Chapter 23
Expression and Characterization of a Thermophilic Trehalose Synthase from *Meiothermus ruber* CBS-01 in *Pichia pastoris*

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Abstract Trehalose synthase (TreS) was proved to catalyze the reversible reaction of maltose into trehalose by intramolecular transglucosylation. In this work, a yeast expression system was constructed to express TreS from *Meiothermus ruber* CBS-01 in the eukaryotic *Pichia pastoris* expression system. The *TreS* gene with 6× His tag at the 3′ end was subcloned into the eukaryotic expression vector pPIC3.5K. Then the constructed vector was integrated into *Pichia pastoris* strain KM71. The recombinant was induced by sterile methanol and the bioactive TreS was expressed successfully intracellular. After optimizing culture conditions, we got approximately 150 mg/L recombinant protein. It was the first time to express TreS from *M. ruber* CBS-01 in eukaryotic expression system. The purified TreS was also characterized in details.

Keywords *Meiothermus ruber* CBS-01 · Optimal expression and characterization · *Pichia pastoris* · Trehalose synthase (TreS)

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23.1 Introduction

Trehalose is a non-reducing disaccharide widely found in various organisms [1]. It has been reported that this sugar is heat- and acid-stable, and can protect organisms or tissues against environmental stresses such as dryness, osmotic stress, heat or cold shock, and so on [2, 3]. Hence, trehalose has been widely used in cosmetic industry, food industry, and in medical industry [4, 5].

Up to now, there are five pathways involved in trehalose biosynthesis [6]. Among all of these, the trehalose synthase (TreS) can convert maltose into trehalose in only one-step reaction and the substrate of the enzyme is inexpensive. Therefore, the enzyme is suitable to produce trehalose in industry. Some *TreS* genes have been isolated and cloned from different organisms. Many of them had been purified and/or expressed in *Escherichia coli* [7–11]. However, TreSs reported so far processed a problem of low yield in the original organisms.

Up to the present, some thermophilic TreSs were discovered in some bacteria, such as *Thermus thermophilus* [12], *Thermus aquaticus* [13], and *Meiothermus ruber* [14]. They will be more suitable for industrial application because of the thermophilicity and stability. But there is not any report about the expression of thermophilic TreSs in eukaryotic expression systems.

The methylotrophic yeast *Pichia pastoris* is well known for being effective in producing recombinant proteins. As a eukaryote, *P. pastoris* can perform some co- and/or post-translational modifications of foreign proteins. The proteins expressed in *P. pastoris* were usually folded with the correct disulfide bonds [15]. Additionally, *P. pastoris* needs a low maintenance energy, which was fitted for high density fermentation.

In our previous research, a *TreS* gene was obtained from *Meiothermus ruber* strain CBS-01 [14]. In this work, a yeast expression system was constructed to express TreS of *M. ruber* CBS-01 in eukaryotic expression system. Under optimal culture conditions, the recombinant protein was expressed and purified. Furthermore, the properties of the enzyme were characterized in details.

23.2 Materials and Methods

23.2.1 Strains, Plasmids, and Regents

E. coli DH5α, *P. pastoris* KM71 (Darmstadt, German) were used for cloning and expression, respectively. Intracellular expression vector pPIC3.5K was purchased from Invitrogen (California, USA).

E. coli DH5α was cultured in LB broth (100 μg/mL ampicillin). The medium MD was used for selection of transformant, the medium YPD, BMGY, and BMMY were used for *P. pastoris* culture and induction, respectively. All the

media were prepared following the methods mentioned in the introduction of Pichia Expression Kit (Invitrogen, USA)

Binding buffer and elution buffer were used for protein purification by NTA-Ni column as described previously [16]. Washing buffer (10 mmol/L potassium phosphate buffer (pH 6.5)) was used for protein purification by Hi-trap Q column.

The restriction enzymes and Taq polymerases were obtained from Takara (Dalian, China). Columns for protein purification were purchased from GE (Tokyo, Japan). Glucose, maltose, and trehalose were got from Sigma (St.Louis, MO). The other chemicals and reagents were of analytical grade.

23.2.2 Construction of Expression Plasmid

The *TreS* gene of *M. ruber* CBS-01 was amplified from pET21a-TreS with primers pPICF (5′-CGGAATTCGCGAGTATGGGTGTGGATCCTCTTTGG, *Eco*R I restriction enzyme site underlined) and pPICR (AATGCGGCCGCCTA **GTGGTGGTGATGATGGTG**GCGGGCCCGTTCCTTCCACC, *Not* I restriction enzyme site underlined and a 6× His tag sequence in bold). The ORF is 2913 bp and encodes 970 amino acid residues. The amplified DNA was ligated into *Eco*R I-and *Not* I-digested pPIC3.5K to produce pPIC3.5K-TreS for intracellular expression.

23.2.3 **Pichia pastoris** *Transformation, Expression, and Activity Detection of TreS*

The purified vector pPIC3.5K-TreS was linearized by *Sal* I. The linearized DNAs were transformed into *P. pastoris* strain KM71 by electroporation following the guide of the Pichia expression kit (Invitrogen, USA). Then the positive transformants were selected on the MD plates. The recombinant strain KM71/pPIC3.5K-TreS was identified by PCR using primers 5′AOXI and 3′AOXI.

Identified transformants were cultured in 5 ml BMGY medium at 30 °C for 2 days. Cells were harvested by centrifugation at 3000 g for 10 min, and then were resuspended in 5 ml BMMY medium and grown for another 2 days. For inducing of the expression of TreS, sterile methanol was added into the BMMY medium to a final concentration of 0.5 % (v/v) every day. After intracellular expression, the culture was centrifuged at 3000 g for 5 min and the supernatant was decanted. The cell pellet was suspended in 1 ml washing buffer and cells were disrupted by shaking with glass beads for 5 times of 1 min shaking, with 1 min intermission on ice. After incubated at 60 °C for 1 h and centrifuged at 10000 g for 10 min, the supernatant was analyzed by SDS-PAGE. At the same time, a same volume of

50 mmol/L maltose solution in washing buffer was added and the mixture was incubated at 50 °C for 60 min to detect the activity of TreS.

The activity of TreS was analyzed by measuring the amount of trehalose produced from maltose. The production was assayed by high performance liquid chromatography (HPLC). HPLC was performed as follows: Hypersil-NH_2 column Ø 4.6 * 250 mm; 80 % acetonitrile- 20 % water eluent (v/v); flow rate 1.0 ml/min; column temperature 30 °C; evaporative light scattering detection.

23.2.4 *Optimized Expression of TreS in* Pichia pastoris

Each single clone of *P. pastoris* transformants was cultivated and induced as described above. To yield a highest level of TreS, the cultivations were carried out at different conditions including the final concentration of methanol, initial cell density for induction, the times to add methanol every day, and the induction time.

All data were obtained at least 3 times and were shown as mean ± SD.

23.2.5 *Purification of TreS and Enzyme Characterization*

A single clone of recombinant strain was inoculated in 5 ml BMGY medium, grown overnight at 3 °C with shaking. A 1 mL of culture was inoculated into 100 mL the same medium and grown at 30 °C for 24 h with 200 r/min. Cells were then harvested by centrifuging for 5 min at 3000 g. The cell pellets were resuspended in 50 mL of BMMY medium in a 500-mL shake flask and cultured under the optimum condition for TreS expression. At the end of incubation, the culture broth was centrifuged at 3000 g for 5 min and the supernatant decanted. The cells were grinded by liquid nitrogen, and then suspended in washing buffer. The supernatant was centrifuged at 10000 g for 10 min and then filtered through 0.22 μm filter. The crude enzyme was purified using NTA-Ni column chromatography as described previously [16]. The eluted solution was loaded on a Hi-trap Q ion-exchange column (200 * 10 mm) equilibrated with a washing buffer. The protein was eluted with a linear gradient of 0–1.0 mol/L NaCl in washing buffer. The active fractions were pooled, then concentrated and desalted by Amicon Ultra-4 centrifugal filter. The protein concentration was quantified using Bradford's method [17]. The purified enzymes were analyzed with 10 % SDS–PAGE. The kinetic parameters, effect of temperature, and pH on the activity and stability of TreS were measured by the methods described previously [16].

23.3 Results

23.3.1 *Expression and Activity Detection of TreS in* Pichia pastoris

After induction using methanol, the expression of TreS was analyzed by SDS-PAGE and the activity of enzyme was detected. In contrast with the negative control, a single protein band around 110 kDa was clearly visible by SDS-PAGE analysis of crude extract from cell pellet for KM71/pPIC3.5K-TreS.

The activity of TreS was determined by HPLC. After cell-free extracts of KM71/pPIC3.5K-TreS reacted with 2 % (w/v) maltose at 50 °C for 60 min, trehalose and a small amount of glucose were detected by HPLC (data not shown). All of the above results indicated that TreS was expressed successfully intracellular in *P. pastoris* KM71.

23.3.2 *Optimized Expression of TreS in* Pichia pastoris

23.3.2.1 The Optimal Harvest Time of TreS Expression in *Pichia pistoris*

To determine the optimal time for TreS production, a time-course analysis on the TreS accumulation was carried out within 168 h. At each time point, the expression samples were withdrawn to analyze the activity of TreS. The accumulation of TreS was proportional to its activity, so the activity of TreS was used to estimate its expression level. As shown in Fig. 23.1, the sample of 96 h after induction showed the highest activity. A plateau was reached beyond this time point, which indicated that 96 h is the best time to harvest the cells.

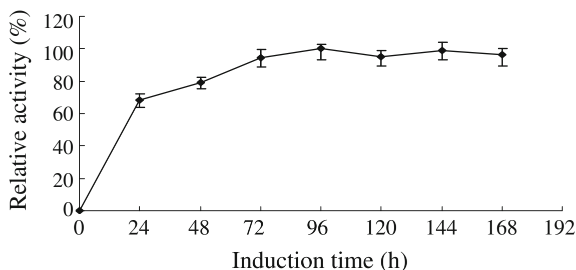

Fig. 23.1 The effect of induction time on the accumulation of TreS in *P. pastoris* KM71

23.3.2.2 Effect of Inoculum Concentration at Induction Phase on TreS Production

Generally, a high density of cells resulted in an increase of recombinant protein production. However, high cell densities mean that oxygen and nutrition will become limiting factors. The initial culture was adjusted to different OD_{600} before induction. We observed that when the density of biomass from BMGY culture reached about $OD_{600} = 12$ (as shown in Fig. 23.2), a culminating point could be achieved in the accumulation of TreS. And inoculum densities above $OD_{600} = 12$ did not result in obvious increase in TreS accumulation.

23.3.2.3 Effect of Methanol Addition on the Induction of TreS

During the expression of TreS, the addition of methanol into the media was performed once every 12 or 24 h to keep induction. The TreS production induced by different concentrations of methanol was compared to determine the optimal condition. When the concentration of methanol in the medium was below 0.75 % (v/v), there was no inhibition on the growth of yeast cells. The TreS accumulation was proportional to the concentration of methanol and peaked at 0.75 % (Fig. 23.3). When the concentration of methanol was too high (above 0.75 %), the production of TreS was suppressed, and the yield of the enzyme was reduced. Therefore, TreS expression reached the highest level by the addition of methanol to a final concentration of 0.75 % each 12 h.

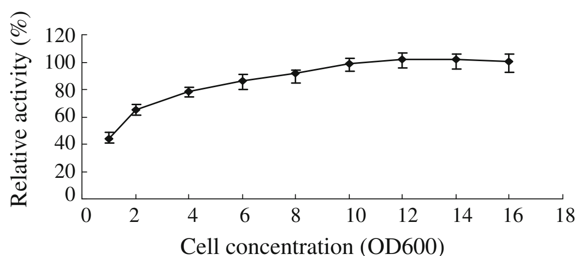

Fig. 23.2 The effect of cell density before induction on the accumulation of TreS in *P. pastoris* KM71

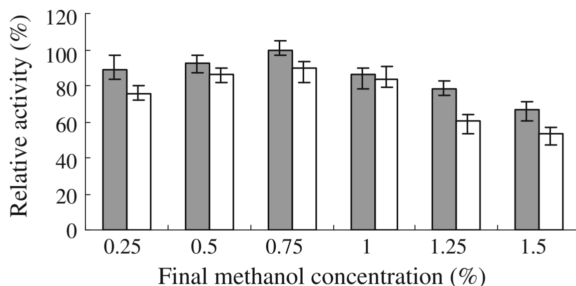

Fig. 23.3 The effect of methanol concentration and adding frequency on the accumulation of TreS in *P. pastoris* KM71. Methanol was added every 12 h (*solid bar*) or 24 h (*open bar*)

23.3.2.4 Optimal Expression and Purification of TreS

Under optimal culture conditions, the TreS expressed abundantly. The recombinant TreS was purified and analyzed by SDS-PAGE which revealed a single protein band around 110 kDa consisted with the deduced molecular weight of TreS (data not shown). The protein concentration was approximately 2 mg/mL. And the yield of the expression of TreS in *P. pastoris* was about 150 mg/L.

23.3.3 Kinetics Analysis of TreS Expressed in Pichia pastoris

The kinetic parameters of TreS expressed in *P. pastoris* were showed in Table 23.1. The K_m value using maltose as substrate was nearly to that using trehalose as substrate, implying that TreS from *M. ruber* CBS-01 expressed in *P. pastoris* had the same affinity to maltose and trehalose. However, the enzyme had approximately twofold conversion rate (k_{cat}) and catalytic efficiency (k_{cat}/K_m) using maltose as substrate to that using trehalose as substrate, which led the priority to produce trehalose.

23.3.4 Effects of Temperature and pH on the Activity and Stability of TreS Expressed in Pichia pastoris

The optimum temperature for TreS expressed in *P. pastoris* was about 50 °C. Besides, TreS showed outstanding thermo-tolerance, and it could maintain more than 90 % of its activity within a temperature range of 0–60 °C (Fig. 23.4a).

As shown in Fig. 23.4b, TreS expressed from *P. pastoris* showed the highest activity at pH 6.5. And between pH 5.0–8.0, the enzyme could highly maintain the original activity. This indicated that TreS has both good adaptability and survivability against pH.

The purified TreS from *P. pastoris* was thermostable and acid tolerant, which made it suitable to produce trehalose in industry.

And all the results above showed that TreS from *M. ruber* was successfully expressed in *P. pastoris*.

Table 23.1 Kinetic parameters of the recombinant TreS expressed in *Pichia pastoris* KM71

Substrate	K_m (mmol/L)	V_{max} (mmol/L·min)	k_{cat} (s^{-1})	k_{cat}/K_m (L/mol·s)
Maltose	97.2 ± 3.5	943.5 ± 28.6	174.7 ± 5.3	1798.2 ± 10.7
Trehalose	95.6 ± 6.9	424.9 ± 22.4	78.7 ± 4.1	823.9 ± 16.2

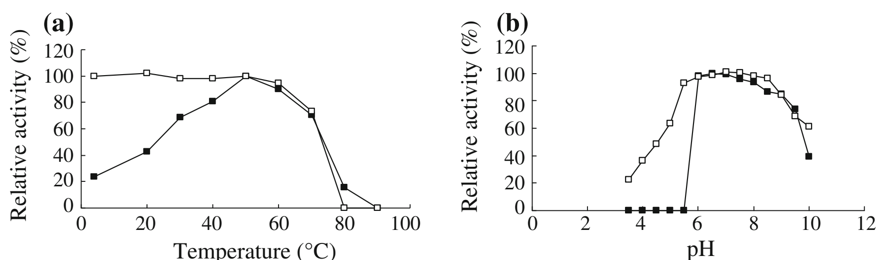

Fig. 23.4 Effects of temperatures (**a**) and pH (**b**) on the activity (*solid square*) and stability (*open square*) of TreS expressed in *P. pastoris* KM71, respectively

23.3.5 Effects of Metal Ions on the Activity of TreS Expressed in **Pichia pastoris**

Table 23.2 showed the effects of metal ions on the activity of purified TreS expressed in *P. pastoris*. The activity of the enzyme was inhibited strongly by 10 mmol/L divalent and trivalent cations in our experiment except Mg^{2+}. However, we did not find any metal ion which can increase the enzyme activity remarkably.

23.3.6 The Stability of TreS Expressed in **Pichia pastoris** *During Storage*

The hetero-expressed TreS was stored at 4 °C for some time and then the residual activities were examined. In was shown that TreS can reserve 98.2 % of its activities after 60 days of storage at 4 °C, indicating that the TreS expressed by *P. pastoris* was very stable during storage.

Table 23.2 Effects of metal ions on the activity of TreS expressed in *P. pastoris* KM71

Reagent concentration	Relative activity (%)										
	None	Na^{+}	K^{+}	Zn^{2+}	Fe^{2+}	Ca^{2+}	Cu^{2+}	Mg^{2+}	Mn^{2+}	Co^{2+}	Al^{3+}
1 mmol/L	100	99.1	98.6	75.3	102.2	68.5	61.3	103.7	81.6	92.8	94.2
2 mmol/L	100	102.5	101.3	86.4	97.6	53.1	65.4	101.3	70.4	79.2	0
10 mmol/L	100	103.7	104.0	0	0	8.8	0	95.1	0	0	0

23.4 Discussion

Due to the outstanding advantage of TreS in producing trehalose, there have been lots of TreS isolated from different organisms, and most of them were expressed successfully in *E. coli*. However, *E. coli* and other prokaryotic expression systems have several disadvantages. For example, in these expression systems, the foreign protein is prone to form inclusion body during fermentation, and the expression level is not very high. Moreover, the expression vector is easy to lose [15]. On the other hand, there is little reports to show that the TreS was expressed in eukaryotic expression systems.

In our precious work, a *TreS* gene was cloned from the thermophilic bacteria *M. ruber* CBS-01 and expressed in *E. coli*. However, the expression level in *E. coli* was low (about 5 mg/L), which greatly hindered it to be applied in industrial production. Considering the notable advantages of *P. pastoris* in expressing foreign proteins, we tried out the *P. pastoris* expression system to produce TreS to investigate the character of the enzyme in eukaryotic cells.

Using the intracellular eukaryotic expression system, we optimized the inducing condition of TreS in *P. pastoris*. Under the optimal condition, the KM71/pPIC3.5K-TreS produced approximately 150 mg/L recombinant TreS, which was almost 30 times of that in *E. coli* expression system. This provided an experimental basis for further industrial production of TreS.

One factor that affects expression of foreign proteins in *P. pastoris* is the Mut phenotype of the recombinant strain. KM71/pPIC3.5K-TreS constructed in this study was Mut^S. In principle, Mut^S recombinant is better for intracellular expression because the strain produces little alcohol oxidase, and the purification of foreign proteins will be easier [18]. For secreted expression, Mut^+ is a preferable choice. However, there is not a specific relationship between them, so both Mut^+ and Mut^S recombinants are useful as one phenotype may favor better expression of the foreign proteins than the other. Therefore, we will investigate and the expression of TreS in the Mut^+ recombinant of *P. pastoris* GS115 system in our further study. Besides, it was demonstrated that multiple copy integration of recombinant genes into the genome of *P. pastoris* could enhance the expression of foreign proteins [19]. So we will screen the recombinants of multiple inserts of *TreS* and test whether mutli-copy of *TreS* can increase expression at the same time.

We have investigated the expression of secreted TreS in both *E.coli* and *P. pastoris,* previously. Unfortunately, no activity of TreS was detected in the culture supernatant after induction. It is probable that the TreS is a naturally endocellular protein in *M. ruber*. However, the soluble fraction of protein extract after cell breaking showed TreS activity. It was indicated that the TreS was expressed endocellularly, but failed to be secreted to the medium. The amino acid sequence was predicted as a hydrophilicity protein by Expasy (http://www.expasy.ch/tools/protscale.html). There was no predicted transmembrane region in the protein which may inhibit the protein to secrete.

The TreS expressed in *P. pastoris* was a thermostable protein which was easy to be purified from non-thermophilic proteins expressed in *P. pastoris*. At same time, it could maintain 90 % of its activity after incubating at pH 5.5–pH 9.0. The storage of TreS expressed in *P. pastoris* was convenient. Hence, the TreS reported here is potential to be applied in industry.

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