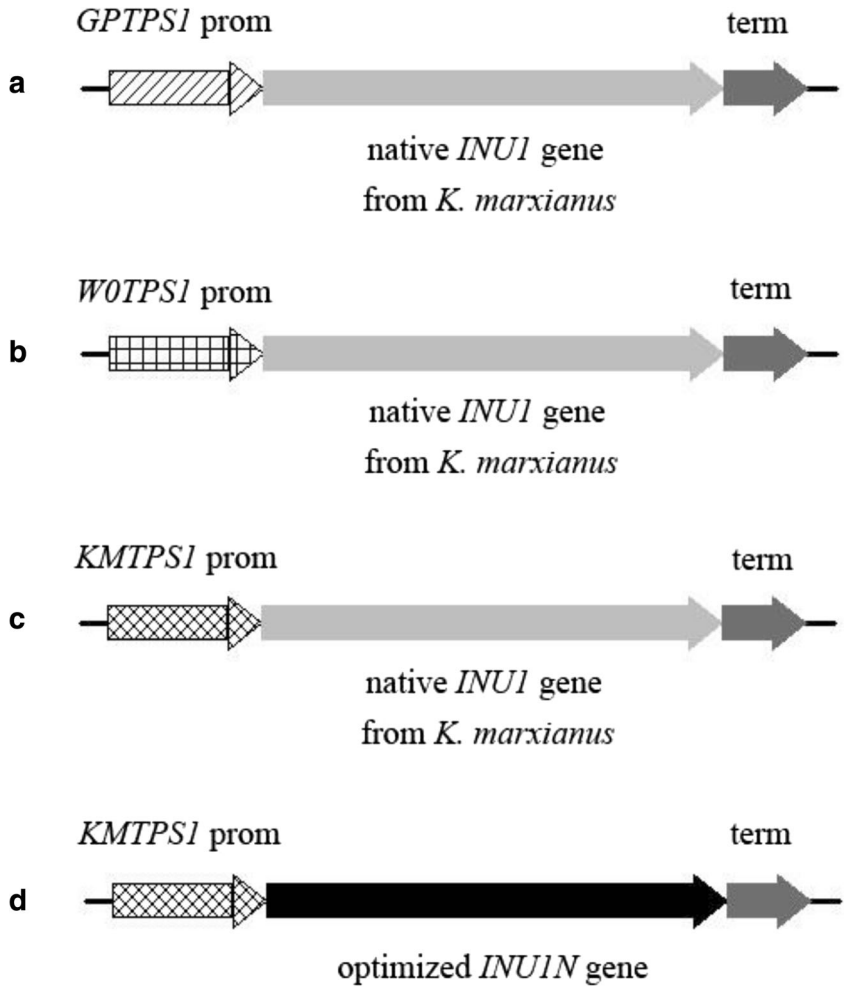

Fig. 1 The linear DNA fragments (A, B, C, and D) which were used to transform the yeast strains. (A) The promoter (*GPTPS1* prom) of the *TPS1* gene from *G. pullulans* 17-1 which was the psychrotolerant yeast isolated from a sea sediment in Antarctica + the native *INU1* gene from *K. marxianus* KM-0. (B) The promoter (*W0TPS1* prom) of the *TPS1* gene from *Saccharomyces* sp. W0 which was the high ethanol producing yeast + the native *INU1* gene from *K. marxianus* KM-0. (C) The promoter (*KMTPS1* prom) of the *TPS1* gene from *K. marxianus* KM-0 + the native *INU1* gene from *K. marxianus* KM-0. (D) The promoter (*KMTPS1* prom) of the *TPS1* gene from *K. marxianus* KM-0 + the optimized *INU1N* gene obtained in this study. term, terminator

digested with the enzyme *Not*I. The recovered fragments carrying the expression cassettes were transformed into the competent cells of *K. marxianus* KM-69 (the glucose-derepressed mutant) by lithium acetate methods, respectively (Sambrook et al. 1989). The transformants were spread on the YPD plates supplemented with 100 μg/mL of geneticin and grown at 28 °C for 96 h. The untransformed *K. marxianus* KM-69 was used as a control. The different transformant colonies were named N9, N26, N70, N96, N120, P49, P29, W1, W104, K20, K31, KN16, and KN32, respectively, and used in the subsequent investigations.

Cultivation of the yeast cells at a flask level

Two wire loops of the yeast cells of *K. marxianus* KM-0, the glucose-derepressed mutant KM-69, and the different transformants obtained above were taken from the fresh slants and transferred to the 250-mL Erlenmeyer flasks containing 50.0 mL of the sterile YPD medium, respectively. The flasks were aerobically incubated at 28 °C and 180 rpm for 12 h. The seed culture (OD_{600nm} = 3.0) was transferred to the new 250-mL Erlenmeyer flasks containing 50.0 mL of the sterile inulinase production medium. The flasks were aerobically incubated at 28 °C and 180 rpm for 48 h. Inulinase activity in the cultures was measured as described below. At the same time, OD value of the cultures was measured at 600 nm using a spectrophotometer. The assays were carried out in triplicate and all the data were the average of the three independent experiments.

Determination of inulinase activity

Inulinase activity in the cultures and supernatants obtained earlier was determined according to Gong et al. (2007). One inulinase unit (U) was defined as the amount of enzyme that produces 1.0 μM of reducing sugar per minute under the assay conditions used in this study. The determinations were carried out in triplicate and all the data were the average of the three independent experiments.

Fluorescent real-time PCR

A fluorescent real-time RT-PCR assay was carried out according to the methods described by Liu et al. (2011). The primers KIS and KIX were designed according to the inulinase gene

sequence (GenBank accession no. X57202.1) and the optimized *INU1N* gene (GenBank accession no. KR612231) (Supplementary file 1). The primers K26S and K26X were designed according to the 26S rDNA gene sequence (GenBank accession no. AB617981) (Supplementary file 1). The measurements were carried out in triplicate and all the data were the average of the three independent experiments.

Inulinase production and cell growth by the recombinant yeast KM-KN16 through batch and high-cell-density fed-batch fermentations

A batch fermentation and a high-cell-density fed-batch fermentation were carried out in a 10-L fermenter (BIOQ-6005-6010B, Huihetang Bio-Engineering Equipment (Shanghai) CO-LTD). The seed cultures were prepared as described above. One hundred forty milliliters of the seed culture (OD_{600nm} = 3.0) was transferred into 7.0 L of the inulinase production medium. The fermentation began with a batch growth phase at 28 °C and pH 4.5. The pH was maintained using sterile 12% ammonium hydroxide. A high level of dissolved oxygen was maintained at approximately 3–5% during the entire process using an agitation rate of 450 rpm and an aeration rate of 800 L/h. After the level of total sugar was decreased to low (below 20.0 g/L), a continuous inulin feeding was carried out at 12, 18, 24, 30, 36, 42, 48, 54, 60, 66, 72, 78, 84, 90, 96, and 102 h of the fermentation until the amount of total sugar reached about 30.0 g/L after each feeding. The fed-batch fermentation was continued for 108 h. Only 10.0 mL of the culture was collected in the interval of 6 h and was centrifuged at 5000×*g* and 4 °C for 5 min, and inulinase activity and cell density in the culture, the amounts of residual total sugar, and residual reducing sugar in the supernatant obtained were determined as described above and below. The fermentations were carried out in triplicate and all the data were the average of the three independent experiments.

Determination of reducing sugar and total sugar in the fermented media

A total reducing sugar and a reducing sugar in the culture were determined by using the Nelson–Somogyi method (1966). The determinations were performed in triplicate and all the data were the average of the three independent experiments.

Making of the crude inulinase preparation

The cell cultures from the fermenter mentioned above were centrifuged at 5000×*g* and 4 °C for 10 min and the supernatants obtained were lyophilized at 0 °C using a vacuum freeze-drier for 24 h. Inulinase activity in the solids (the crude inulinase preparation) formed during the lyophilization was determined as described above.

Inulin hydrolysis by the crude inulinase preparation

The extract of Jerusalem artichoke tubers was prepared according to the method described by Wang et al. (2013) and the final concentration of total sugar was adjusted to 328.2 g/L. An inulin solution (332.0 g/L) was prepared by dissolving 332.0 g of inulin into distilled water and the final volume was 1.0 L. A small amount of the crude inulinase preparation was added to the extract and the inulin solution and the final inulinase concentration was 75 U/g of the substrate, respectively. The suspensions were incubated at pH 4.5–5.0 and 50 °C for 8 h. The amount of total reducing sugars in the hydrolysates was determined using the Nelson–Somogyi method (Spiro 1966). The amount of total sugars was determined as reducing sugars after acid hydrolysis in 0.1 N HCl at 100 °C for 1 h. The extents of inulin hydrolysis (%) were calculated as (the amount of reducing sugars released the amount of total sugars) × 100. D-Glucose was assayed calorimetrically using a glucose oxidase and peroxidase kit (Boehringer Mannheim GmbH, Mannheim, Germany). D-Fructose was measured using a Fructose Assay Kit (Sigma-Aldrich, USA). The hydrolysates were also withdrawn and identified to ascertain the extent of hydrolysis by ascending thin-layer chromatography (Silica gel 60, MERCK, Germany) with the solvent system of n-butanol–ethanol–water (5:3:2) and a detection reagent comprising 20.0 g/L diphenylamine in acetone–850.0 g/L phosphoric acid–aniline (50:5:1, *v*/*v*/*v*) (Gong et al. 2007).

Statistical analysis

Statistical tests (two-tailed paired *t* test and two-tailed two-sample *t* test) were performed using a Statistical Package for the Social Sciences (SPSS) (ver.19; IBM Corporation, USA) with significance set at $\alpha = 0.05$. The results of two independent experiments, each carried out in duplicate (means ± SD), are presented in all the figures and tables shown in this study.

Results and discussion

Optimization of the codons in the *INU1* gene

The analysis by using the graphical codon usage analyzer revealed that the native *INU1* gene from *K. marxianus* contained many rare codons, including instances of CTC, CTT, GGA, GCG, and GGC, which shared only 20 and 10% relative adaptiveness in *K. marxianus*, respectively (Supplementary file 3). Therefore, these rare codons of the native *INU1* gene were replaced by the preferred ones (TTG,

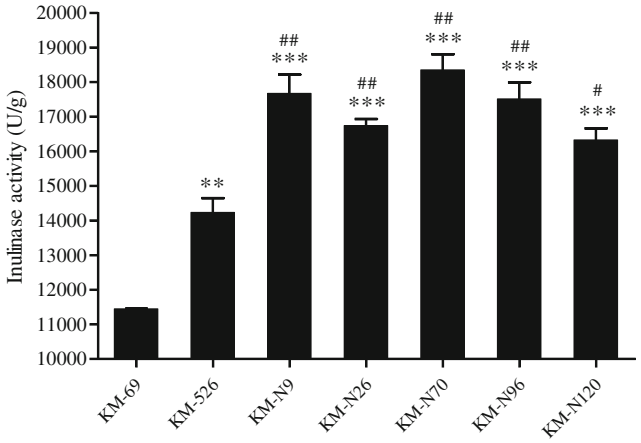

Fig. 2 The specific inulinase activities of the different transformants: the strain KM-69 was the *mig1* disruptant; the strain KM-526 was the overexpressing mutant of the native *INU1* gene; the strains KM-N9, KM-N26, KM-N70, KM-N96, and KM-N120 were the transformants carrying the optimized *INU1N* gene. Data are given as mean ± SD. $n = 3$. $^{**}P < 0.01$, $^{***}P < 0.001$: significant difference compared with the KM-69 group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$: significant difference compared with the KM-526 group. U/g means inulinase activity per gram of cell dry weight

GCT, GGT) which appeared more common in *K. marxianus* through the PCR techniques (Supplementary file 3). Consequently, the optimized gene *INU1N* obtained shared a 99.10% nucleotide sequence identity to the native *INU1* gene (Supplementary file 3). Afterwards, the genes *INU1N* and *INU1* directed by the promoter of the native *INU1* gene from *K. marxianus* were inserted into the expression vectors to construct pMD-KM-rDNA-G418-INU1N and pMD-rDNA-G418-INU1, respectively (Supplementary file 2), as described in "Materials and methods." After the linear DNA fragments carrying the *INU1N* and *INU1* genes were integrated into the chromosomal DNA and expressed in the glucose-derepressed mutant *K. marxianus* KM-69, inulinase activities of the transformants N9, N26, N70, N96, and N120 were determined. The data in Fig. 2 showed that all the transformants had higher inulinase activity than *K. marxianus* KM-69 and *K. marxianus* KM-526 overexpressing the native *INU1* gene. Especially, the inulinase activity produced by the transformant KM-N70 was 251.4 U/mL while the inulinase activities produced by *K. marxianus* KM-526 and *K. marxianus* KM-69 were only 214.1 and 160.4 U/mL, respectively (Fig. 2). This meant that expression of the optimized *INU1N* gene resulted in the transformant KM-N70 by which inulinase activity was obviously enhanced. In contrast, expression of the optimized exo-inulinase gene *INU1* from a marine yeast *Meyerozyma guilliermondii* in *Saccharomyces* sp. W0 made a recombinant yeast Y13 with the optimized gene *INU1Y* produce 43.84 U/mL of inulinase activity while that produced by a recombinant yeast EX3 with the native gene *INU1* was only 31.39 U/mL (Liu et al. 2014). 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. Similarly, expression of a native and an optimized endo-inulinase gene (*EnInu*) from *Aspergillus niger* CICIM F0620 in *Pichia pastoris* allowed a recombinant endo-inulinase activity produced by a yeast carrying the optimized endo-inulinase gene to be 4.18 times higher than that produced by a yeast carrying the native endo-inulinase gene (He et al. 2014). The codon optimization of a native *A. niger* α-glucosidase-encoding gene (*aglu*) resulted in a significant enhancement of α-glucosidase (AGL) production (10.1 U/mL) (Liu et al. 2012). They believe that this is due to enhanced translation efficiency as well as more stable mRNA secondary structure. However, expression of an exo-inulinase gene *inuKM* from *K. marxianus* PT-1 in *S. cerevisiae* strain JZ1C only rendered the mutant JZ1C-inuKM to produce 2.73 U/mL of extracellular inulinase activity (Yuan et al. 2013a, b). All these results demonstrated that the expression of the codon-optimized gene *INU1N* was indeed greatly enhanced in *K. marxianus* KM-N70 compared to that of the native gene *INU1* from *K. marxianus* KM-0.

Optimization of the promoters for the inulinase gene expression and inulinase production

It has been known that expression of the *TPS1* and *TPS2* genes in the yeasts *S. cerevisiae* and *G. pullulans* 17-1 can be enhanced when the yeasts are grown in the extreme environments, such as high temperature (Zhang et al. 2013). This is due to the fact that the promoters of the genes have a CCCCT (C4T) sequence and an AGGGG (AG4) sequence and such C4T sequence and AG4 sequence are regarded as the essential components of these stress–response sequences (Zhang et al. 2013). So a transcriptional activator can bind to the promoters of the *TPS1* and *TPS2* genes in *S. cerevisiae* and *G. pullulans* 17-1 to activate expression of these two genes. In contrast, expression of the *TPS1* and *TPS2* genes in the yeasts *K. marxianus* and *Saccharomycopsis fibuligera* is constitutive because the genes have no such a C4T sequence and an AG4 sequence in their promoters so that expression levels of the *TPS1* and *TPS2* genes in the yeasts *K. marxianus* and *S. fibuligera* grown under the normal conditions are always kept high (Zhang et al. 2013; Chi et al. 2009a, b). In order to know if the different promoters from different *TPS1* genes in yeasts can influence expression of the *INU1* gene, first, the *INU1* promoter in the plasmid pMD-rDNA-G418-INU1 (Supplementary file 2) mentioned above was replaced by the *TPS1* promoters from *Saccharomyces* sp. W0 (the high-ethanol-producing yeast), *G. pullulans* 17-1 (the psychrotolerant yeast), and *K. marxianus* KM-0 (the high-inulinase-producing yeast) (A, B, and C in Fig. 1), respectively. Then, effects of the different promoters from the different *TPS1* genes in different yeasts on expression of the native *INU1* gene and inulinase production were examined by transforming the linear DNA fragments A, B, and C shown

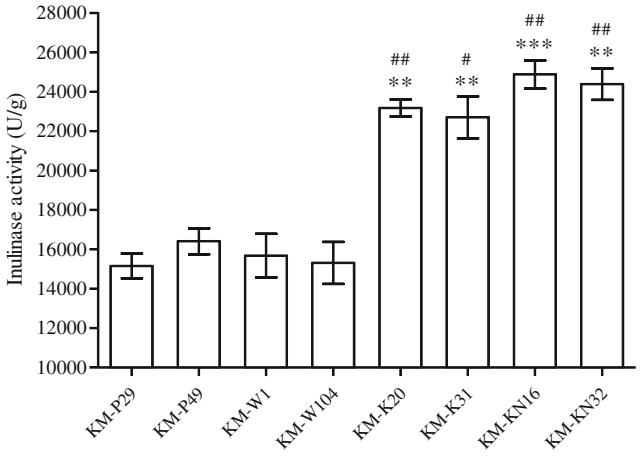

Fig. 3 The specific inulinase activities of the different mutants: the strains KM-P29 and KM-P49 were the mutants carrying the promoter of the *TPS1* gene from the psychrotolerant yeast *G. pullulans* 17-1; the strains KM-W1 and KM-W104 were the mutants carrying the promoter of the *TPS1* gene from the high-ethanol-producing yeast *Saccharomyces* sp. W0; the strains KM-K20 and KM-K31 were the mutants carrying the promoter of the *TPS1* gene from *K. marxianus* KM-0. The strains KM-KN16 and KM-KN32 were the mutants carrying the optimized *INU1N* gene directed by the *TPS1* promoter from *K. marxianus* KM-0. Data are given as mean ± SD, $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$: significant difference compared with the KM-P49 group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$: significant difference compared with the KM-W1 group. U/g means inulinase activity per gram of cell dry weight

in Fig. 1 into the glucose-derepressed mutant *K. marxianus* KM-69. The results in Fig. 3 indicated that inulinase activities produced by the strains KM-K20 and KM-K31 which carried the linear DNA fragment C in Fig. 1 were much higher than those produced by strains KM-P49 and KM-P29 which carried the linear DNA fragment A and by strains KM-W1 and KM-W104 which carried the linear DNA fragment B. This demonstrated that the promoter from the *TPS1* gene in *K. marxianus* KM-0 was much better than any other promoters used in this study. Therefore, the native *INU1* promoter in the plasmid pMD-KM-rDNA-G418-INU1N (Supplementary file 2) mentioned above was replaced by the *TPS1* promoter from *K. marxianus* KM-0, and effects of the promoter on expression of the optimized *INU1N* gene and inulinase production were examined by transforming the linear DNA fragment D (Fig. 1) into the competent cells of the glucose-derepressed mutant *K. marxianus* KM-69. It can be seen from the data in Fig. 3 that the KM-KN16 strain having the linear DNA fragment D (Fig. 1) yielded the highest inulinase activities (338.5 U/mL, 25,030 U/g of cell dry weight). It has been reported that cell–cell flocculation of *S. cerevisiae* was also induced by its own *TPS1* promoter which directed the expression of the flocculin gene (Li et al. 2012). After expression of the *INU1* gene with its own promoter from *K. marxianus* using the integrative cassette, inulinase activity produced by a recombinant strain K/INU2 was 114.9 U/mL under the aerobic conditions while the inulinase activity produced by its wild-type strain was only 52.3 U/mL (Yuan et al. 2013a, b). Overexpression of an exo-inulinase gene from *K. marxianus* NCYC2887 in a *S. cerevisiae* △*gal80* strain made a recombinant strain produce inulinase activity of 34.6 U/mL (Lim et al. 2010). Moreover, overexpression of the *INU1* gene from *M. guilliermondii* strain 1 in *Saccharomyces* sp. W0 rendered the different transformants to produce 34.2 and 34.8 U/mL of extracellular inulinase activities (Wang et al. 2011; Zhang et al. 2010). The inulinase activity of 40.5 U/mL produced by *K. marxianus*YS-1 in an optimized medium has been obtained at a shake flask level (Singh et al. 2006). This meant that the KM-KN16 strain which had the optimized *INU1N* gene directed by the *TPS1* promoter from *K. marxianus* KM-0 could produce much higher inulinase activity than any other yeast strains obtained so far at a flask level.

The transcriptional levels of the *INU1* gene in different yeast strains

The transcriptional levels of the *INU1* gene in the strain KM-69 (the glucose-derepressed mutant), the strain KM-526 overexpressing the native *INU1* gene, the strain KM-N70 overexpressing the optimized *INU1N* gene, the strain KM-K20 overexpressing the native *INU1* gene directed by the *TPS1* promoter from *K. marxianus* KM-0, and the strain KM-KN16 overexpressing the optimized *INU1N* gene directed by the *TPS1* promoter from *K. marxianus* KM-0 were measured as described in "Materials and methods"; it can be clearly observed from the data in Fig. 4 that the transcriptional level of the optimized *INU1N* gene directed by the *TPS1* promoter from *K. marxianus* KM-0 was the highest. These results were consistent with those in Fig. 3.

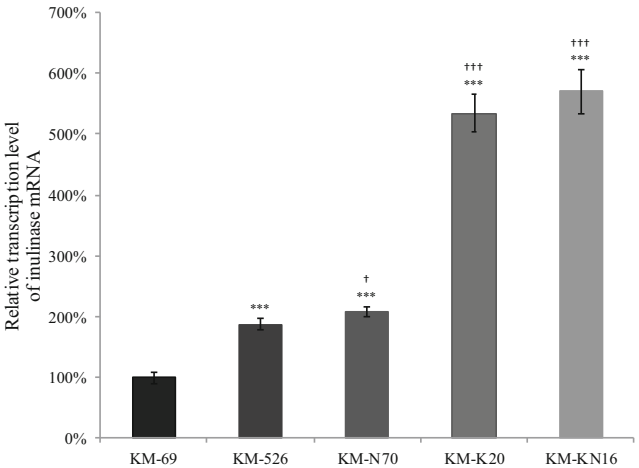

Fig. 4 The transcriptional levels of the *INU1* gene in the different yeast strains. The yeast strains were the same as those shown in Fig. 5. Data are given as mean ± SD, $n = 3$. $^{***}P < 0.001$: significant difference compared with the KM-69 group; $^{\#}P < 0.05$), $^{\#\#\#}P < 0.001$: significant difference compared with the KM-526 group

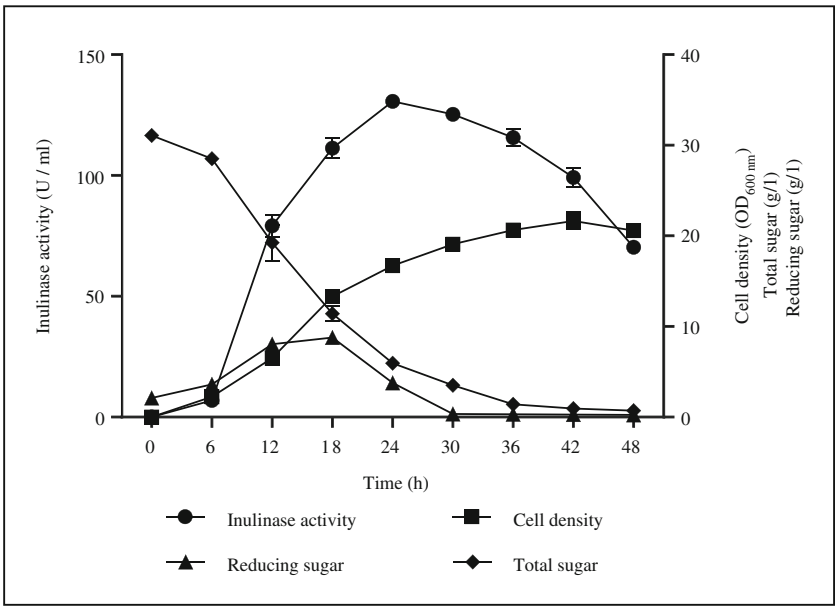

Fig. 5 Time course of inulinase production, cell growth, and the changes in reducing sugar and total sugar when the KM-KN16 strain was grown in the inulin production medium during the 10-L batch fermentation. Data are given as mean ± SD, $n = 3$

Inulinase production by the KM-KN16 strain during 10-L batch fermentation

As shown in Fig. 3, the KM-KN16 strain carrying the optimized *INU1N* gene directed by the *TPS1* promoter from *K. marxianus* KM-0 had 338.5 U/mL of inulinase activity and the expression level of the *INU1N* gene in this yeast strain was also the highest (Fig. 4). Therefore, time course of inulinase production, cell growth, and the changes in the amounts of reducing sugar and total sugar by the KM-KN16 strain during the 10-L batch fermentation was tested. The results in Fig. 5 revealed that within 24 h of the cultivation, the KM-KN16 strain could produce 374.3 U/mL of inulinase activity, leaving 0.66 g/L of total sugar and 0.27 g/L of reducing sugar in the culture and the OD_{600nm} value of the culture was 23.4 within 48 h of the fermentation. It has been reported that the maximum inulinase activity produced by *Kluyveromyces* sp. Y-85 was only 59.5 U/mL during a fermentation (Wei et al. 1998). This meant that the recombinant yeast strain KM-KN16 obtained in this study also produced a very high level of inulinase activity (374.3 U/mL) within the very short time (24 h) during the fermentation.

Inulinase hyperproduction by the KM-KN16 strain during the high-cell-density fed-batch fermentation

It has been well documented that enzyme production can be greatly enhanced by a high-cell-density fermentation (Zhang

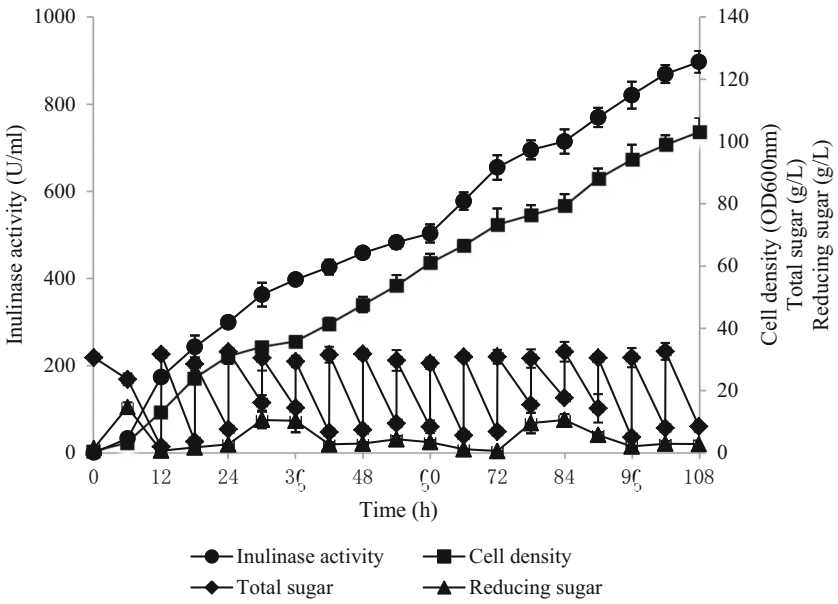

Fig. 6 Time course of inulinase production, cell growth, and the changes in reducing sugar and total sugar by the KM-KN16 strain during the high-cell-density fed-batch fermentation. Data are given as mean ± SD, $n = 3$

Table 1 Inulin hydrolysis by the crude inulinase preparation

Items	Hydrolysate of inulin	Hydrolysate of the extract of the tubers of Jerusalem artichoke
Total sugar	332.4 g/L ± 2.4	328.2 g/L ± 1.8
Hydrolysis degree	90.3% ± 1.2	91.1% ± 2.2
Total glucose	41.0 g/L ± 0.8	48.3 g/L ± 0.7
Total fructose	256.1 g/L ± 5.2	250. 5 g/L ± 4.2

Inulin was hydrolyzed at pH 4.5–5.0 and 50 °C for 8 h and the added inulinase units were 75 U/g of the substrate; data are given as mean ± SD, $n = 3$

et al. 2015). Therefore, inulinase hyperproduction by the KM-KN16 strain was also performed in the 10-L fermenter using the techniques for the high-cell-density fed-batch fermentation as described in "Materials and methods." The

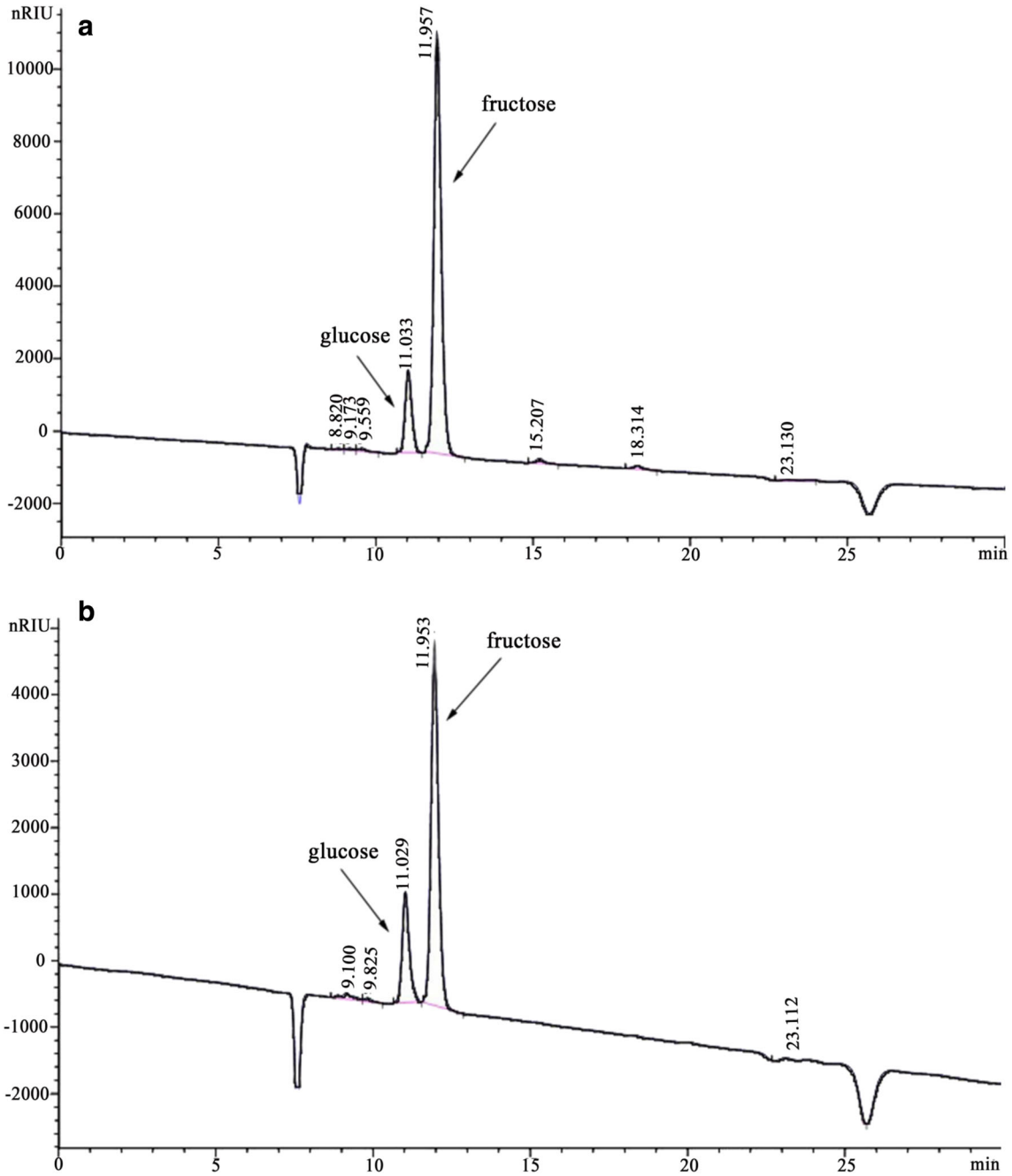

Fig. 7 Analysis of the hydrolyssates of inulin (A) and the extract of the tubers of Jerusalem artichoke (B) by HPLC

results in Fig. 6 showed that within 108 h of the fermentation, the KM-KN16 strain could produce 896.1 U/mL of inulinase activity and OD_{600nm} value of the culture reached 108, leaving 8.37 g/L of total sugar and 2.73 g/L of reducing sugar in the fermented medium. An extracellular inulinase (more than 2 g/L of inulinase) was also yielded in a high-cell-density fed-batch cultivation of the yeast *K. marxianus* CBS 6556 and its final cell mass exceeded 100 g/L (Hensing et al. 1994). It has been reported that expression of an optimized endo-inulinase gene (*EnInu*) from *Aspergillus niger* CICIM F0620 in *P. pastoris* allowed a mutant to produce 1349 U/mL of the recombinant endo-inulinase activity (He et al. 2014). However, when the recombinant *P. pastoris* was applied to an industry for a large scale of endo-inulinase production, the process was very complicated (Zhang et al. 2009). Furthermore, the results in Fig. 6 demonstrated that the high-cell-density fermentation was one of the efficient ways to promote enzyme production and cell growth.

All the results mentioned above demonstrated that the KM-KN16 strain constructed in this study was the best yeast strain for exo-inulinase production and had highly potential applications in biotechnology.

The crude inulinase preparation

The crude inulinase preparation obtained in this study had inulinase activity of 18,699.8 ± 736.4 U/L of the crude preparation (data not shown).

Inulin hydrolysis

After inulin and the inulin in the extract of the tubers of Jerusalem artichoke were hydrolyzed by the crude inulinase preparation as described in "Materials and methods," it was found that 90.3% of 332.4 g/L of inulin was hydrolyzed to produce 41.0 g/L of glucose and 256.0 g/L of fructose (Table 1) while 91.1% of 328.2 g/L of the inulin in the extract of the tubers of Jerusalem artichoke was hydrolyzed to produce 48.3 g/L of glucose and 250.5 g/L of fructose by the crude inulinase preparation (75 U/g of the substrate) within 8 h (Table 1). The results in Supplementary file 2 showed that the hydrolysates contained major monosaccharides and a trace amount of trisaccharides. Analysis of the hydrolysates using HPLC found that the hydrolysate of inulin contained 83.7% fructose and 16.3% glucose (Fig. 7A) while the hydrolysate of extract of the tubers of Jerusalem artichoke contained 75.9% fructose and 24.1% glucose (Fig. 7B). Indeed, most of the hydrolysates from inulin and extract of the tubers of Jerusalem artichoke contained about 85% fructose and 15% glucose (Chi et al. 2011). This meant that inulin could be efficiently hydrolyzed to produce the ultra-high-fructose syrup by using the crude inulinase preparation obtained in this study.

Conclusions

Expression of the optimized *INU1N* gene directed by the promoter of the native *TPS1* gene from *K. marxianus* KM-0 rendered the recombinant KM-KN16 strain to produce 374.3 U/mL of inulinase activity within 24 h during the 10-L fermentation, while during the high-cell-density fed-batch fermentation, the recombinant KM-KN16 strain could produce 896.1 U/mL of inulinase activity and OD_{600nm} value of the culture reached 108. Therefore, the codon optimization, selection of the suitable promoter, and high-cell-density fermentation were very important for high level of inulinase production by the genetically modified strain KM-KN16. We believe that the produced inulinase and the recombinant KM-KN16 strain will have highly potential applications. The crude inulinase preparation obtained in this study had 18,699.8 ± 736.4 U/g of inulinase activity, which was the highest among all the inulinase activities produced by yeast, fungal, and bacterial strains obtained to date. The prepared fructose syrup contained over 85% fructose.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

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