JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

Sk. Z. and Vardharajula et al. 2013 : 3 (1) 63-68

ISOLATION AND IDENTIFICATION OF A THERMOTOLERANT PLANT GROWTH PROMOTING *PSEUDOMONAS PUTIDA* PRODUCING TREHALOSE SYNTHASE

Ali Sk.Z.[1] and Sandhya Vardharajula*[2]

Address(es): Dr.Ali Sk.Z.,
[1]Department of Microbiology, Agri Biotech Foundation, Acharya N.G. Ranaga Agricultural University, Rajendranagar, Hyderabad-500030, Andhra Pradesh, India. Telephone: +91 04-24002147/24009011/64562146.
[2]Department of Dryland Cereals, International Crops Research Institute for the Semi-Arid-Tropics, Patancheru, Hyderabad, Andhra Pradesh, India.

*Corresponding author: skzali28@gmail.com

ARTICLE INFO

Received 26. 2. 2013
Revised 26. 6. 2013
Accepted 28. 6. 2013
Published 1. 8. 2013

Regular article

ABSTRACT

A thermotolerant plant growth promoting *Pseudomonas* isolate growing at 40°C producing trehalose synthase (TreS) was isolated from rhizosphere soil under semi arid conditions of India. Trehalose synthase was extracted; purified and enzymatic activity was examined at various temperatures and pH. The optimum temperature and pH was 38°C and pH 7.5 and the activity declined at above or below the optimum pH and temperature. The enzyme was active on maltose and trehalose among saccharides tested. The enzyme had a higher catalytic activity for maltose with a trehalose yield of 72% than for trehalose where 30% yield of maltose was achieved, indicating maltose as preferred substrate. The isolate showed multiple plant growth promoting traits (indole acetic acid (IAA), phosphate solubilization, siderophore and ammonia) both at ambient (28°C) and high temperature (40°C). Based on phenotypic and 16SrRNA analysis the isolate was identified as *Pseudomonas putida* (Accession No. GU396283).

Keywords: Trehalose synthase (TreS), *Pseudomonas putida*, thermotolerance , PGP traits

INTRODUCTION

A changing environment creates conditions that can be stressful for microorganisms (**Schimel *et al.*, 2007**). Temperature is one of the most important factors influencing bacterial growth and survival in natural environments (**Doetsch and Cook, 1973**). Bacteria have a remarkable capacity for adaptation to environmental stress (**Storz, 2000**). The defense system involves the reorganization of gene expression for adaptation to the altered physiological state, including synthesis of chaperones and other stress related proteins that shield macromolecules and membranes from damage (**Csonka, 1989; Kempf and Bremer, 1998**). In addition to the synthesis of new proteins, most microorganisms synthesize low molecular weight compatible solutes which help in maintaining membrane integrity and protein stability (**Brown, 1990**).
Trehalose is a soluble, non reducing disaccharide of glucose which exists in three isomeric forms α, α- trehalose, α, β- trehalose and β, β- trehalose. Among them, only α, α- trehalose (1-*0*-(α-D-glucopyranosyl)- α-D-glucopyranoside) is the most commol l-1 only occurring isomer and is found in bacteria, fungi, plants and animals (**Elbein, 1974**). Initially, trehalose was thought to serve as a reserve metabolizable substrate (**Lillie and Pringle, 1980**), but recent studies indicated that this sugar instead plays a major role in cell protection against harsh environmental conditions (**Strom and Kaasen, 1993**). Bacterial and yeast cells accumulate trehalose to a very high levels (up to 500 mM) in response to heat shock (**Attfield, 1987**). There are several pathways for the biosynthesis of trehalose and three pathways have been well studied (**De Smet *et al.*, 2000**).The most widely studied and the best-known pathway is *OtsA*/*OtsB* (TPS/TPP) (**Cabib and Leloir, 1958**) which involves condensation of glucose-6-phosphate with UDP-glucose to form trehalose-6-phosphate (T6P), catalyzed by trehalose-phosphate synthase (TPS) and subsequently T6P is dephosphorylate to yield trehalose, catalyzed by threhalose-phosphate phosphatase (TPP). In the second pathway maltose is converted to trehalose *via* trehalose synthase (TreS), which is encoded by *treS* gene (**Lee *et al.*, 2005**).The third pathway involves polyglucose to generate trehalose *via* maltooligosyltrehlaose synthase (TreY) and maltooligosyltrehlaose trehalohydrolyase (TreZ), which are encoded by *treY* and *treZ* (**Maruta *et al.*, 1996**).
In this study we isolated and identified a thermotolerant *Pseudomonas* isolate from rhizosphere soil. It was found that the cell free extracts of the strain showed trehalose synthase activity at various temperatures and pH conditions. Based on phenotypic and 16SrRNA analysis the isolate was identified as *Pseudomonas putida*.

MATERIAL AND METHODS

Isolation and screening for thermotolerance

Isolation of *Pseudomonas* sp. was made from rhizhosphere soil grown under semi arid conditions of India The plants were uprooted along with bulk soil and brought immediately to the laboratory in polythene bags under refrigerated conditions for isolation of rhizobacteria within 48 h of uprooting. The bulk soil was removed by gently shaking the plants. The rhizosphere soil was collected by dipping the roots in sterile normal saline and kept on shaker for 30 min. The soil suspension was serially diluted and appropriate dilutions were spread plated on solid King's B medium. The plates were incubated at 28±2°C and fluorescent colonies were selected for further studies.
In order to screen the strain for thermotolerance, the overnight-grown broth cultures adjusted to optical density of 0.5 at 600 nm were inoculated into trypticase soy broth (TSB) and incubated at different temperatures (from 28°C to 40°C) in a orbital shaking incubator at 120 rpm for 48 h. Optical density (600 nm) of the cultures incubated at different temperatures was recorded.

Morphological and Biochemical characterization

Morphological and biochemical characterization was done by performing different biochemical tests viz. Gram staining, oxidase activity, catalase activity, starch hydrolysis, denitrification, production of indole and acetyl methyl carbinol, citrate utilization, and fermentation of different sugars glycerol, glucose, fructose, mannitol, lactose, galactose, mannose, rhamnose, sorbitol, maltose, sucrose and arginin following Bergey's manual of determinative bacteriology (**Holt *et al.*, 1994**).

Genotypic characterization

For genotypic characterization, bacterial genomic DNA was isolated according to the method of **Chen and Kuo (1993)** and the 16S rRNA gene was amplified using 16S forward primer (5' AGAGTTTGATCCTGGCTCAG 3') and reverse primer (5'AAGGAGGTGATCCAGCCGCA 3'). PCR was carried out in a Veriti

96-well PCR system model 9902 (Applied Biosystems, Singapore) using a reaction volume of 100 µl (deionized water, 78.0 µl; 10 X Taq Pol buffer, 10.0 µl; Taq Polymerase (1U), 1.0 µl; dNTP's (2 mM), 6.0 µl; forward primer (100 ng), 1.5 µl; reverse primer (100 ng), 1.5 µl and target DNA (50 ng), 1.0 µl) under the following conditions one cycle of 94°C for 5 min, 30 cycles of 94°C for 1 min (denaturation), 50°C for 40 sec (annealing) and 72°C for 90 sec (extension), one cycle of 7 min at 72°C and a holding at 4°C. The PCR products obtained were resolved on 1.5% agarose gel electrophoresis. Gels were visualized and photographed in UV gel image acquisition CCD camera (Bio-Vision ++, France). DNA sequencing was conducted at Occimum Bio-Solutions, Hyderabad, India with an Applied Biosystems 3730xl DNA Analyzer. Sequencing of the PCR products was performed using the fluorescence based dideoxy termination method. Sequencing reactions were set up with the Big Dye Terminator V 3.1Cycle Sequencing kit. The 16S rRNA nucleotide sequence corresponding to the genotypes were analyzed for homologies with sequences in the existing database using BLAST searching programme (*ftp://. ncbi.nih.gov/toolbox/ncbi_tools/*).

Heat shock treatment

In order to induce the heat shock the cells were harvested from overnight raised Luria Bertain (LB) broth culture by centrifugation at 5,000 rpm for 10 min and washed twice with sterile 50 mM sodium phosphate buffer (pH 7.0), resuspended in LB broth to a final concentration of 8.2 log CFU/ml. To induce heat shock, the cell suspensions were incubated for 3 h at 40°C in a shaking water bath at 50 rpm respectively. Control cells were incubated for 3 h at 28°C. After heat shock for 3 h, cells were chilled on ice until the temperature dropped to room and used for extraction of intracellular trehalose and trehalose synthase.

Detection of intracellular trehalose

For detection of intracellular trehalose, heat shocked (40°C) and control (28°C) cells were harvested by centrifugation (5,000 rpm for 10 min) and washed with 50 mM potassium phosphate buffer (pH 7.0). The pellets were suspended in 70% methanol (v/v) and heated at 80°C for 10 min in a water bath and the supernatants were collected by centrifugation (10,000 rpm for 15 min) and the lysate were analyzed for sugar analysis by thin layer chromatography (TLC) according to the method of **Managbang and Torzilli (2002)** with little modifications. Standards which consisted of glucose, trehalose, fructose, maltose, mannose and galactose (Sigma-Aldrich) were spotted onto Silica Gel 60 plates (Merck, Germany) and developed with butanol: acetic acid: water (40:10:50 v/v) as mobile phase. The spots were detected by spraying with 1% $KMNO_4$ in 1% NaOH. The rf-values of spots were measured and compared with that of standard sugars.

Purification of crude cell extract

For purification of trehalose synthesizing enzymes, controlled and heat shocked cell pellets were suspended in 5 ml of 5 mM sodium EDTA-100 mM potassium phosphate buffer (pH 7.0) and disrupted in a sonicator at 4°C for 10 short bursts of 10 sec each with an interval of 30 sec on ice to prevent denaturation of cell proteins. The crude cell lysate obtained by centrifugation 12,000 rpm for 15 min at 4⁰C was subjected to purification according to the methods of **Ko *et al.* (1996)** and **Koh *et al.* (1998)**. All the purification steps were carried out at 4⁰C unless otherwise specified. Solid ammonium sulphate $(NH_4)_2 SO_4$) was added to reach 60% saturation, the suspension was stored for 2 h and then centrifuged at 12,000 rpm for 10 min. The resulting precipitate was dissolved in 15 ml of ice- cold 10 mM potassium phosphate buffer, pH 7.0 and dialyzed using nitrocellulose membrane against same buffer. The dialyzed enzyme solution was applied to a DEAE-Sephacel column (2.6 x 10 cm) that had been equilibrated with 10 mM potassium phosphate buffer, pH 7.0 containing 1M KCl. Fractions (3 ml) were collected and an aliquot of each fraction was removed. The eluted enzyme fractions were pooled and solid $(NH_4)_2 SO_4$ was added to reach 60% saturation and stored for 2 h. The enzyme fractions were centrifuged at 12,000 rpm for 15 min and the resulted precipitate was dissolved in 10 ml of 1M KCl and applied to a Phenyl-Sepharose CL-4B column (5 x 20 cm) equilibrated with 10 mM potassium phosphate buffer, pH 7.0. The column was washed with 100 ml of 10 mM potassium phosphate buffer, pH 7.0 and the retained proteins were eluted with a linear gradient of 1 to 0 M $(NH_4)_2 SO_4$ in 200 ml of 10 mM potassium phosphate buffer, pH 7.0.The enzyme fractions were pooled and solid $(NH_4)_2 SO_4$ was added and allowed to stand for 2 h. After centrifugation at 12,000 rpm for 15 min the precipitate was dissolved in 1 ml of 10 mM potassium phosphate buffer, pH 7.0 and applied to Sephacryl S-300 (1.6 X 60cm) column equilibrated with the same buffer and the resulted fractions were used as target enzyme.

Protein assay

Protein content was measured by the method of Bradford (**Bradford, 1976**) with bovine serum albumin as standard protein. Absorbance at 280 nm was used for monitoring protein in column elutes.

Enzyme assay

The activity of trehalose synthase was assayed by measuring trehalose produced from maltose. The reaction mixture, consisting of 10 mM potassium phosphate buffer (pH 7.0) and 10 mM maltose, 10mM trehalose and the enzyme (5µg) in the final reaction volume of 5 ml. The reaction mixture was incubated for 1 h at 40⁰C and heated for 10 min in a boiling water bath to stop the reaction. The supernatant was collected by centrifugation at 10,000 rpm for 10 min and assayed by high performance liquid chromatography (HPLC). One unit of enzyme activity was defined as the amount of enzyme catalyzes the formation of 1 µmol of trehalose per minute. The relative enzyme activity (%) was defined as the percentage of enzyme activity in the control.

Optimum temperature and pH of trehalose synthase

The optimum temperature of the purified enzyme was determined by incubating the reaction mixture at various temperatures ranging from 30°C to 42°C for 1 h and the assay was carried out as mentioned above. The optimum pH of trehalose synthase was determined at various pH ranging from 5.0-9.0 using citrate buffer (pH 3.0-6.0), phosphate buffer (pH 6.0-8.0) and glycine buffer (pH 8.5-10.0). The enzyme was incubated at various pH values at 38°C for 1 h and the assay was carried out as mentioned above.

Thermal and pH stability of trehalose synthase

The temperature stability of TreS was determined by incubating 2 ml purified enzyme with 2 ml of 10 mM potassium phosphate buffer without substrate at different temperatures ranging from 30°C to 42°C for 1 h and then the trehalose assay was performed to determine their residual activity. For pH stability 2 ml of purified enzyme was incubated with 2 ml of citrate buffer (pH 3.0-6.0), phosphate buffer (pH 6.0-8.0) and glycine buffer (pH 8.5-10.0), respectively without substrate for 1 h and then trehalose assay was performed to determined their residual activity.

HPLC analysis of trehalose

HPLC analysis of trehalose was carried out at Vimta Labs (Hyderabad, India) with 515 pump and 2410 refractive index detector. The conditions of HPLC were: Ø4.6 * 250 mm Hypersil-NH_2 column (Waters Co., Milford, MA, USA); acetonitrile: water (75:25 v/v) as mobile phase; 2.0 ml/min flow velocity; 30°C column temperature. The trehalose in the sample was measured by comparing the HPLC curves for the samples with the standards; trehalose, glucose and maltose (Sigma).

Screening for plant growth promoting (PGP) traits

The isolate was tested *in vitro* for its multiple PGP traits under both ambient (28°C) and high temperature (40°C). The method of **Gordon and Weber (1951)** was followed for the estimation of indole acetic acid (IAA). Luria broth (LB) amended with 5-mmol tryptophan was inoculated with overnight raised bacterial cultures (0.5 OD at 600 nm) and incubated at 28°C and 40°C for 48 h. One ml of culture was centrifuged (10,000 for 20 min) and supernatant separated. To the supernatant, 4-ml of Salkowsky reagent was added followed by incubation for 1 h at room temperature under dark conditions. Absorbance of the pink colour developed was read at 530 nm. Concentration of the proteins in the pellet was determined (**Bradford, 1976**) and the amount of IAA produced was expressed µg mg^{-1} cell protein. For studying phosphate solubilization, 5 µl of overnight raised culture was spotted on Pikovskaya's agar plates containing 2% tri-calcium phosphate. The plates were incubated at both temperature (28°C and 40°C) for 24 to 72 h, and observed for appearance of zone of solubilization around the bacterial colonies. For quantitative analysis, 5 ml of NBRI-BBP medium (**Mehta and Nautiyal, 2001**) was inoculated in triplicates with 50 µl of bacterial culture (0.5 OD at 600 nm) followed by incubation for 7 days at 28°C and 40°C respectively in incubator shaker at 120 rpm. The cells were harvested by centrifugation at 10,000 rpm for 10 min and the supernatant thus obtained were used for quantitative estimation of phosphate. For testing ammonia production, cultures were raised in 10 ml of peptone water at both temperatures (28°C and 40°C) for 48 h and 1-ml of Nesseler's reagent was added. Development of yellow to brown colour indicated production of ammonia (**Dey *et al.*, 2004**). For siderophore production, 1µl of overnight raised culture in Luria broth was spotted on Chrome Azurol S (CAS) agar plates and incubated at both temperatures for 48 h. Plates were observed for the appearance of orange halo around the bacterial colony (**Schwyn and Neilands, 1987**). For HCN production, the culture were streaked on King's B medium amended with 0.4% of glycine and Whatman no.1 filter paper disc soaked in 0.5% picric acid (in 2% sodium carbonate) was placed in the lid of petri plate. The plates were sealed with parafilm and incubated at 28°C and 40⁰C for 4 days for development of deep orange colour (**Bakker and schipper, 1987**).

RESULTS AND DISCUSSION

Isolation and identification of thermotolerant *Pseudomonas sp.*

Pseudomonas sp. was isolated from rhizosphere soil grown under semi arid conditions of India. The isolate formed circular, butyrous, elevated, translucent colonies with yellowish green fluorescent pigmentation under UV light (365nm). Cell morphology and size was recorded from light microscope (Olympus-CX31, Japan). Cells were Gram-negative rods measuring 0.25 x 2.5 µm, motile and the isolate could grow at a temperature of 40°C similar to, a high temperature tolerant-strain Pseudomonas putida NBRI0987 isolated from the drought-exposed rhizosphere of chickpea (*Cicer arietinum* L. cv. Radhey) (**Srivastsva *et al.*, 2008**). Under conditions where temperature exceed the normal growth range, cells experience stress due to the damaging effect of heat on intracellular macromolecules, such as heat-sensitive enzymes and to cell membranes (**Henle *et al.*, 1982**) that force them into dormancy or cause death (**Schimel *et al*., 2007**). In response, microorganisms have a variety of evolutionary adaptations and physiological acclimation mechanisms that allows them to survive and remain active in the face of environmental stress. The isolate showed positive reactions for citrate, catalase and oxidase, but negative reactions for starch, aesculin, urea hydrolysis, gelatin hydrolysis, indole, methyl red, voges proskauer production. Hydrolysis of arginine, casein and tween 80 is positive and assimilates glucose, maltose, fructose and glycerol (table 1).

Table 1 Phenotypic characterization of *Pseudomonas* isolate

Characteristic	*Pseudomonas* isolate
Motility	+
Indole	-
Methyl red	-
Voges- Proskauer	-
Citrate	+
Oxidase	+
Catalase	+
Starch	-
Denitrification	-
Glycerol	+
Galactose	-
Glucose	+
Fructose	+
Mannose	-
Rhamnose	-
Mannitol	-
Sorbitol	-
Maltose	+
Lactose	-
Sucrose	-
Arginin	+
Casein	+
Tween80	+
Aesculin	-
Urea	-

+ positive, - negative

For molecular identification, PCR amplification of 16S rRNA gene was carried out and the amplicon (~1500bp) was sequenced. The 16S rRNA gene sequence was BLAST analyzed using 16S rRNA sequence database of NCBI to determine sequence similarity. Alignment with the sequences available in the GenBank database showed 100% sequence similarity to *Pseudomonas putida* (DQ060242). The sequence was submitted to GenBank under the accession number GU396283.

Intracellular trehalose detection and enzyme assay

When *Pseudomonas* isolate exposed to heat shock at 40°C for 3h the intracellular accumulation of trehalose has been detected compared to control cells shown by TLC (figure 1).

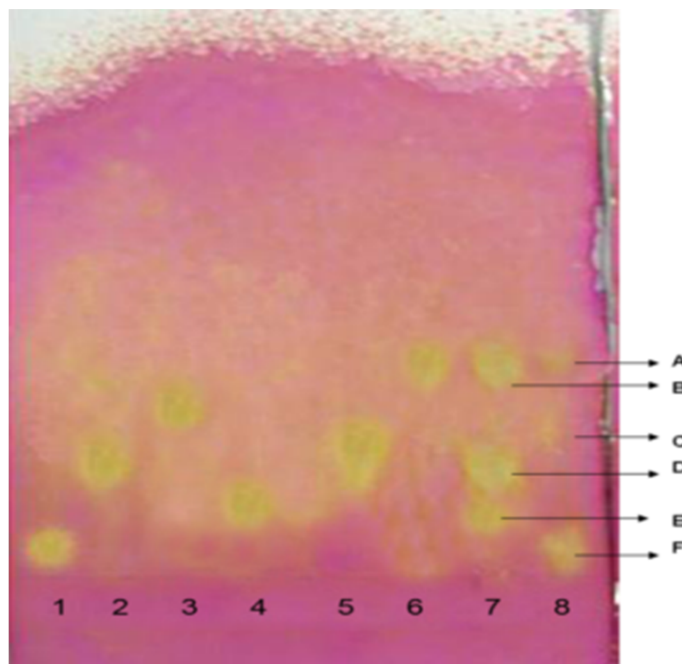
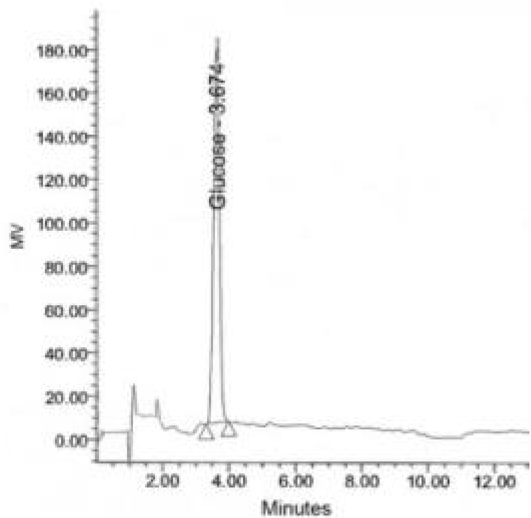
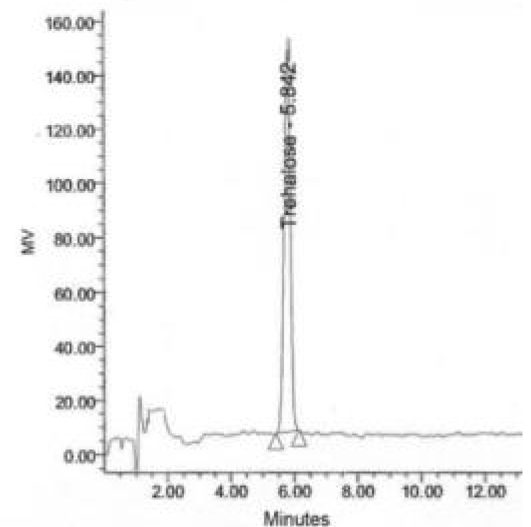
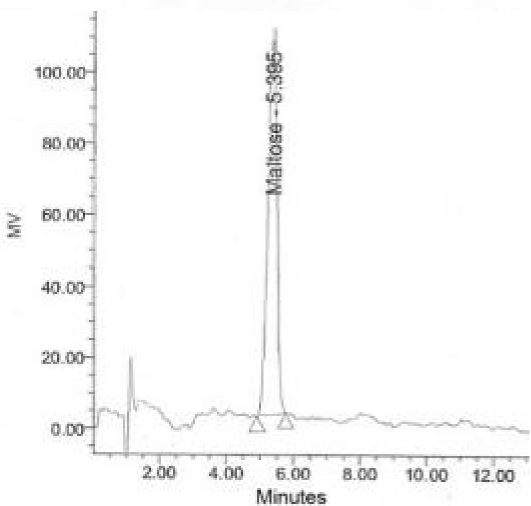


Figure 1 Thin layer chromatography (TLC) of the intracellular sugar extracts from thermotolerant *Pseudomonas putida*. (1 to 6, standard sugars) 1, trehalose; 2, fructose; 3, maltose; 4, mannose; 5, galactose; 6, glucose; 7, control cell extract; 8, heat shock cell extract; (A to E, spots) A and B, glucose; C, maltose; D, galactose; E, mannose; F, trehalose

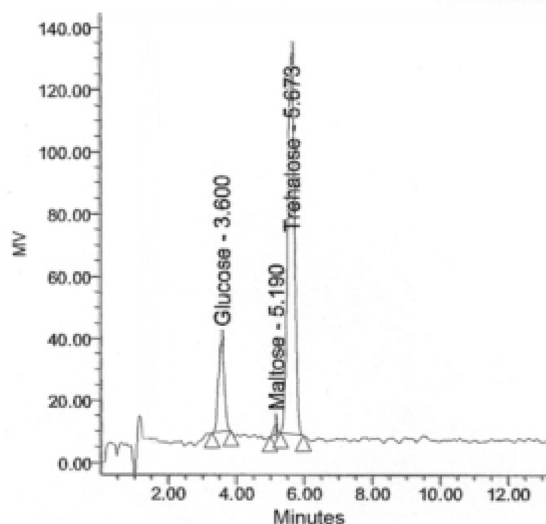
Trehalose assay was carried out using purified enzyme preparations which catalyzed the reversible interconversion of maltose to non-reducing trehalose or vice versa by intramolecular transglucosylation reaction. When maltose was used as a substrate, three major peaks appeared on the chromatograms obtained from HPLC as shown in figure 2. One was unreacted maltose, second was identified as trehalose due to conversion of maltose to trehalose and third identified as glucose produced during the conversion as byproduct. Retention time for trehalose formed due to trehalose synthase corresponded closely to those of standard solutions (± 0.05 min). Production of trehalose by stressed cells appears to be a critical adaptation that protects the microorganisms against wide varieties of lethal conditions and several trehalose synthase have been isolated from various bacteria (**Nishimoto *et al*., 1996a; Nishimoto *et al*., 1996b; Tsusaki *et al*., 1996; Tsusaki *et al*., 1997; Lee *et al*., 2005; Chen *et al*., 2006**).







(A)

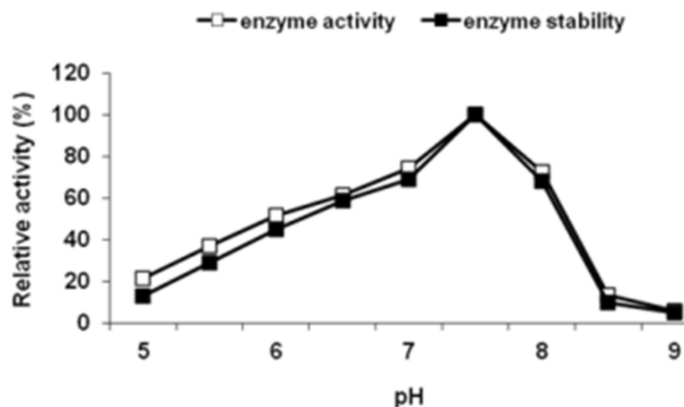


(B)

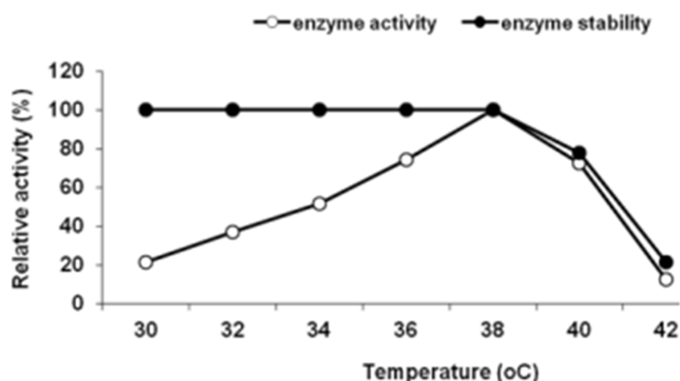
Figure 2 High performance liquid chromatography (HPLC) of the reaction products by purified trehalose synthase from thermotolerant *Pseudomonas putida*. (A) chromatogram with standard sugars (glucose, maltose, trehalose); (B) chromatogram showing reaction products

Similarly the purified extract obtained from controlled cells had not showed the conversion of maltose to trehalose and vice- versa. **Attifield (1987)** demonstrated that bacterial and yeast cells accumulate trehalose to very high levels (up to 500 mM) in response to heat shock allowing the cells to survive and remain active under thermal stress. When the strain exposed to heat shock at 40°C for 3 h the cells respond to heat shock by synthesizing trehalose synthase resulted in the intracellular accumulation of trehalose whereas, under controlled conditions (28°C), trehalose accumulation has not been detected indicates heat shock act as inducer for trehalose synthase expression producing trehalose allowing isolate to survive under high temperature stress.

A total yield of 72% was attained at optimum pH (7.5) and temperature (30 °C) when maltose was used as a substrate whereas in the reverse reaction the conversion of trehalose to maltose was a limited reaction where 30% yield of maltose was achieved along with small amount of glucose release indicating the equilibrium of the enzyme favorable towards the synthesis of trehalose. According to **Nishimoto *et al*., (1996 a, b), Lee *et al*., (2005), Chen *et al*., (2006)** TreS producing less or no glucose have relatively higher production rate of trehalose of about 70% to 80%. The enzyme was completely inactive on other substrates; maltotriose, maltotetraose, maltopentaose, maltohexaose, glucose and cellobiose. The purified trehalose synthase was stable between pH 6.0-8.0 (figure 3a) and temperature between 30°C to 40°C (figure 3b).



(A)



(B)

Figure 2 (A) Effect of pH on the activity and stability of trehalose synthase from thermotolerant *Pseudomonas putida*. The enzyme activity of TreS at various pH was studied at 38°C using 10 mM/l maltose as a substrate. The stability of trehalose synthase was studied at various pH values (pH 5.0-9.0) for 1 h at 38°C. The residual activity was measured at pH 7.5. The open Square (□) and solid square (■) represents effect of pH on the activity and stability of TreS respectively. (B) Effect of temperature on the activity and stability of trehalose synthase from thermotolerant *Pseudomonas putida* AKMP7. The enzyme activity of TreS at various temperatures was studied at pH 7.5 using 10 mM maltose as a substrate. The stability of trehalose synthase was studied at various temperatures (30°C to 42°C) for 1 h at pH 7.5. The residual activity was measured at 38°C. The open circle (○) and solid circle (●) represents effect of temperature on the activity and stability of TreS respectively**.**

The results showed that the enzyme was more stable at pH 7.5, similar to optimum pH reported for trehalose synthase from *Pimelobacter sp* R48 (**Nishimoto *et al*., 1996a**), *P. putida* H76 (**Ma *et al*., 2006**) and *T. aquaticus* ATCC33923 (**Nishimoto *et al*., 1996 b**). Similarly optimum temperature of the enzyme was 38°C (table 2), which was higher than optimum temperature showed by *Pimelobacter* sp R48 (**Nishimoto *et al*., 1996a**), *P. stutzeri* (**Lee *et al*., 2005**) and *P. putida* H76 (**Ma *et al*., 2006**). Moreover a decrease in the enzyme activity was observed above and below these optimum conditions (temperature and pH). The isolate possessed plant growth promoting traits such as IAA (42.9 mg mg^{-1} protein under ambient (28°C) and 25 mg mg^{-1} protein under high temperature (40°C), phosphate solubilization (63µl ml^{-1} ambient and 46µl ml^{-1} under high temperature), siderophore and ammonia positive both under ambient and under high temperature indicating its additional activity under different temperatures.

Table 2 Comparison of enzymatic properties of trehalose synthase from different sources

	***P. putida* This study**	***Meiothermus ruber***	***Thermus aquaticus* ATCC33923**	***Pimelobacter* sp. R48**	***Pseudomonas stutzeri***	***P.putida* H76**	***Picrophilus torridus***
Optimum pH	7.5	6.5	6.5	7.5	8.5-9.0	7.4	6.0
Optimum Temperature (°C)	38	50	65	35	20	35	45
pH stability	6.0-8.0	4.0-8.0	5.5-9.5	6.0	9.0	6.6-7.4	5.0- 7.5
Thermostability (°C)	42°C (1 h)	60 (5 h)	80(1 h)	30(1 h)	30(1 h)	35(1 h)	60(1 h)
References	This study	Zhu et al. 2010	Nishimoto et al. 1996b; Tsusaki et al. 1997	Nishimoto et al. 1996a; Tsusaki et al. 1996	Lee et al. 2005	Ma et al. 2006	Chen et al. 2006

CONCLUSION

Pseudomonas putida responds to high temperature by synthesizing trehalose synthase which resulted in the intracellular accumulation of trehalose allowing isolate to survive under high temperature. Selection and use of thermotolerant plant growth promoting rhizobacteria, with multiple PGP activities for the facilitation of plant growth in stressed environments, will be a highly important area for future research.

Acknowledgments: The authors are grateful to Indian Council of Agricultural Research (ICAR), New Delhi for providing the financial assistance in the form of network project on “Application of Microorganisms in agriculture and Allied Sectors” (AMAAS).

REFERENCES

ATTFIELD, P.V.1987. Trehalose accumulates in *Saccharomyces cerevisiae* during exposure to agents that induce heat shock response. *Federation of European Biochemical Societies Letters,* 225, 259-263.

BAKKER, A.W., SCHIPPER, B.1987. Microbial cyanide production in the rhizosphere in relation to potato yield reduction and *Pseudomonas* sp. Mediated plant growth stimulation. *Soil Biology and Biochemistry,*19,451–457.

BRADFORD, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry,*72, 248-254.

BROWN, A.D.1990. Microbial water stress physiology. Principles and perspectives, Wiley, New York.

CABIB, E., LELOIR, L.F.1958. The biosynthesis of trehalose-phosphate. *Journal of Biological Chemistry,* 23, 259-275.

CHEN, W.P., KUO, T.T.1993. A simple and rapid method for the preparation of Gram-negative bacterial genomic DNA. *Nucleic Acids Research,* 21, 2260.

CHEN, Y.S., Lee, G.C., Shaw, J.F.2006. Gene cloning, expression and biochemical characterization of a recombinant trehalose synthase from *Picrophilus torridus* in *Escherichia coli*. *Journal of Agricultural and Food Chemistry*, 54, 7098-8104.

CSONKA, L.M.1989. Physiological and genetic responses of bacteria to osmotic stress. *Microbiological Reviews,* 5, 121-147.

DE SMET, K.A.L., WESTON, A., BROWN, I.N., YOUNG, D.B., ROBERTSON, B.D. 2000. Three pathways for trehalose biosynthesis in Mycobacteria. *Microbiology,* 146,199-208.

DEY, R., PAL, K.K., BHATT, D.M., CHAUHAN, S.M.2004. Growth promotion and yield enhancement of pea nut (*Arachis hypogaea* L.) by application of plant growth promoting rhizobacteria. *Microbiological Research*, 159, 371–394.

DOETSCH, R.N., COOK, TM.1973. Introduction to bacteria and their ecobiology. University Park Press, Baltimore.

ELBEIN, A.D.1974. The metabolism of α, α- trehalose. *Advances in Carbohydrate Chemistry and Biochemistry,* 30, 227-256.

GORDON, A.S., WEBER, R.P.1951. Colorimetric estimation of indole acetic acid. *Plant Physiology,* 26, 192–195.

HENLE, K.J., NAGLE, W.A., MOSS, A.J., HERMAN, T.S. 1982. Polyhydroxy compounds and thermotolerance: a proposed concatenation. *Radiation Research,* 92, 445-451.

HOLT, J.G., KRIEG, N.R., SNEATH, P.H.A., STALELY, J.T., WILLIAMS, S.T. 1994. Bergey’s manual of determinative bacteriology, 9th edn. Williams and Wilkins, Baltimore.

KEMPF, B., BREMER, E.1998. Uptake and synthesis of compatible solutes as microbial stress responses to high-osmolality environments. *Archives of Microbiology,*170, 319-330.

KO, J.H., KIM, C.H., LEE, D.S., KIM, Y.S. 1996. Purification and characterization of a thermostable ADP-glucose pyrophosphorylase from *Thermus caldophilus* GK-24. *Biochemical Jounal,* 319, 977–983.

KOH, S., SHIN, H.J., KIM, J.S., LEE, D.S., LEE, S.Y. 1998. Trehalose synthase from maltose by thermostable trehalose synthase from *Thermus caldophilus*. *Biotechnology Letters,* 20, 757-761.

LEE, J.H., Lee, K.H., Kim, C.G., Lee, S.Y., Kim, G.J., Park, Y.H., Chung, S.O.2005. Cloning and expression of a trehalose synthase from *Pseudomonas stutzeri* CJ38 in Escherichia coli for the production of trehalose. *Applied Microbiology and Biotechnology*, 68, 213-219.

LILLIE, S.H., Pringle, J.R. 1980. Reserve Carbohydrate Metabolism in *Saccharomyces cerevisiae*: Responses to Nutrient Limitation. *Journal of Bacteriology,* 143, 1384-1394.

MA, Y., XUE, L., SUN, D.2006. Characteristics of trehalose synthase from permealized *Pseudomonas putida* cells and its application in converting maltose into trehalose. *Journal of Food Engineering,*77, 342-347.

MANAGBANAG, J.R., Torzilli, A.P.2002. An analysis of trehalose, glycerol and mannitol accumulation during heat and salt stress in a salt marsh isolate of *Aureobasidium pulluans*. *Mycologia,* 94, 384-391.

MARUTA, K., HATTORI, K., NAKADA, T., KUBOTA, M., SUGIMOTO, T., KURIMOTO, M.1996. Cloning and sequencing of trehalose biosynthesis genes from *Rhizobium sp*. M-11. *Bioscience, Biotechnology, Biochemistry,* 60,717-720.

MEHTA, S., NAUTIYAL, C.S.2001. An efficient method for qualitative screening of phosphate solubilizing bacteria. *Current Microbiology,* 43, 51–56.
NISHIMOTO, T., NAKANO, M., NAKADA, T., CHAEN, H., FUKUDA, S., SUGIMOTO, T., KURIMOTO, M., TSUJISAK, Y.1996a. Purification of a novel enzyme trehalose synthase, from *Pimelobacter* sp. R48. *Bioscience, Biotechnology, Biochemistry,* 60, 640-644.
NISHIMOTO, T., NAKADA, T., CHAEN, H., FUKUDA, S., SUGIMOTO, T., KURIMOTO, M. 1996b. Purification and characterization of a thermostable trehalose synthase from *Thermus aquaticus*. *Bioscience, Biotechnology, Biochemistry,* 60, 835-839.
SCHIMEL, J., BALSER, T.C., WALLENSTEIN, M. 2007. Microbial stress-response physiological and its implications for ecosystem function. *Ecology,* 88,1386-1394.
SCHWYN, B., NEILANDS, J.B.1987. Universal chemical assay for the detection and determination of Siderophore. *Analytical Biochemistry,*160, 47–56.
SRIVASTAVA, S., Yadav, A., Seem, K., Mishra, S., Chaudhary, V., Nautiyal, C.S.2008. Effect of high temperature on Pseudo*monas putida* NBRI0987 biofilm formation and expression of stress sigma factor RpoS. *Current Microbiology,* 56, 453-457.
STROM, A.R., KAASEN, I.1993. Trehalose metabolism in *Escherichia coli*: stress protection and stress regulation of gene expression. *Molecular Microbiology,* 8, 205-210.
STORZ, G.2000. Hengge-Aronis R. edn. Bacterial stress responses. ASM Press. Washington DC.
TSUSAKI, K., NISHIMOTO, T., NAKADA, T., KUBOTA, M., CHAEN, H., FUKUDA, S., SUGIMOTO, T., KURIMOTO, M.1996.ical Cloning and sequencing of trehalose synthase gene from *Pimelobacter* sp. R48.*Biochimca et Biophysica Acta,*1290,1-3.
TSUSAKI, K., NISHIMOTO, T., NAKADA, T., KUBOTA, M., CHAEN, H., FUKUDA, S., SUGIMOTO, T., KURIMOTO, M.1997. Cloning and sequencing of trehalose synthase gene from *Thermus aquaticus*. *Biochimca et Biophysica Acta,*1334,28-32.