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Enhanced expression of endoinulinase from *Aspergillus niger* by codon optimization in *Pichia pastoris* and its application in inulooligosaccharide production

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Abstract In the present study, the endoinulinase gene (*EnInu*) from *Aspergillus niger* CICIM F0620 was optimized according to the codon usage of *Pichia pastoris* and both the native and the optimized gene were expressed in *P. pastoris*. Use of the optimized gene resulted in the secretion of recombinant endoinulinase activity that reached 1,349 U ml^{-1}, 4.18 times that observed using the native gene. This is the highest endoinulinase activity reported to date. The recombinant enzyme was optimally active at pH 6.0 and 60 °C. Moreover, inulooligosaccharides production from inulin was studied using the recombinant enzyme produced from the optimized gene. After 8 h under optimal conditions, which included 400 g l^{-1} inulin, an enzyme concentration of 40 U g^{-1} substrate, 50 °C and pH 6.0, the inulooligosaccharide yield was 91 %. The high substrate concentration and short reaction time described here should reduce production costs distinctly, compared with the conditions used in previous studies. Thus, this study may provide the basis for the industrial use of this recombinant endoinulinase for the production of inulooligosaccharides.

Keywords Endoinulinase · *Pichia pastoris* · Codon optimization · Inulooligosaccharides · Industrial production

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Introduction

Inulin is composed of linear chains of β-2, 1-linked *D*-fructofuranose molecules that contain a glucose residue linked through a sucrose-type linkage at the reducing terminal [6]. Inulin, which is the second largest non-structural natural polysaccharide after starch [6, 11], is present as a reserve carbohydrate in the roots and tubers of plants, such as Jerusalem artichoke, chicory, dahlia, and yacon. Inulin is resistant to digestion by gastrointestinal enzymes because of the presence of fructose in the β-configuration [12]. Therefore, it is inedible and the development of an inulin-processing industry will not reduce human food sources. Due to its renewable, inexpensive, and abundant source, inulin has received much attention as an excellent natural raw material in recent years.

Inulinases, which can hydrolyze the β-2, 1 linkage of inulin, are divided into the exoinulinases (EC 3.2.1.80) and the endoinulinases (EC 3.2.1.7), which display different cleavage patterns [7]. The exoinulinases digest inulin into individual fructose (or glucose) molecules, beginning at the non-reducing end, while the endoinulinases selectively hydrolyze internal linkages to yield non-digestible inulooligosaccharides (IOS) with a degree of polymerization (DP) of 2–9. These IOS are among the most popular functional food components because they are low-calorie, bifidogenic, and have health-promoting properties [7]. There are numerous reports of high-level exoinulinase expression, while reports of high-level endoinulinase expression are quite fewer [14, 15, 19, 24]. The expression levels of these endoinulinases are elevated, but they are still relatively low and unsuitable for the large-scale production of IOS. In addition, although there have been many reports of the high-level conversion of inulin to IOS, these processes may not be commercially viable, due to their low substrate

concentration (<200 g l^{-1}), long reaction time, and large quantity of enzyme consumption, all of which make production less profitable [9, 13, 22, 23].

It has been reported that endoinulinases from *Aspergillus niger* have superior thermal stability at the optimum temperature for enzyme activity, >55 °C [21], which is conducive to industrial application. *Aspergillus niger* endoinulinase also hydrolyzes inulin specifically into IOS, yielding very small amounts of glucose, fructose, and sucrose byproducts, which simplifies downstream processing. The *Pichia pastoris* expression system is one of the most widely used eukaryotic expression systems. It has many advantages, including high-level expression of the target enzyme and efficient secretion of extracellular proteins [2, 17]. Heterologous expression of enzymes in *P. pastoris* can be enhanced by a variety of different techniques and parameters, including codon optimization [16]. Codon optimization is a method that converts the codons present in a target sequence into codons more frequently used by the expression host [26]. Increasing evidence indicates that the choice of synonymous codons impacts protein production by improving correlation with tRNA levels in the host cell, and changing the secondary structure of the expressed mRNA [1]. In this study, the expression of endoinulinase from *A. niger* CICIM F0620 in *P. pastoris* using the native gene and a codon-optimized construct were both investigated, followed with the basic characterization of the recombinant endoinulinase. Finally, the conditions of the biotransformation of inulin to IOS were optimized, including substrate concentration, pH, and temperature, aimed at establishing a conversion process suitable for industrial IOS production.

Materials and methods

Strains, vectors, reagents, and media

The strain of *A. niger* CICIM F0620 was from China Center for Type Culture Collection (CCTCC). *Pichia pastoris* KM71 and the expression vector pPIC9K were obtained from Invitrogen (Carlsbad, CA, USA). The EZ-10 Spin Column Plasmid Mini-Prep kit, agarose gel DNA purification kit, restriction enzymes, PCR enzyme "Primer STARTM" and T4 DNA ligase were purchased from TakaRa (Otsu, Japan). *Escherichia coli* strain JM109 (Agilent Stratagene, Santa Clara, CA, USA) was used for propagation and manipulation of plasmids. DNA primer synthesis and DNA sequencing were performed by Shanghai Sangon Biological Engineering Technology & Services Co. Ltd. (Shanghai, China). YPD, MD, G418, BMGY, BMMY, and BSM media were prepared according to the manufacturer's instructions for the cultivation of yeast from the Multi-Copy *Pichia* Expression Kit (Invitrogen, Carlsbad, CA, USA). Unless stated otherwise, all the chemicals were of analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Codon optimization and synthesis of the modified gene

The gene encoding endoinulinase (*EnInu,* GenBank Accession No. XM_001395842) was cloned from *A. niger* CICIM F0620. A signal peptide was detected using the SignalP 4.0 server (http://www.cbs.dtu.dk/services/SignalP/). The codon usage of the DNA sequence of *EnInu* was analyzed using the graphical codon usage analyzer (http://gcua.schoedl.de/) and optimized by replacing the codons predicted to be less frequently used in *P. pastoris* with the frequently used ones by DNA works software (http://mcl1.ncifcrf.gov/dnaworks/) without altering the encoded amino acid sequence. Modifications were made throughout the sequence, including the addition of two restriction sites, *Eco*R I and *Not* I, to the forward and reverse end, respectively. The mRNA structure and free energy of mRNA folding were analyzed using the program RNA Structure 5.2. The optimized *EnInu* gene (*EnInuop*) was synthesized by Genewiz (Suzhou, China).

Construction of expression plasmid

Synthetic DNA encoding the mature region of *EnInuop* without the predicted signal sequence was digested with *Eco*R I and *Not* I, gel-purified, and ligated into pPIC9K, yielding the recombinant plasmid pPIC9K-*EnInuop*, which was verified by restriction analysis and sequencing. The native *EnInu* was subjected to the same manipulations, forming the plasmid pPIC9K-*EnInu.*

Transformation of *P. pastoris* and expression of endoinulinase

The recombinant plasmids pPIC9K-*EnInuop* and pPIC9K-*EnInu* were linearized by *Sac* I and transformed into *P. pastoris* KM71 by electroporation using a Gene Pulser (Eppendorf, Hamburg, Germany) electroporator. The transformants were preliminarily selected at 30 °C on MD agar plates and further screened on YPD agar plates with G418 at a final concentration of 2 mg ml^{-1} for 2–4 days. Recombinant *P. pastoris* with multiple copies of the target gene was obtained. For expression, colonies on G418 plates were grown in 10 ml of YPD medium at 30 °C for 24 h, then inoculated into 50 ml of BMGY medium and shaken (200 rpm) at 30 °C for 24 h. The cells were harvested by centrifugation at 8,000 rpm for 5 min and resuspended in 25 ml of BMMY containing an initial methanol concentration of 1 % (v/v). To maintain induction, methanol was

supplemented every 24 h to a final concentration of 1.5 % (v/v) throughout the induction phase. After 5 days of induction, the cells were pelleted by centrifugation at 8,000 rpm for 5 min, and the supernatant was assayed for inulinase activity.

High cell-density fermentation

High cell-density fermentation was carried out in a 3-l bioreactor (New Brunswick Scientific, Edison, NJ, USA). The bioreactor contained 1 l of BSM medium plus 4.35 ml of PTM1 trace salts solution. The fermentation began with batch growth phase at 30 °C and pH 5. The pH was maintained using 100 % ammonium hydroxide. The level of dissolved oxygen was maintained at approximately 20 % during the entire process by a cascaded control of agitation rate and aeration rate. After the level of dissolved oxygen increased, continuous glycerol feeding was carried out until the OD_{600} reached 150. When the dissolved oxygen increased again, a methanol solution was added to the bioreactor. Methanol online control station (FC2002, East China University of Science and Technology) was used to supplement the methanol mixing with PTM1 (12.5 ml PTM1 per liter methanol) to maintain the methanol concentration at 1 %. The induction temperature was 27 °C. The fed-batch fermentation was continued for 144 h and samples were collected every 12 h.

Assay of inulinase activity

The reaction mixture contained 600 μl of 6 % (w/v) inulin derived from chicory (Sangon, Shanghai, China) dissolved in 50 mM sodium acetate buffer (SA buffer, pH 6.0), 10 μl of 0.5 M $CaCl_2$, and 150 μl of appropriately diluted enzyme. The reaction was incubated at 55 °C for 10 min and terminated by being placed in boiling water for 5 min. The amount of reducing sugar liberated was determined using the DNS method [18], measuring the absorbance at 540 nm using a spectrophotometer (Unico, Shanghai, China). One unit of inulinase activity was defined as the amount of enzyme required to produce 1 μmol of reducing sugar per minute under the assay conditions.

Purification of recombinant endoinulinase

The culture supernatant of engineered *P. pastoris* was obtained by centrifugation at 10,000 rpm for 20 min at 4 °C, and then $(NH_4)_2SO_4$ was slowly added to the culture supernatant, with stirring, to a final concentration of 70 % (w/v). The precipitated protein was collected and dissolved in buffer A (20 mM acetate, pH 5.5), and then dialyzed against 2 l of buffer A overnight. The sample was filtered (0.22 μm) and loaded onto a DEAE-cellulose column (2.6 × 50 cm, Sigma, St. Louis, MO, USA) pre-equilibrated with buffer A. The column was eluted at a flow rate of 0.6 ml min^{-1} using a five-column-volume linear gradient of 0–0.4 M NaCl in buffer A. The fractions containing inulinase activity were pooled and then dialyzed against 1 l of buffer A at 4 °C overnight. The dialyzed sample was subjected to mono Q (5 × 50 cm, GE, Fairfield, CT, USA) chromatography using a procedure similar to the one described above. The purified enzyme was stored at −20 °C.

Characterization of the recombinant enzyme

The optimal temperature for activity of the purified recombinant enzyme was investigated by measuring activity at temperatures ranging between 30 and 80 °C in 50 mM acetate buffer, pH 6.0. Since the pH of acetate buffer is temperature-dependent, the pH of each buffer was adjusted to 6.0 at the temperature at which it was used [3]. The substrate was preincubated at each temperature for 5 min. The thermostability of the enzyme was determined by incubating it in 50 mM acetate buffer (pH 6.0) in a 55 °C water bath. Samples were taken and assayed for residual activity every 2 h. The influence of pH on inulinase activity was determined at 55 °C using 50 mM buffers containing acetate (pH 4.0–7.0) or Na_2HPO_4–KH_2PO_4 (pH 6.0–9.0). The pH stability was determined by incubating the enzyme in the buffers described above for 24 h at 4 °C, and then assaying for residual activity at pH 6.0.

Inulin hydrolysis by crude recombinant enzyme

Inulin for the study of enzymatic degradation was purchased from Sigma (St. Louis, MO, USA). Inulin hydrolysis was carried out under various conditions including substrate concentration, pH, and temperature, in a stoppered 100-ml flask incubated in a rotary shaker. To investigate the effect of substrate concentration on IOS production, reactions were carried out with an inulin concentration of 200, 400, or 600 g l^{-1}, using an enzyme concentration of 40 U g^{-1} substrate at 60 °C and pH 6.0. To test the effect of pH on IOS production, reactions were carried out in buffers of pH from 4.0 to 9.0, under conditions of 400 g l^{-1} inulin as substrate, 60 °C, pH 6.0, and enzyme concentration of 40 U g^{-1} substrate. To investigate the influence of temperature on IOS production, enzyme reactions were carried out at different temperatures ranging from 30 to 70 °C under conditions of 400 g l^{-1} inulin as substrate, pH 6.0, and enzyme concentration of 40 U g^{-1} substrate. Samples were taken at regular intervals, at which point the hydrolysis was stopped by heating the samples in a boiling water bath for 5 min.

HPLC analysis

The products of enzymatic hydrolysis were analyzed by HPLC (HITACHI, Japan) using a NH_2 column (4.6 × 250 mm, Thermo, Germany) and a HITACHI L-2490 RI detector. The eluent was a mixture of 72 % acetonitrile and 28 % water, and the flow rate was 0.8 ml min^{-1}. Total IOS yield was estimated as the sum of all IOS with DP ranging from 2 to 9. DP 2–4 were quantified by an external standard method and others were estimated by the ratio of their peak areas to that of DP 2–4.

Miscellaneous methods

Protein concentration was determined by the Bradford method using BSA (bovine serum albumin) as the standard. The optical density at 600 nm (OD_{600}) was monitored using a UV-2450 ultraviolet–visible spectrophotometer (Shimadzu, Kyoto, Japan). SDS-PAGE was performed on a 12 % polyacrylamide gel. The gel was visualized with 0.25 % Coomassie Brilliant Blue R-250.

Results and discussion

Synthesis of a codon-optimized gene and expression in *P. pastoris*

Analysis by graphical codon usage analyzer revealed that the native *EnInu* is harboring nearly 12 % codons, including instances of GGG (Gly), UCG (Ser), CCG (Pro), and CUC (Leu), which shared <10 % usage in *P. pastoris*. These rare codons were replaced by preferred ones which appear more common in *P. pastoris* (Table 1), and the G+C content

Table 1 Comparison of codon usage between *EnInu* and *EnInuop*

AA	Codon	Host fraction	*EnInu*	*EnInuop*	AA	Codon	Host fraction	*EnInu*	*EnInuop*
Arg	AGA	0.48	0	14	Val	GUA	0.16	4	0
	AGG	0.16	0	0		GUC	0.23	10	8
	CGG	0.1	7	0		GUG	0.19	9	7
	CGA	0.05	3	0		GUU	0.42	12	20
	CGU	0.17	2	0	Ser	AGC	0.09	5	0
	CGC	0.05	2	0		AGU	0.15	10	10
His	CAC	0.43	7	5		UCA	0.18	6	12
	CAU	0.57	4	6		UCC	0.20	10	12
Gly	GGA	0.33	10	14		UCG	0.09	12	0
	GGC	0.14	16	0		UCU	0.29	7	16
	GGG	0.10	11	0	Thr	ACA	0.24	15	12
	GGU	0.44	8	31		ACC	0.26	19	12
Ala	GCA	0.23	7	9		ACG	0.11	11	0
	GCC	0.26	15	10		ACU	0.40	4	25
	GCG	0.06	4	0	Cys	UGC	0.36	1	1
	GCU	0.45	10	17		UGU	0.64	1	1
Leu	CUA	0.11	3	0	Pro	CCU	0.35	6	9
	CUC	0.08	8	0		CCC	0.15	6	0
	CUU	0.17	2	6		CCA	0.42	5	16
	UUA	0.16	1	5		CCG	0.09	8	0
	UUG	0.33	6	17	Glu	GAA	0.56	10	12
	CUG	0.16	15	7		GAG	0.44	11	9
Asp	GAC	0.42	14	15	Lys	AAA	0.47	5	6
	GAU	0.58	21	20		AAG	0.53	8	7
Asn	AAC	0.52	16	12	Met	ATG	1.00	9	9
	AAU	0.48	7	11	Trp	UGG	1.00	16	16
Ile	AUA	0.18	4	4	Tyr	UAC	0.53	7	6
	AUC	0.31	9	7		UAU	0.47	4	5
	AUU	0.50	9	11	Phe	UUC	0.46	10	9
Gln	CAA	0.61	9	14		UUU	0.54	9	10
	CAG	0.39	13	8	Stop	UGA	–	1	1

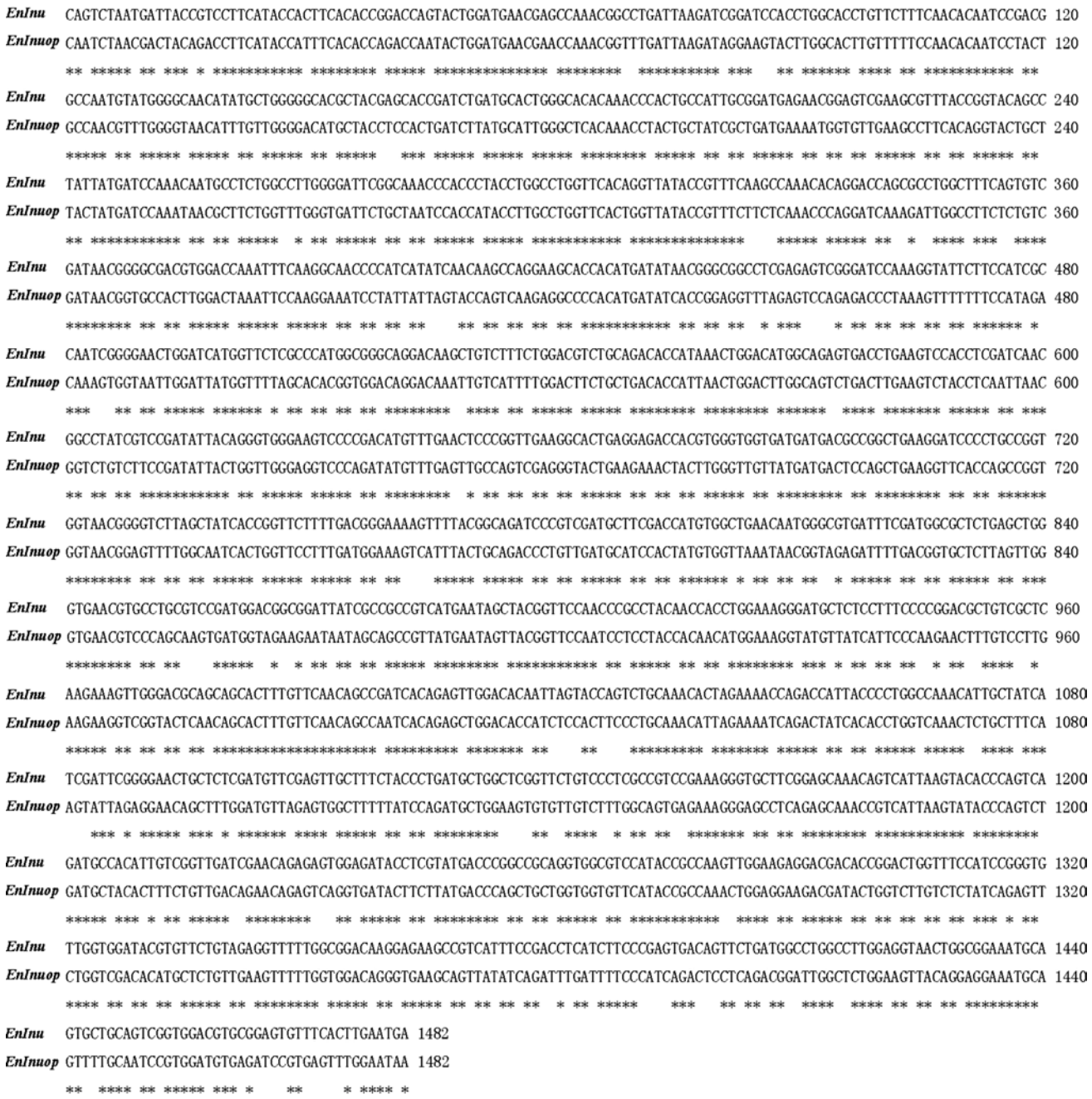

Fig. 1 Alignment of the nucleotide sequences of *EnInu* and *EnInuop* starting from the first residue of the mature protein. Identical nucleotides are marked with an *asterisk*

was adjusted to an appropriate range of 44.4–54.3 %, which is much closer to the G+C content of *P. pastoris*. In addition, the mRNA secondary structure around the start codon was eliminated. The free energy of folding of the resulting mRNA was increased from −512.3 to −423.9 kcal mol^{-1} according to the program RNA Structure 5.2, which indicated less stable mRNA secondary structure after codon optimization. The optimized gene (*EnInuop*) shared 75.30 % nucleotide sequence identity with the native gene (*EnInu*) (Fig. 1). The *EnInuop* and *EnInu genes* were each inserted into the expression vector pPIC9K, which was linearized and used to transformed *P. pastoris* KM71. A control strain was constructed using the empty pPIC9K vector.

As an initial test of expression, both *EnInu*- and *EnInuop*-containing colonies were grown in shake flasks, and expression was induced for 120 h using methanol. The

highest inulinase activity obtained from an *EnInuop*-containing strain reached 26.5 U ml^{-1}, which was 1.59-fold that of the highest-expressing *EnInu*-containing strain (16.7 U ml^{-1}). No inulin hydrolase activity was detected in the culture supernatant of the control strain under the same culture conditions.

Zhang et al. [25] have identified an exoinulinase, inuA1, from *A. niger* AF10 that has an amino acid sequence that is almost the same as that of *EnInuop,* except for residue Ser 443. Despite this substantial identity, no invertase activity was detected using EnInuop. This suggests that residue Ser 443 plays a key role in determining the cleavage pattern of inulinase.

Enzyme production and purification

The expression efficiency of the engineered *P. pastoris* was further explored in a 3-l bioreactor. After methanol induction fermentation in a 3-l bioreactor for 120 h, the protein concentration in supernatant increased 215 %, from 0.7 to 2.21 g l^{-1}, and the activity of EnInuop in the supernatant reached 1,349 U ml^{-1}. This represents a 418 % increase compared with expression experiments using the native gene, which produced a supernatant containing 260 U ml^{-1} (Fig. 2a). Previously, several successful attempts have been made to identify, clone, and express endoinulinase gene from various hosts including molds and bacteria [12]. Wang et al. [24] expressed endoinulinase from *A. niger* 9891 in *P. pastoris* GS115, the activity of the recombinant enzyme reached 291 U ml^{-1} by optimization of the fermentation in a 7-l of bioreactor. The endoinulinase gene (*EnIA*) from *Arthrobacter* sp. S37 was expressed in *Yarrowia lipolytica* Po1 h with the endoinulinase activity of 16.7 U ml^{-1} [15]. Although previous recombinant expressions for endoinulinase were much higher compared with their native strains [7, 12], they still could not meet the needs of industrial application well. In this study, the production of EnInuop reached 1,349 U ml^{-1} after codon optimization according to the codon bias of *P. pastoris* and high cell-density fermentation, which was much higher than the expression systems mentioned above. To our knowledge, this is the highest expression level of endoinulinase reported so far [7, 12].

The recombinant inulinase was purified as the procedures shown in Table 2. By ammonium sulphate precipitation, DEAE-cellulose column chromatography and Mono Q column chromatography, the enzyme was purified to homogeneity with a 2.2-fold increase in the specific activity with a yield of about 21.3 % and had a specific activity of 1,260 U mg^{-1} protein. SDS-PAGE showed a single band around 59 kDa (Fig. 2b).

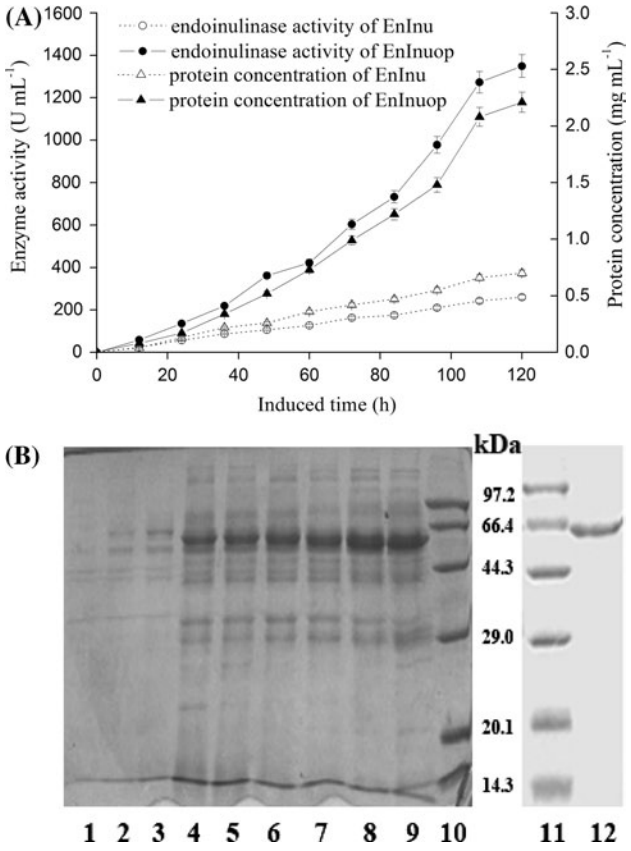

Fig. 2 **a** Time profiles for batch cultivations of recombinant *P. pastoris* in a 3-l bioreactor. *Error bars* correspond to the standard deviation of three independent determinations. **b** SDS-PAGE analysis of culture supernatant of *P. pastoris* KM71/pPIC9K-*EnInuop* in a 3-l bioreactor during the induction phase. *Lanes 1–9* Culture supernatant of recombinant *P. pastoris* after methanol induction for 0, 12, 36, 48, 60, 84, 96, 108, and 120 h, respectively. *Lanes 10–11* Protein weight markers. *Lane 12* Purified endoinulinase

Characterization of the purified recombinant enzyme

The temperature profile showed that under assay conditions used, the temperature for optimal activity of the endoinulinase was determined to be 60 °C (Fig. 3a), which was in accordance with the optimum inulinases temperatures of 45–60 °C from other molds [12]. Moreover, the enzyme retained 54 % of its activity after 8 h at 50 °C, and retained 55 % activity after 4 h at 60 °C (Fig. 3b), which displayed good thermostability among the inulinase family [4, 7]. Since temperatures ≥60 °C ensure proper solubility of inulin and prevent microbial contamination [20], thermostability of the endoinulinase at 60 °C is definitely beneficial for industrial applications.

The effects of pH on the activity and stability of the recombinant endoinulinase were also determined. As

Table 2 Purification of endo-inulinase from recombinant *P. pastoris*

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Purification fold	Yield (%)
Crude extract	120	67,450	562	1.0	100
$(NH_4)_2SO_4$ precipitation	59.0	42,966	728	1.3	63.7
DEAE-cellulose	22.9	23,877	1,043	1.9	35.4
Mono Q	11.4	14,367	1,260	2.2	21.3

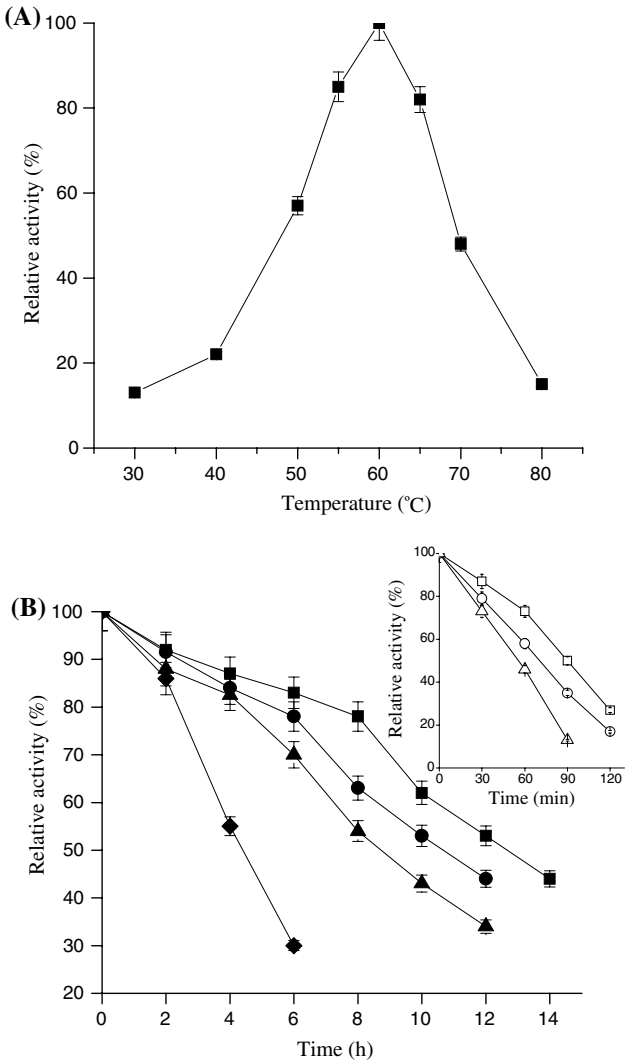

Fig. 3 Effects of temperature on activity and stability of recombinant endoinulinase. **a** Temperature optimum. The activity of recombinant endoinulinase at 60 °C was defined as 100 %. **b** Thermostability of the enzyme, 30 °C (*filled square*), 40 °C (*filled circle*), 50 °C (*filled triangle*), 60 °C (*filled diamond*), 65 °C (*open square*) 70 °C (*open circle*), and 80 °C (*open triangle*). The activity of recombinant endoinulinase without heat treatment was defined as 100 %. *Error bars* correspond to the standard deviation of three independent determinations

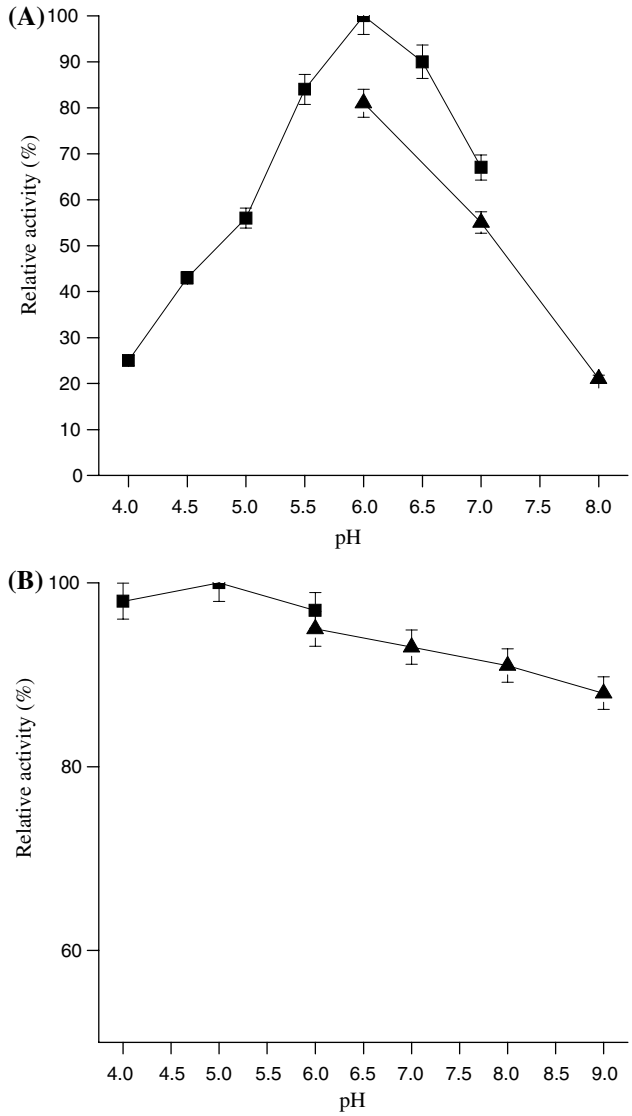

Fig. 4 pH optimum (**a**) and stability (**b**) of recombinant endoinulinase expressed from the codon-optimized gene. *Error bars* correspond to the standard deviation of three independent determinations

shown in Fig. 4a, the enzyme exhibited the highest activity at pH 6.0, which was similar to the native endoinulinase from *A. niger* [21]. The enzyme was stable over a pH range of 4.0–9.0, retaining >85 % of its initial activity after incubation at 4 °C for 24 h (Fig. 4b). Unlike the endoinulinase from *Aspergillus ficuum* JNSP5-06, which was sensitive to pH and lost its activity dramatically beyond the range of 4.5–6.0 [5], the enzyme in this

study was stable over a wide pH range of 4.0–9.0, retaining >85 % of its initial activity after incubation at 4 °C for 24 h (Fig. 4b). These results showed that the recombinant endoinulinase was potentially to be effectively useful in the preparation of IOS.

Optimization of biotransformation reaction conditions

After the endoinulinase was characterized, further investigations on the conditions of inulin hydrolysis were performed to analyze the economic effectiveness of the enzyme on IOS production. As shown in Fig. 5a, the maximum yield (79 %) of IOS was reached after 8 h of hydrolysis using 400 g l^{-1} inulin. The IOS yield was also high using 200 g l^{-1} inulin, and was achieved using a shorter reaction time. However, the low substrate concentration reduced production efficiency, leading to increased costs. Therefore, 400 g l^{-1} inulin was used for IOS production. Figure 5a also shows that inulin was over-hydrolyzed to generate some fructose after 8 h, causing a decrease in IOS [10], which was also confirmed by the following results. Results showed that pH had a critical effect on IOS production (Fig. 5b). Although the optimum pH for the recombinant enzyme was 6.0, a shift to the acidic range (e.g., pH 5.0) was more favorable for IOS formation. The highest yield (87 %) achieved at pH 5.0 was 1.45 times the lowest yield (60 %) at pH 7.0. As shown in Fig. 5c, the optimum temperature for inulin hydrolysis of the endoinulinase was 50 °C, with the maximum yield of IOS 91 %, which is acceptable in large-scale applications compared with the commercially available endoinulinase [6].

Although high yields of IOS have been previously reported, the substrate concentration in those studies, <200 g l^{-1}, is too low for efficient large-scale production. In the present study, 400 g l^{-1} of substrate was investigated, even though it has been reported that high concentrations of inulin may inhibit endoinulinase activity [8]. The results showed that the highest yield of 91 % was achieved with an enzyme concentration of only 40 U g^{-1} inulin for 8 h, which will result in substantially reduced costs, compared with the results of previous studies. In addition, the hydrolysis products were mainly IOS with DP 2–5, which are far more functional than the counterparts of other DP (Fig. 6).

Intriguingly, a previous report described under optimal conditions, using a partially purified endoinulinase from *A. ficuum*, an IOS yield >50 % was observed after 72 h, whereas using Jerusalem artichoke juice as a substrate, the maximum IOS production increased to 80 % after 72 h [10]. The amino acid sequence of the enzyme used in this study is several residues longer than the endoinulinase of *A. ficuum*. Thus, the N-terminal residues may play an important role in the biotransformation reaction of the enzyme. Further studies are needed to explore this issue.

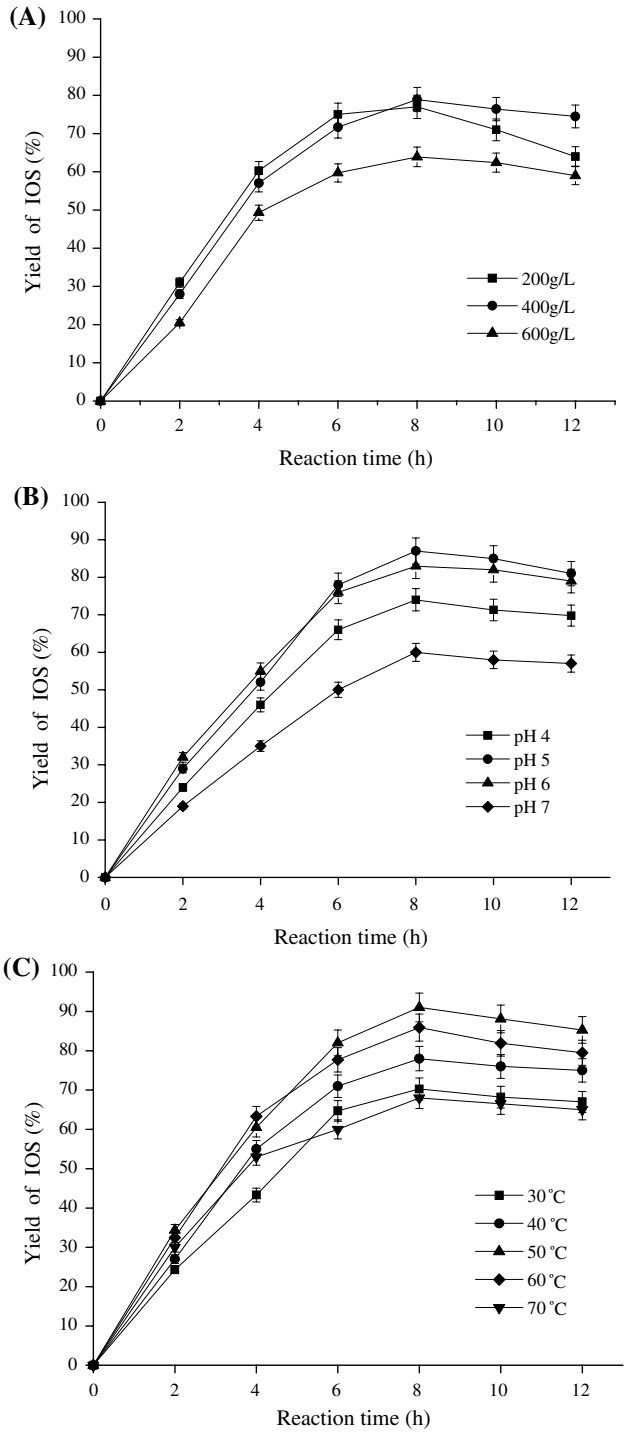

Fig. 5 Effects of substrate concentration (**a**), pH (**b**), and temperature (**c**) on the production of IOS by the recombinant endoinulinase expressed from the codon-optimized gene. *Error bars* correspond to the standard deviation of three independent determinations

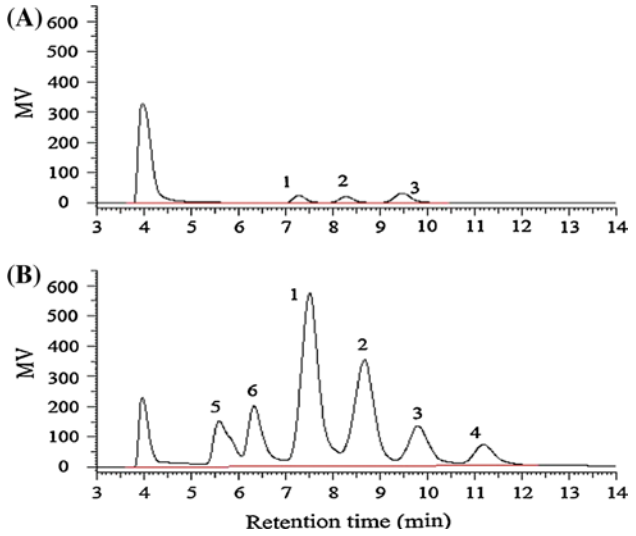

Fig. 6 Analysis of hydrolysis products by HPLC. **a** Standard (commercial IOS). Peak 1, kestose (DP 2); Peak 2, nystose (DP 3); Peak 3, 1F-fructofuranosyl nystose (DP 4). **b** Hydrolysis products formed by recombinant endoinulinase, Peak 1, DP 2 and trimer of fructose; Peak 2, DP 3 and tetramer of fructose; Peak 3, DP 4 and pentamer of fructose; Peak 4, DP 5 and hexamer of fructose; Peak 5, glucose and fructose; Peak 6, sucrose and dimer of fructose

Conclusions

This study represents the first time that codon optimization technology has been used to enhance the production of endoinulinase. After codon optimization and high cell-density fermentation in *P. pastoris* KM71, the expression level of the endoinulinase from *A. niger* CICIM F0620 reached 1,349 U ml^{-1}, which is the highest yield reported to date. In addition, the yield of IOS from the enzymatic hydrolysis of inulin using recombinant EnInuop was 91 %, using a substrate concentration of 400 g l^{-1}, which is a good, cost-effective feature. Our studies may provide the foundation for the application of endoinulinase to the industrial production of IOS.

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