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Cloning and expression of a Trehalose Synthase from *Pseudomonas putida* KT2440 in *Bacillus subtilis* W800N for the production of Trehalose

Tengfei Wang[1,2]*, Kun Dai[2], Hongjuan Liu[2], Shiru Jia[1] and Ruiming Wang[2]

[1]Key Laboratory of Industrial Fermentation Microbiology, Ministry of Education, Tianjin University of Science and Technology, TEDA, Tianjin 300457, PR China. 2Key Laboratory of Shandong Microbial Engineering, QILU University of Technology, Jinan, Shandong 250353, PR China.

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Trehalose synthase which catalyze inexpensive maltose to trehalose by one-step hydrolysis reaction, was considered to be a potential biocatalyst for trehalose production. In our work, the DNA encoding trehalose synthase with 6×his-tag was ligated with pGEM-T easy vector, transformed into *E.coli* DH5á and screened. After sequence analysis, the PCR products was digested by *Bam*HI and *Aat*II and ligated into pHT01 by using T4 DNA ligase to generate the recombinant vector pHT01-TreS. The purified pHT01-TreS was electrotransformed into *B.subtilis* W800N. The recombinant TreS protein was expressed and purified by nickel affinity chromatogaraphy. The optimum temperature and pH were 25°C and pH 8.0, respectively. The pure enzyme was stable in pH 6.0 to 9.0. It activity increased by Na^+, K^+ and Mg^{2+}, but it was greater inhibited by Zn^{2+} and Ca^{2+}.

Key words: Trehalose synthase, Gene cloning, Protein expression, *Bacillus subtilis* W800N, *Pseudomonas putida* KT2440, Conversion**.**

Trehalose can be widely found in nature (Elbein *et al*., 2003). It has been accepted as a novel food ingredient under the GRAS terms in the U.S. and the EU (Schiraldi *et al*., 2002). It is a non-reducing disaccharide, which is composed of two glucose molecular by an α, α- 1, 1 - glycosidic linkage. It has a lot of desirable characteristics such as anti-desiccation, anti-freezing, heat shock resistance and osmotic stress resistance. Therefore, it has been widely used as an additive or stabilizer in food, medicine, cosmetic (Satpathya *et al*., 2004).

As reported, there are five main types of trehalose biosynthesis pathway: TreS (trehalose synthase, EC5.4.99.16), pathway, TreT pathway, TreS pathway, TPS/TPP pathway and TreY/TreZ pathway (Kouril *et al*., 2008). TreS pathway is considered to be a convenient, economical biocatalyst process, and it is suitable for industrial production of trehalose. TreS can convert low-cost maltose into trehalose in one-step reaction. So many strains have been identified and characterized for industrial production of trehalose, such as *Pseudomonas stutzeri* CJ38 (Lee *et al*., 2005), *Enterobacter hormaechei* (Yue *et al*., 2009), *Thermobifida fusca* (Wei *et al*., 2004), *Corynebacterium glutamicum* ATCC13032 (Kim *et al*., 2010), *Arthrobacter aurescens* CGMCC 1.1892 (Wu *et al*., 2009), *Meiothermus ruber* CBS-01 (Zhu *et al*., 2010), *Mycobacterium smegmatis* (Pan *et al*., 2004), *Pseudomonas putida* (Ma *et al*., 2006). The gene of TreS in different strains have been cloned and expressed in *E.coli* systems (Lee *et al*., 2005; Yue *et al*., 2009; Kim *et al*., 2010; Wu *et al*. 2009; Zhu *et al*. 2010). According to reports,

* To whom all correspondence should be addressed.
Kun Dai is the co-first author
E-mail: wangtengfei1981@163.com

the gene of TreS is rarely cloned and expressed in *Bacillus subtilis* systems. Comparing with *Escherichia coli*, the *Bacillus subtilis* (Gram-positive bacteria) as the host has many advantages. It was free of endotoxin and has generally regarded as safe status. Drug Administration of USA (FDA) has made it an attractive expression host to produce proteins of commercial interest (Harwood *et al.*, 1990). Therefore, It can be used in food, enzyme and pharmaceutical industries and can replace *Escherichia coli* for proteins expressed.

In our research, we cloned the trehalose synthase gene from *Pseudomonas putida* KT2440 by primer containing 6× histidine tag, expressed in *B.subtilis*W800N, and also characterized its main enzyme features. The report could afford some useful information for the Scale-up fermentation on TreS and meeting the industrial production of trehalose in food and medicine.

MATERIALS AND METHODS

Bacterial strains and plasmids

Pseudomonas putida KT2440 (ATCC47054) was used to provide DNA template. *Escherichia coli* DH5α (Invitrogen Co.) was used as a DNA manipulation host, *Bacillus subtilis*W800N (Novagen, Germany), the protease-negative mutant, was used as an expression host. Plasmid pGEM-T Easy Vector (Takara Co.) was used as a TA cloning vector. Plasmid pHT01 (Novagen, Germany) with ampicillin and chloramphenicol-resistant genes, P*grac* promoter, a shuttle vector for *E.coli* and *B.subtilis*, was used for the expression of TreS.

Gene cloning and expression vector construction

The genomic DNA from *Pseudomonas putida* KT2440 was prepared with bacteria genome DNA extracting kit. The following two primers were designed and synthesized for amplification of the TreS gene (gi = 1042893, NCBI). The forward primer was designed as 5'- (*GGATCC*ATGACCC AGCCCGACCCGTC)-3', [with a *Bam*HI (*italic*) cutting site at the 5'-terminal and cloning full sequence of TreS gene]. The reverse primers with 6×his-tag were 5'-(*GACGTC*TCAGTGGTGGT GGTGGTGGTGGAACATGCCCGCTGCTG)-3', [with a *Aat*II (*italic*) cutting site at the at the 3'-terminal]. By using these primers, the TreS was amplified by polymerase chain reaction (PCR) (2720 Thermal Cycler, Applied Biosystems, Foster, CA) and purified by using a QIA quick polymerase chain reaction purification Kit (Qiagen, Valencia, CA), ligated with pGEM-T easy vector, transformed into *E.coli* DH5α and screened. After sequence analysis, the PCR products was digested by *Bam*HI and *Aat*II and ligated into pHT01 by using T4 DNA ligase to generate the recombinant vector pHT01-TreS. The purified pHT01-TreS was electrotransformed into *B.subtilis* W800N by using a Micro Pulser (Bio-Rad, Hercules, CA) at 2.0 KV with a 0.2 cm cuvette for expression.

Protein express and purification

The recombinant strain was cultured on LB agar containing 10 µg/mL Chloramphenicol at 37°C for 12h, was picked and inoculated into 100 mL of LB broth containing 10 µg/mL Chloramphenicol with constant shaking (200 rpm) at 37°C for 10 h, 1% (v/v) of the cultures was inoculated to 500 mL of fresh super-rich broth (2.5% bacto tryptose, 2% yeast extract, and 0.3% K_2HPO_4, pH7.5) with constant shaking (200 rpm) at 37°C, when the absorbance of OD_{600} reached 0.6, isopropyl α-D-1-thiogalactopyranoside (IPTG, final concentration 0.5 mmol/L) was added for induction, and the cultivation was continued for additional 24 h at 30°C or 37°C. The cells were harvested by centrifugation at 6000×g for 10 min at 4°C. The wet cell pellet was suspended in 10 mmol/L potassium phosphate buffer (pH8.0) and cells were cyclically disrupted two times by high-pressure homogenizer (APV-2000, Germany) at 1200 bar. The insoluble cell debris were removed by centrifugation at 6000×g for 20 min 4°C. The recombinant TreS was purified using nickel-nitrilotriacetic acid affinity chromatography (Ni-NTA, Qiagen) as the manufacturer recommended. The purified enzymes were analyzed on 15% SDS-PAGE and protein concentration was determined by the method of Bradford using BSA as a standard.

The recombinant TreS characterization

The function of the recombinant TreS was analyzed by HPLC (Asker *et al*., 2009), using 100 g/L maltose as a substrate. The recombinant trehalose synthase activity was measured by the amount of trehalose produced from maltose. One unit (U) of trehalsoe synthase was defined as the amount of enzyme required to produce 1 µmol trehalose per hour under the specified conditions.

The optimal pH was determined by measuring the activity of purified TreS at pH 3.0-10.0 (pH 3.0-6.0, 20 mM citrate buffer; pH 6.0-8.0, 20 mM sodium phosphate buffer; pH 7.0-9.0, 20 mM Tris-HCl buffer; and pH 9.0-11.0, 20 mM sodium carbonate buffer). The optimal temperature of purified TreS in 10 mM sodium phosphate buffer (pH 8.0) was measured at 10-65°C by using maltose substrate. The pure TreS in various pH values of buffer (as shown above) was incubated at 25°C for 60 min to determine the pH stability. An equal volume of 10 mM sodium phosphate buffer (pH 8.0) was added to maintain the pH at 8.0. The TreS in 10 mM phosphate buffer (pH8.0) was incubated at 10-65°C for 60 min and then chilled in ice water immediately for 5 min to determine the thermal stability. The effects of common metal ions on the activity of TreS were determined in an assay buffer containing 5.0 mmol/L, 10.0 mmol/L, 15.0 mmol/L metal ions under the standard reaction conditions. The TreS was stored at 4°C, and the activities were examined after 1, 5, 10, 15 and 20 days with the standard reaction conditions.

RESULTS AND DISCUSSION

Expression and purification of recombinant TreS

The recombinant expression vector pHT01-TreS was transformed into *B.subtilis*W800N strain, and then TreS was produced by induction of T7 polymerase. The soluble proteins in the cell were analyzed with SDS-PAGE after an IPTG induction of 24 h at 30°C and 37°C. A clearly visible band, a recombinant protein about 76 kDa, was observed. The product accounts for about 2.6% of total soluble cell proteins, measured with thin charomatography scanner.

To determine whether the gene encoded a functional TreS, we purified the recombinant protein from the supernatant of cell lysate shown by the single band in Figure 1, Crude protein concentration in the supernatant of the cell lysate was 2.49 mg/mL, and the purified TreS concentration was 47.6 μg/mL.

To identify the function of TreS, the purified enzyme by nickel nitrilotriacetic affinity chromatography was incubated with maltose or trehalose as a substrate, HPLC analysis of the reaction mixtures showed that TreS catalyzed the conversion of maltose into trehalose, and vice

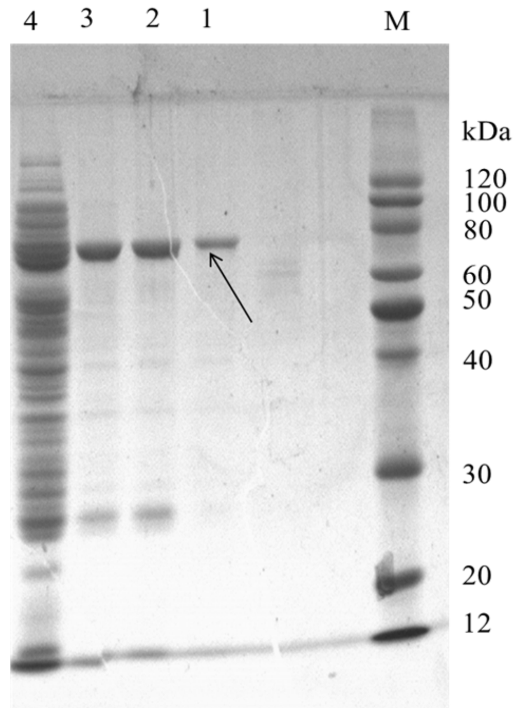

Fig. 1. SDS-PAGE analysis of the purified recombinant protein Arrow shows the position of purified TreS protein. *Lane 1* purified recombinant TreS; *Lane 2* and *Lane 3* Eluted protein solution; *Lane 4* total cell lysate of *B.subtilis* transformed with pHT01-TreS; *Lane M* protein standard marker (12, 20, 30, 40, 50, 60, 80, 100, 120).

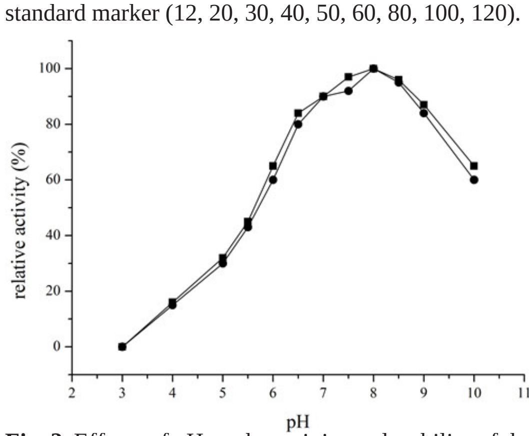

Fig. 2. Effects of pH on the activity and stability of the recombinant TreS. The enzyme activity of TreS at various pH was studied at 25°C in 100 mM phosphate buffer (pH 3.0-10.0) for 60 min, using 100g/L maltose as a substrate. To examine the stability of TreS, the enzymes were preincubated at various pH values (pH 3.0-10.0) for 60 min at 25°C. The residual activities were measured at pH 8.0. The square (■) represents effects of pH on the activity of TreS; the triangle (●) represents effects of pH on the stability of TreS. Data are averages of three independent experiments.

versa, which suggested TreS was an active TreS.

Effects of pH and temperature on the activity and stability of recombinant Trehalose

The pH dependence of TreS was studied at various pH values ranging from pH 3.0 to pH 10.0. To determine the pH stability, the recombinant enzyme was preincubated at various pH values (pH 3.0-10.0) for 60 min before the residual activity was measured at pH 8.0, immediately. The optimum pH for TreS was pH 8.0, but it remained highly stable within a pH range from 6.5 to 9.0 (Figure 2).

The effects of temperature on TreS activity were determined at various temperature ranging from 15°C to 65°C. To determine the stability against thermal denaturation, the recombinant enzymes were preincubated at various temperature (15-60°C) for 60 min and cooled immediately to assay the residual activity at 25°C. The optimum temperature was 25°C, and the enzyme remained stable up to 40°C (Figure 3).

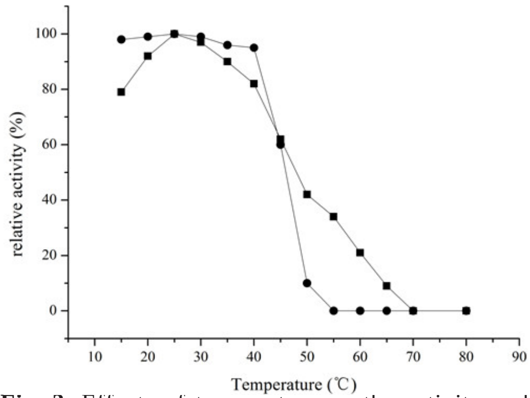

Fig. 3. Effects of temperature on the activity and stability of the recombinant TreS. The enzyme activity of TreS at various temperature was studied in 100 mM phosphate buffers (pH8.0) for 60 min, using 100g/L maltose as a substrate. To examine the thermal stability of TreS, the enzymes were preincubated at various temperatures (20-60°C) for 60 min at pH 8.0. the residual activities were measured at 25°C. The square (■) represents effects of temperature on the activity of TreS; the triangle (●) represents effects of temperature on the stability of TreS. Data are averages of three independent experiments

Effects of metal ions and storage time at 4 °C on the activity of recombinant Trehalose

The addition of 5 mM, 10 mM, 15 mM Na^{2+}, Mg^{2+}, K^{+}, greatly increased the activity, showing 110-130% of the initial activity whereas the addition of 5 mM, 10 mM, 15 mM Ca^{2+} and Zn^{2+} inhibited the enzyme activity interestingly and the enzyme activity was inhibited more strongly with the increase of Ca^{2+} and Zn^{2+} ion concentration. TreS showed 45% of the initial activity in the presence of 15 mM Zn^{2+} which leaded to sharp decrease in the activity of TreS (Table 1).

The activities of TreS stored at 4°C for various days were relatively stable. It is indicated that the recombinant TreS was very stable at cold storage. In liquid, the relative activity of TreS could maintain more than 80% in the 20 days of storage at 4°C.

Conversion of maltose to trehalose by the purified recombinant TreS

The purified enzyme was incubated in 100 mM phosphate buffer (pH8.0) at 25°C for 0-12 h, using 100g/L maltose as a substrate. All the reactions were stopped by boiling them for 10 min

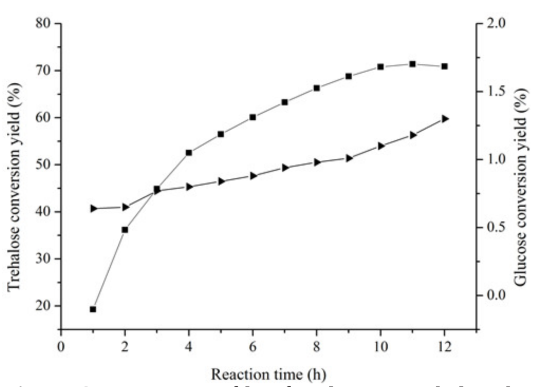

Fig. 4. Conversion profile of maltose to trehalose by TreS. The conversion profile was obtained by incubating the enzyme (47.5 µg/mL) at 25°C, pH 8.0 for 0-12 h, by using 100 g/L maltose as a substrate. Then, the reaction mixture was analyzed by HPLC. Square (■): Trehalose; triangle (▶): Glucose. Data are averages of three independent experiments.

Table 1. Effects of common metal ions on the activity of TreS

Reagent	Relative activity (%)		
	5 mM	10 mM	15 mM
Control	100	100	100
$CaCl_2$	90.6	70.5	54
$MgCl_2$	123	122.8	113.7
NaCl	124.8	125	126.1
KCl	124.2	131.2	118
$ZnSO_4$	85.6	64.3	45

Data are averages of three independent experiments.

Table 2. Comparison of enzymatic properties of TreS expressed in *E.coli* and *B.subtilis*

Trehalose synthase strains	Expression strain	Expression vector	Optimum temp. (°)	Optimum pH
Pseudomonas putida KT2440	*B.subtilis* W800N	pHT01	25	8.0
Pseudomonas stutzeri CJ38	*E. coli* NM522	pUC18	15	8.5-9.0
Enterobacter hormaechei	*E.coli* BL21 (DE3) plysS	pET30a	37	6
Corynebacterium glutamicum ATCC13032	*E.coli* MC1061	pTKNd6xH	35	7.0
Arthrobacter aurescens CGMCC 1.1892	*E.coli* BL21 (DE3) plysS	pET30a	35	6.5
Meiothermus ruber	*Rosetta gami* (DE3)	pET21a	50	6.5

before the samples were analyzed by HPLC. The maximum conversion yield of maltose into trehalsoe reached 71% (g/g) with 1.3% (g/g) glucose by-production at 25°C in 100 g /L maltose substrate (5 mL) for 12 h by pure enzyme (47.5 µg) (Figure 4).

CONCLUSIONS

In this paper, we confirmed that the TreS gene (gi = 1042893) was cloned and expressed in *B.subtilis* W800N. The recombinant TreS was the solution and could catalyze the conversion of maltose to trehalose and vice versa. The purified recombinant TreS showed its best activity at pH 8.0 and 25°C. It could convert about 71% (g/g) maltose to trehalose in the 100g/L maltose concentration at 25°C for 12 h, accompanied by about 1.3% (g/g) glucose as a byproduct. It was reported that most TreS enzymes could produce glucose, but the glucose as a by-product were more than the recombinant TreS in our work.

In comparison with other previously reported trehalsoe synthases (Table 2), the purified recombinant TreS was more alkali tolerant. It could maintain 90% of its activity after incubating at pH 7.0-9.0, but the purified recombinant TreS was stable from 15°C to 40°C and was not thermostable. In our research, one interesting result was observed from the characterization of this enzyme, which was not reported in other enzymes before. The recombinant *B.subtilis* W800N whole cell lysates was directly used to convert maltose into trehlose, the conversion yield was optimal at 50°C with the concentration of 300 g/L maltose substrate by 0.1g (DCW) cell lysates for 24 h, the conversion yield reached to 64% (g/g) in 40 mL solution (pH 8.0). So the cell lysate may improve the reaction temperature tolerance of the recombinant enzyme, it was possible that the protective proteins in cells prevents degeneration of the recombinant enzyme. The recombinant TreS activity subtstantially increased by 5 mM, 10 mM Na^+, K^+ and Mg^{2+}, but the activity was inhibited by Zn^{2+} and Ca^{2+}, Therefore, sodium phosphate buffer solution of pure water preparation are beneficial to improve the enzyme activity.

In industrial production, there were three key factors that the cost was reduced, the process was simply and endotoxin free. As shown above, we used the cell lysates directly to convert maltose into trehalose at 50°C with pH 8.0, the cost of enzyme purification could be effectively saved, and the energy consumption can effectively be reduced in the whole conversion process. And at pH8.0 and 50°C, the microbial growth was inhibited and avoided harmful microorganisms contamination. Hence, the recombinant TreS from *B.subtilis* W800N was very suitable for industrial production of trehalose.

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