

Cloning, Expression and Characterization of a Trehalose Synthase Gene From *Rhodococcus opacus*

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Abstract Trehalose is a unique disaccharide capable of protecting proteins against environmental stress. A novel trehalose synthase (TreS) gene from *Rhodococcus opacus* was cloned and expressed in *Escherichia coli* Top10 and BL21 (DE3) pLysS, respectively. The recombinant TreS showed a molecular mass of 79 kDa. Thin layer chromatography (TLC) result suggested that this enzyme had the ability to catalyze the mutual conversion of maltose and trehalose. Moreover, high-performance liquid chromatography (HPLC) result suggested that glucose appeared as a byproduct with a conversion rate of 12 %. The purified recombinant enzyme had an optimum temperature of 25 °C and pH optimum around 7.0. Kinetic analysis revealed that the K_m for trehalose was around 98 mM, which was a little higher than that of maltose. The preferred substrate of TreS was maltose according to the analysis of k_{cat}/K_m . Both 1 and 10 mM of Hg^{2+} , Cu^{2+} and Al^{3+} could inhibit the TreS activity, while only 1 mM of Ca^{2+} and Mn^{2+} could increase its activity. Five amino acid residues, Asp²⁴⁴, Glu²⁸⁶, Asp³⁵⁴, His¹⁴⁷ and His³⁵³, were shown to be conserved in *R. opacus* TreS, which were also important for α -amylase family enzyme catalysis.

Keywords *Rhodococcus opacus* · Trehalose synthase · Trehalose · *Escherichia coli*

Abbreviations

Amp	Ampicillin
ATCC	American type culture collection
EMBL	The European Molecular Biology Laboratory
HPLC	High-performance liquid chromatography
IPTG	Isopropyl β -D-1-thiogalactopyranoside
LB	Luria–Bertani
PCR	Polymerase chain reaction
SDS-PAGE	SDS polyacrylamide gel electrophoresis
TLC	Thin layer chromatography
TreS	Trehalose synthase

1 Introduction

Trehalose composed of two glucose molecules is a non-reducing disaccharide, which has been isolated from many organisms ranging from bacteria, yeast, fungi to higher and lower of animals and plants [7]. Trehalose and its derivatives can play a role as protectants against various physical and chemical stresses, such as cold, heat, dehydration and oxygen radical [22]. Moreover, trehalose works as an important cell constituent of many *Mycobacteria* and *Corynebacteria* strains [2]. Due to its properties, it can be used as a useful supplement in industrial applications, like foods, cosmetics and pharmaceutical industries [23].

Five distinct biosynthetic pathways can lead to the formation of trehalose: (a) TPS-TPP [3]; (b) TreP [25]; (c) TreT [28]; (d) TreY-TreZ [8]; and (e) TreS [30]. TreS is an enzyme that allows one-step formation of trehalose from maltose through intramolecular transglycosylation. In summary, this pathway is capable of being used in the industrial manufacture of trehalose [29, 31]. Members of

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the *Rhodococcus* genus have a marked ability to metabolize a wide variety of xenobiotic compounds, and can survive in extreme environment, such as *R. opacus* PD630 could withstand water stress [1]. The formation of biosurfactants by rhodococci has been studied well, and several trehalose-containing glycolipids that often occur as a complex mixture during growth of rhodococci were structurally clarified. Certain trehalose lipids from rhodococci also played an important role in many asides [10, 21].

Rhodococcus opacus B4 was isolated in 2005, which had a tolerance to organic solvents like benzene, toluene, ethyl-benzene, xylene and styrene and could survive in the extreme environment for at least 5 days [19, 20, 26]. This tolerance probably attributed to glycolipids, typically trehalose mycolates, which were the parts of its own cell envelope [27].

The genome sequence of *R. opacus* B4 (EMBL AP011115) was published 3 years ago. Nevertheless, very little work has been presented on the biochemical and biophysical properties of its putative TreS. In our study, a novel TreS gene was cloned from the genomic DNA of *R. opacus* ACCC 41021 and overexpressed under the control of T7 promoter in *Escherichia coli*. We studied the biochemical function and enzyme features of this TreS. To our knowledge, this is the first report on TreS from *Rhodococcus* genus and would provide helpful information for the studies both on TreS and on *Rhodococcus* in the future.

2 Materials and Methods

2.1 Bacterial Strains, Plasmids and Cultivation

Rhodococcus opacus was obtained from the American Type Culture Collection (ATCC 41021). The pGEM Easy-T Vector System (Promega A3600, USA) was used as a cloning vector. The pRSET-B (Novagen, USA) plasmid was used as an expression vector, which contained a T7 promoter and a hexahistidine-tag. The bacterial strains *E. coli* Top10 and BL21 (DE3) pLysS (Novagen, USA) were used as hosts for target gene clone and expression, respectively. The *E. coli* strains were grown either in Luria–Bertani (LB) broth or on LB agar supplemented with 100 µg/mL ampicillin (Amp) at 37 °C. The *R. opacus* strain was cultured at 26 °C in ISP-1 medium (glucose 0.4 %, yeast extract 0.4 %, malt extract 1 % and CaCO₃ 0.2 %).

2.2 Construction of Recombinant Expression Vector

The genomic DNA of *R. opacus* ATCC 41021 was obtained by a bacteria genome DNA extracting kit (Tiangen Biotech, China) and worked as the template for amplification of target gene. The oligonucleotide primers for amplification

were designed as 5'-GAAGATCTATGCCCCTGGAAGATTTCCTCGTC-3' (*Bgl*II cleavage site underlined) and 5'-CCAAGCTTTCCTGTTGCACCGCCTGTT-3' (*Hind*III cleavage site underlined). The amplified DNA fragments were linked with pGEM Easy-T vector and transformed into *E. coli* Top10. The correct transformants were digested with *Bgl*II and *Hind*III, then being linked with pRSET-B that had been digested with the corresponding restriction enzymes. The correct recombinant vector was transformed into *E. coli* BL21 (DE3) pLysS to express recombinant TreS.

2.3 Expression of the Recombinant TreS

The recombinant *E. coli* BL21 (DE3) pLysS containing the TreS gene was cultured in 1 L LB broth supplemented with 100 µg/mL Amp and grew until reaching OD₆₀₀ of 0.6. Then, isopropyl β-D-1-thiogalactopyranoside (IPTG, final concentration 0.5 mM) was added to induce the protein expression, and incubation was continued for 6 h at 25 °C. The cells were harvested by centrifugation at 4,000 rpm for 10 min at 4 °C and the resulting cell pellet was stored at -75 °C.

2.4 Protein Purification

Each pellet was resuspended in 20 mL lysis buffer (50 mM K₂HPO₄-KH₂PO₄, pH 8.0, 0.5 M NaCl and 10 % glycerol). Phenylmethanesulfonyl fluoride (PMSF, final concentration 1 mM) and lysozyme (0.4 mg/mL) were supplemented. After half-hour incubation on ice, cells were lysed using a sonicator (Scientz JY92-DN, China). After that, 10 % Triton X-100 (0.05 %, final concentration), MgCl₂ (final concentration 1 M), and DNase (10 µg/mL) were supplemented. Cells were lysed after incubation again on ice. The lysates were harvested by centrifugation (6,000 rpm, 4 °C and 20 min), and then applied to NTA nickel-ion column (Novagen, USA), which had been washed twice with 10 bed volumes of lysis buffer. The column containing TreS was washed by lysis buffers containing different concentrations (20–500 mM) of imidazole at the flow speed of 0.2 mL/min. Finally, the solution containing target protein was concentrated by ultrafiltration (Millipore UFC803024, BJXJKSW, and China) following the manufacturer's instructions.

2.5 Electrophoretic Methods: SDS-PAGE, Native-PAGE of the Recombinant TreS

Protein samples from expression and purification (10 µL) were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on polyacrylamide gel (12 %) in accordance with Laemmli [12]. Electrophoresis was performed on 1.5 mm vertical gel slabs at 24 mA for 4 h. Gels were stained with Coomassie Brilliant blue G250

after electrophoresis. The standard protein mixtures obtained from TransGen Biotech (Beijing, China) were used as reference.

The oligomerization degree of the recombinant TreS was determined by native gel electrophoresis employing a 12 % gel according to native Laemmli system [12] by using native protein marker (TIANGEN Biotech CO, China). The Bradford method [4] was used to quantify the recombinant protein with bovine serum albumin as a standard.

2.6 Activity Assay of TreS

The enzymatic activity of TreS was identified by the method of thin layer chromatography (TLC). The reaction mixtures containing either 90 mM maltose or trehalose and 100 μ L enzyme solution were performed at 25 °C for 1 h. To stop the enzymatic reaction, the reaction mixtures were heated at 100 °C for 10 min. The products (1 μ L) were injected onto silica gel plates (HPTLC 10 \times 20 cm, Merck, Germany) and a hair drier was used to dry the plates. To separate the samples, a solvent system of 1-butanol/pyridine/water (4/5/1) was used. The plates were dried again by a hair drier after 1 h. Sugars were detected by spraying the plate with 20 % (v/v) sulfuric acid in methanol. To view the results, the plate was heated in 105 °C for 5–10 min. The BandScan software was used to quantify the reaction mixtures. One unit of enzyme activity was defined as the amount of enzyme that generated 1 mmol trehalose per minute. HPLC system equipped with a Dionex 2500 system was used to confirm and quantify the products of different reactions. Ten pmol of reaction samples were injected into the system at a flow speed of 10 μ L per minute.

The effects of metal ions on the activity of TreS were determined in an assay buffer containing 1 or 10 mmol/L metal ions under the standard reaction conditions.

2.7 Effects of Temperature on TreS

A reaction mixture containing 90 mM maltose was incubated with 100 μ L enzyme solution at different temperatures (15–70 °C) to identify the effects of temperature on TreS activity. To study the stability of TreS, reaction mixtures were pre-incubated at different temperatures (15–70 °C) for 1 h, and then examined the residual activities under the standard assay. All reactions were carried out at pH 7.0. To stop the enzymatic reaction, the reaction mixtures were heated in 100 °C for 10 min. The mixtures were analyzed by TLC and HPLC.

2.8 Effects of pH on TreS

A reaction mixture (pH 3–11) containing 90 mM maltose was incubated with 100 μ L enzyme solution at 25 °C for

1 h to identify the effects of pH on TreS activity. To study the stability of TreS, the reaction mixtures were pre-incubated at different pHs (pH 3–11) for 1 h, and then the residual activities were examined under the standard assay. To stop the enzymatic reaction, the reaction mixture was heated in 100 °C for 10 min.

2.9 Determination of Kinetic Parameters

The K_m , V_{max} , and k_{cat} of the TreS were analyzed according to the method of Lineweaver and Burk [15]. The analyses of Kinetic parameters were carried out under the conditions of pH 7.0 and 25 °C for 1 h in 100 mmol/L potassium phosphate buffer containing different substrates (maltose, trehalose, and glucose) at different concentrations (1–50 mM).

3 Results

3.1 Cloning of TreS Gene From *R. opacus*

Since trehalose has been detected in *R. opacus* [1], the biosynthesis of trehalose was assumed in this organism. Besides, *R. opacus* B4 was reported to have the TreS gene according to its genomic DNA sequence (EMBL AP011115). Hence, we designed primers according to the TreS gene sequence of *R. opacus* B4. As a result, we successfully isolated the TreS gene from *R. opacus* ACCC 41021, which had a length of 1,857 bp and encoded 618 amino acids. The TreS gene had been submitted to the GenBank database, and the Accession Number was KC473564. This TreS showed 96, 93, and 81 % homology with the TreS genes from *Rhodococcus jostii* RHA1 (gi = 110816552), *R. opacus* B4 (gi = 226359415), and *Pseudonocardia dioxanivorans* CB1190 (gi = 326948588), respectively. On the other hand, this gene produce showed 99, 98, 96, and 79 % identify with *R. opacus* M213 (gi = 419961084), *Rhodococcus jostii* RHA1 (gi = 111023004), *Rhodococcus opacus* B4 (gi = 226365509), and *Pseudonocardia dioxanivorans* CB1190 (gi = 331695578), respectively. The product of TreS gene had no signal peptide according to the analysis of its amino acid sequence, which suggested that this amino acid sequence was an intracellular protein.

3.2 Expression and Purification of the TreS

With the help of T7 promoter, the recombinant strain transformed with plasmid pRSET-B that carried the TreS gene had successfully expressed our aim gene and a hexabistidine-tagged. The enzyme was purified by NTA nickel-chelat chromatography, and most of TreS was eluted with lysis buffer consisting of 100 mM imidazole, 50 mM K_2HPO_4 - KH_2PO_4 , 0.5 M NaCl and 10 % glycerol. This

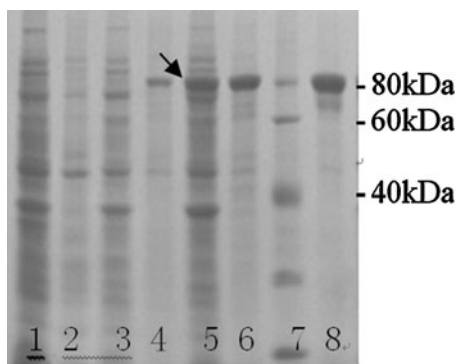


Fig. 1 SDS-PAGE analysis for the recombinant protein. *Lane 1, 2:* Total cell proteins of control group in supernatant and deposit, inducing for 4 h by IPTG; *Lane 3, 4:* Total cell proteins of positive group in supernatant and deposit, after inducing for 0 h by IPTG; *Lane 5, 6:* Total cell proteins of positive group in supernatant and deposit, after inducing for 6 h by IPTG; *Lane 7:* Protein molecular weight marker (80, 60, 40, 30, 20 kDa); *Lane 8* The purified protein

recombinant protein showed a 76 kDa band on SDS-PAGE gel (Fig. 1), which was a little larger than 71 kDa predicted by Compute pI/Mw (http://web.expasy.org/compute_pi/). This phenomenon was due to the extra parts coming from the pRSET-B vector. However, in native condition, TreS was present in a tetramer oligomeric state with a molecular weight of 290–300 kDa (date not shown), which kept pace with the TreS gene coming from *Meiothermus ruber* [31]. The fusion protein reached its peak expression level at 25 °C and induced for 6 h with 0.5 mM IPTG. The fusion protein accounted for approximately 45 % of total protein content in the bacterial lysates (analyzed by BandScan). The cellular extract of *E. coli* harboring TreS had a specific activity of 3.2 U/mg, which was greater than 2.822 and 0.726 U/mg coming from *S. solfataricus* KM1 and MT4 enzymes [24].

3.3 Activity of the Recombinant Enzyme

The products of reactions were showed in Fig. 2. TLC results revealed that this recombinant enzyme had the ability to catalyze both maltose and trehalose, moreover, these phenomenon were also verified by the analysis of HPLC. To quantify the trehalose production, the reaction mixtures were analyzed by HPLC. HPLC results showed additional that glucose appeared as a byproduct, which was in line with the TreS coming from *Arthrobacter aureescens* [29]. The HPLC results also showed the transformation efficiency of trehalose and glucose was 67 and 12 %, respectively.

3.4 Effects of Temperature on TreS

The effects of temperature on TreS were showed in Fig. 3. The optimal temperature (25 °C) of recombinant enzyme

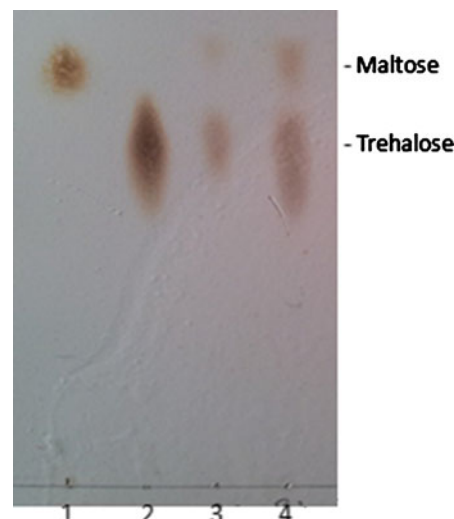


Fig. 2 TLC analysis of reaction products from different substrates by TreS. *Lane 1:* standard molecule of maltose; *Lane 2:* standard molecule of trehalose; *Lane 3:* maltose as the substrate; *Lane 4:* trehalose as the substrate. The reaction mixtures containing 90 mM substrate and 100 μ L enzyme solutions were performed at 25 °C for 1 h

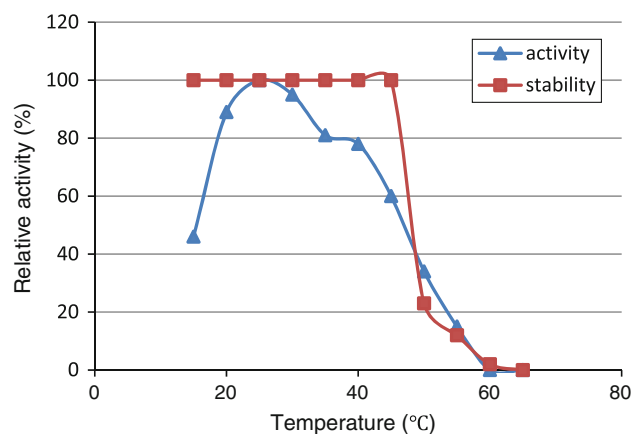


Fig. 3 Effects of temperature on TreS with maltose as the substrate. The effects of various temperatures (15–70 °C) on the activity of TreS were incubated at pH 7.0 for 1 h, using maltose as the reaction substrate. To study the stability of TreS, reaction mixtures were pre-incubated at different temperatures (15–70 °C) for 1 h, and then examined the residual activities

was lower than many other TreS such as the one (50 °C) coming from *Meiothermus ruber* [31] and the other (35 °C) coming from *Corynebacterium glutamicum* [9]. Upon prolonged incubation of TreS at the temperature range of 15–70 °C, the stability of TreS remained constant at the temperature range of 15–45 °C, while the enzymatic activity of TreS descended dramatically in 3 h when the temperature was above 60 °C. TreS catalyzed both transglycosylation reaction to produce trehalose and hydrolysis reaction to split maltose to glucose [9]. The trehalose yield increased along with a lower temperature, due to the

hydrolysis reaction decreased [6]. The structural flexibility of TreS decreased at a lower temperature according to Koh et al. [11], thus, the reaction temperature should be reduced to get the best conversion yield.

3.5 Effects of pH on TreS

The optimal pH was obtained by the analysis of activity at the pH range of 3–11 (Fig. 4). The pH dependence of the TreS activity is a typical bell-shaped curve suggesting the enzymatic hydrolysis depends upon two ionizable amino acid residues. And the pH optimum of this enzyme was around pH 7.0. Upon prolonged incubation of TreS in the pH range of 4.0–6.5 and 10.5–11.0 the enzyme lost 30–60 % of its activity in 1 h, whereas in the pH range of 6.5–8.5, the enzyme activity remains constant. The enzyme maintained the highest stability at pH 7.0–7.5, which was consistent with the properties of other TreS [9].

3.6 Effects of Metal Ions and Reagents

It's well known that an enzyme activity was frequently affected by metal ions and reagents [5]. To detect the influences of metal ions and reagents on TreS activity, we tested enzyme activity under the conditions containing different metal ions and reagents (1 or 10 mM), shown in Fig. 5. TreS activity could be suppressed by 1 mM Hg^{2+} , Cu^{2+} , SDS and Al^{3+} , while increased by 1 mM Ca^{2+} and Mn^{2+} . But almost all metals and reagents inhibited the enzyme activity when the concentration reached 10 mM, except for the chelator EDTA, which suggested that the enzyme was not sensitive to EDTA.

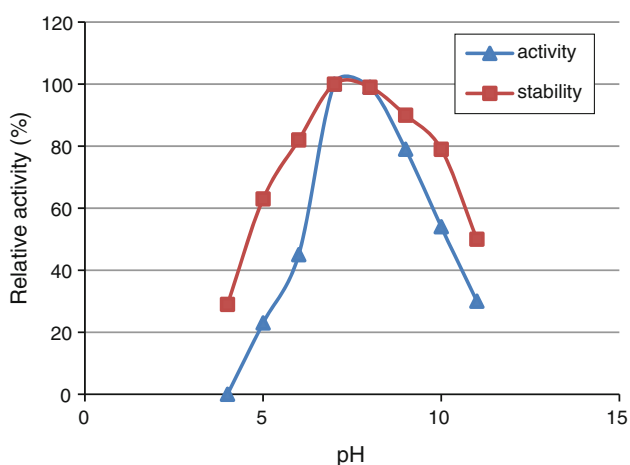


Fig. 4 Effects of pH on TreS with maltose as substrate. The effects of different pHs (pH 3–11) on the activity of TreS were studied at 25 °C for 1 h, using maltose as the reaction substrate. To study the stability of TreS, the reaction mixtures were pre-incubated at different pHs (pH 3–11) for 1 h, and then examined the residual activities

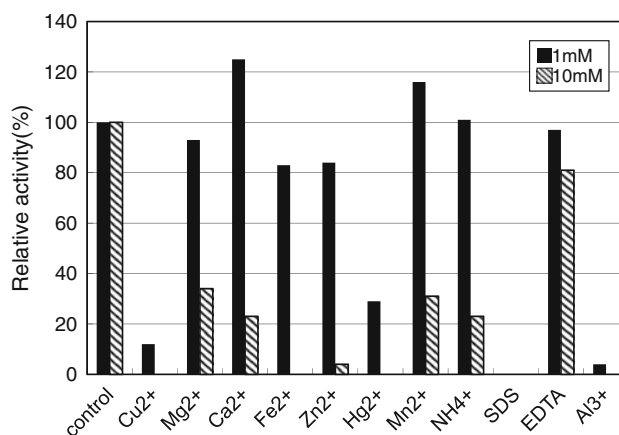


Fig. 5 Effects of metal ions and reagents on TreS. The enzyme was incubated with 1 or 10 mM of different substrate metal ions and reagents at 25 °C for 1 h, using maltose as the substrate

3.7 Kinetic Parameters

Reactions of the recombinant TreS with different substrates were performed (Table 1). The enzyme TreS had a higher affinity for maltose than trehalose according to their K_m values. It had 4.3-fold higher enzyme efficiency toward maltose than trehalose according to k_{cat}/K_m , which indicated that the reaction equilibrium trend was producing trehalose. With glucose adding in, the K_m presented a five-fold increase, while k_{cat}/K_m decreased by six-fold, implying that glucose could inhibit the formation of trehalose.

4 Discussion

Two TreS genes, 1,860 bp gene (gi = 226359415) and 2,190 bp gene (gi = 226359415), were previously known about the genomic DNA of *R. opacus* B4 (EMBL AP011115). In our study, we successfully cloned the 1,857 bp gene from *R. opacus* ACCC 41021 according to the 1,860 bp gene, which encoded 618 amino acids. It was more similar to other known TreS genes than the 2,190 bp gene [16, 31]. To our known, members of *Rhodococcus* had the ability to generate biosurfactant molecules [13]. The molecules were mainly glycolipids, especially trehalose monomycolates, trehalose dimycolates, trehalose trimycolates, as well as octa-acylated derivatives of trehalose [14, 18, 21]. Furthermore, the extracts of rhodococcal cell envelope were normally represented by trehalose mycolates [27]. According to this phenomenon, *R. opacus* was a good candidate to isolate the gene encoding an enzyme for synthesizing trehalose.

Besides, this TreS belonged to the amylase family according to the analysis of *N*-terminal by BLAST [17].

Table 1 Kinetic parameters of TreS reacted with different substrates

Substrate	K_m (mM)	V_{max} (mM/min/mg)	k_{cat} (s^{-1})	k_{cat}/K_m (mM/s)
Maltose	60 ± 1.4	0.911 ± 0.04	75.90 ± 2.1	1.26 ± 0.04
Trehalose	98 ± 2.2	0.329 ± 0.02	27.44 ± 0.9	0.29 ± 0.03
Maltose+glucose	327 ± 4.6	0.998 ± 0.02	66.71 ± 1.3	0.20 ± 0.01

Kinetic parameters are expressed as mean ± SD calculated by non liner regression

Table 2 Comparison of conserved amino acid sequences about α -amylase family

	Region 1	Region 2	Region 3	Region 4
<i>Rhodococcus opacus</i> B4	DLVMNH 147	GFRLDAVPY 244	AEAN 286	FLRNHD 353
<i>Pseudonocardia dioxanivorans</i> CB1190	DLVMNH 133	GFRLDAVPY 230	AEAN 276	FLRNHD 341
<i>Amycolicococcus subflavus</i> DQS3-9A1	DLVINH 148	GFRLDAVPY 247	AEAN 289	FLRNHD 358
<i>Mycobacterium smegmatis</i> str. MC2-155	DLVMNH 133	GFRLDAVPY 230	AEAN 276	FLRNHD 341
<i>Streptosporangium roseum</i> DSM 43021	DLVMNH 120	GFRMDAIPY 217	AEAN 263	FLRNHD 328
<i>Thermomonospora curvata</i> DSM 43183	DLVMNH 120	GFRLDAVPY 217	AEAN 263	FLRNHD 328
<i>Corynebacterium amycolatum</i> SK46	DLVMNH 108	GFRLDAVPY 205	AEAN 251	FLRNHD 316

Conserved residues are highlighted in black

Accession numbers for relative nucleotide sequences are as follows. *Pseudonocardia dioxanivorans* CB1190 gi = 331695578; *Amycolicococcus subflavus* DQS3-9A1 gi = 333921358; *Mycobacterium smegmatis* str. MC2-155 gi = 118469348; *Streptosporangium roseum* DSM 43021 gi = 271969343; *Thermomonospora curvata* DSM 43183 gi = 269127787; *Corynebacterium amycolatum* SK46 gi = 213965532

Based on the result of Clustal Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>), we found five highly conserved regions, which were identical to the α -amylase family (Table 2) [17]. The important residues are Asp²⁴⁴, Glu²⁸⁶, Asp³⁵⁴, His¹⁴⁷ and His³⁵³. Asp²⁴⁴ and Asp³⁵⁴ might function as nucleophiles that attack the bonded anomeric carbon of maltose. Glu²⁸⁶ might play a role in proton donor. His¹⁴⁷ and His³⁵³ might act as the substrate binding site [6]. The role of conserved sites also confirmed in *Thermus caldophilu* [11] and *Meiothermus ruber* [31]. We tested both the effects of temperature and pH on TreS activities and kinetics parameters of TreS to get the properties of this enzyme. Concluding the data from the pH/temperature dependence and stability studies, the optimum temperature of TreS was 25 °C and the optimal pH of TreS was 7.0. Besides, the TreS was stable with the highest activity at 15–30 °C and pH 6.5–8.5 for several hours, which was related to the environmental conditions of this species [24]. It could produce 67 % trehalose and 12 % glucose using maltose as substrate. Lower reaction temperature could decrease the weak hydrolytic property and decrease the synthesis of glucose [11]. Considering the properties of this protein, the *R. opacus* TreS has the potential to be used in industrial applications.

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