present study. In fact, the standard deviation for NADH concentration in normal and malignant breast tissues by TPAF has been found to be quite large (99 ± 37 and 168 ± 49 μm) as studied by others[13]. Further studies with more specimens of varying parameters (e.g. age group, stage of malignancy, etc.) for standardizing the present method prior to having a real clinical application are presently being pursued in our laboratory.

1. Lakowicz, J. R., *Principles of Fluorescence Spectroscopy*, Springer, 2005.
2. Denk, W. *et al.*, *Science*, 1990, **248**, 73–76.
3. Maiti, S. *et al.*, *Science*, 1997, **275**, 530–532.
4. Bird, D. K. *et al.*, *Cancer Res.*, 2005, **65**, 8766–8773.
5. Konig, K. and Riemann, I., *J. Biomed. Opt.*, 2003, **8**, 432–439.
6. Minsky, M., *Scanning*, 1988, **10**, 128–138.
7. Göppert-Mayer, M., *Ann. Phys.*, 1931, **9**, 273–294.
8. Kaiser, W. and Garret, C. G. B., *Phys. Rev. Lett.*, 1961, **7**, 229–231.
9. Xu, C. *et al.*, *Proc. Natl. Acad. Sci. USA*, 1996, **93**, 10763–10768.
10. Centonze, V. E. and White, J. G., *Biophys. J.*, 1998, **75**, 2015–2024.
11. Huang, S. *et al.*, *Biophys. J.*, 2002, **82**, 2811–2825.
12. Palmer, G. M. *et al.*, *Photochem. Photobiol.*, 2003, **78**, 462–469.
13. Heiden, M. G. V. *et al.*, *Science*, 2009, **324**, 1029–1033.
14. Kroemer, G. and Pouyssegur, J., *Cancer Cell Rev.*, 2008, **13**, 472–482.
15. Kaelin Jr, W. B. and Thompson, C. B., *Nature*, 2010, **465**, 562–564.
16. Yu, Q. and Heikal, A. A., *J. Photochem. Photobiol. B: Biol.*, 2009, **95**, 46–57.
17. Masilamani, V. *et al.*, *J. Lumin.*, 2004, **109**, 143–154.

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Cloning, overexpression, purification and characterization of maltooligosyltrehalose synthase from a cyanobacterium, *Anabaena* 7120

Trehalose is a non-reducing sugar with low energy (1 kcal mol^{-1}), withstands 100°C and is functional in the pH range 3.5–10 (ref. 1). Therefore, it is used as a stabilizer and preservative of foodstuff, cryoprotectant for cells in medicine, in cosmetics and various biotechnological purposes[2]. It has also been reported as a signalling molecule of higher plants[3–5], and plays an important role in *Mycobacterium tuberculosis*–host interactions[6] and in Huntingdon's and Alzheimer's diseases[7,8]. There are five types of trehalose biosynthetic pathways reported in various organisms[1]. One of the pathways having maltooligosyltrehalose synthase (MTSase) (*all0167*) and maltooligosyltrehalose trehalohydrolase (MTHase) (*all0168*) enzymes has superiority in trehalose production (up to 80–92%) in many bacteria[2]. A gene cluster constituting trehalose biosynthesizing enzymes, MTSase and MTHase, along with trehalase (*all0168*), has been reported in *Anabaena* 7120 (ref. 9) and is maximally expressed in *Anabaena* 7120 exposed to 150 mM NaCl (refs 10, 11). In addition, increase in trehalose yield may be achieved through manipulation of such enzymes.

The MTSase gene was PCR-amplified and cloned in pGEM-T Easy vector (Promega) and sub-cloned in pET-19b (Novagen) expression vector. The recombinant was transformed in *Escherichia coli* BL21 (DE3) and expressed optimally in isopropyl β-D-1 thiogalactopyranoside (IPTG) (1 mM)-induced cultures (14 h, 28°C). The recombinant MTSase enzyme purified by Ni^{+2}-NTA affinity chromatography showed maximum transglycosylation activity at pH 6 (40°C), with maltohexaose as the substrate. To our knowledge, there are no other reports on the expression, purification and characterization of MTSase from a cyanobacterium.

Anabaena 7120 was grown in BG-11 medium[12] free from combined N-source under continuous tungsten plus fluorescent illumination (14.40 Wm^{-2}) at 28 ± 1°C. *E. coli* NM was used as the host for vectors. The amino acid sequence of MTSase of *Anabaena* 7120 was compared with MTSase from different microbes. Protein sequence databases (Swiss_Port, PIR and GenBank) were searched using the standard search algorithm, BLASTP (NCBI, NIH). The sequences were aligned and a phylogenetic tree was generated based on the neighbour-joining (NJ) algorithm using Clustal X[13].

Genomic DNA from *Anabaena* 7120 was isolated using Marmur method[14]. PCR-amplified *all0167* from the genomic DNA using primers was designed from the nucleotide sequence (http://bacteria.kazusa.or.jp/cyanobase; GenBank accession code, Q8ZOD1). The forward primer (5′ GCG CAT ATG CGA ATT CCT AAA GCT AC 3′) and reverse primer (5′ GCG GGA TCC **TTA** TTC GGC GAT TAA CAG G 3′) contained the restriction

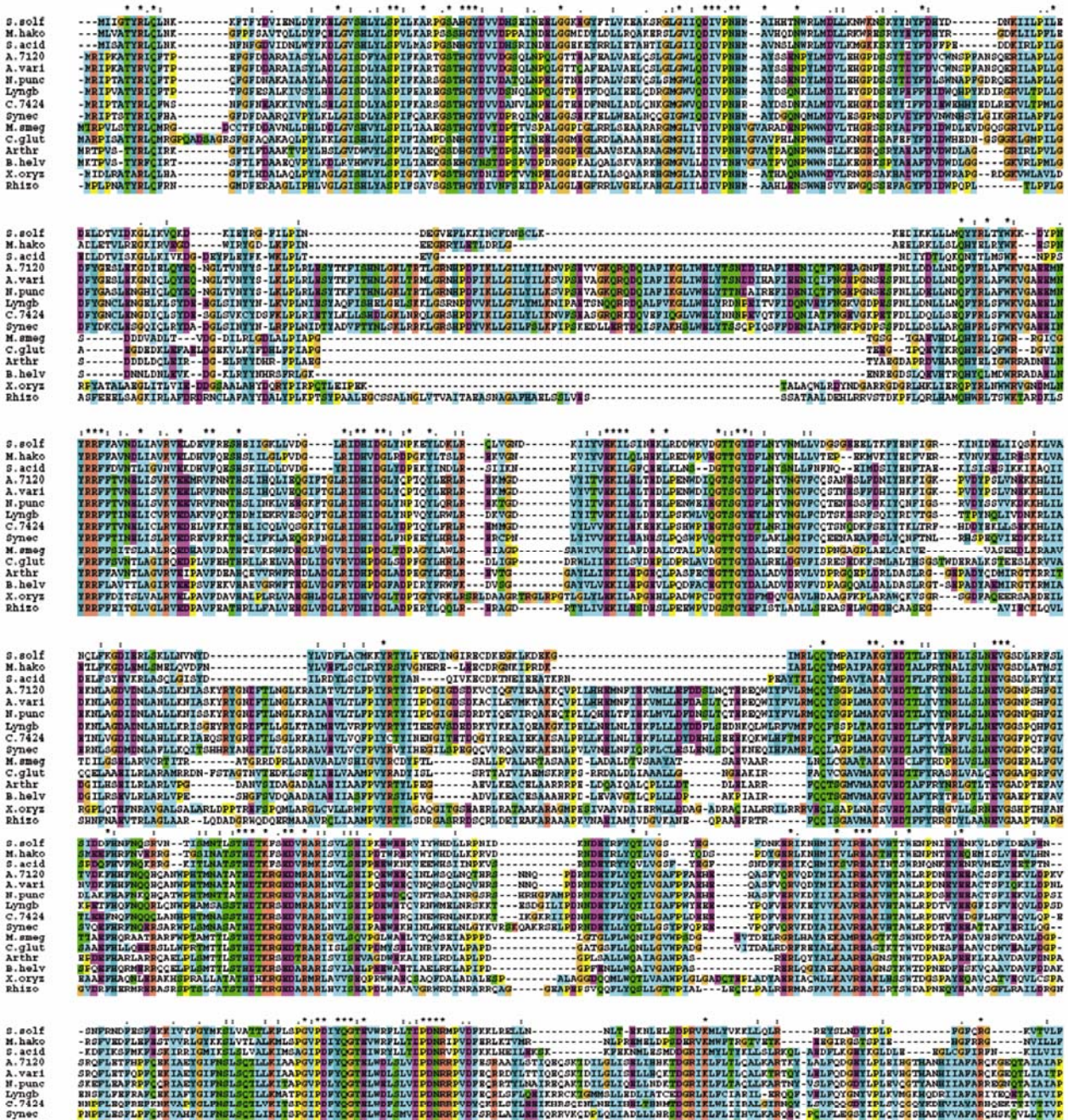


Figure 1. Multiple sequence alignment of amino acids in maltooligosyltrehalose synthase (MTSase) of *Anabaena* PCC 7120 with the same enzyme of selected prokaryotes. *indicates conserved amino acids in toto, colons, high and, dots, low conservation. A.7120, *Anabaena* (*Nostoc* PCC 7120); N. punc, *Nostoc punctiforme*; A. vari, *Anabaena variabilis*, C.7424, *Cyanothece* PCC 7424; Synec., *Synechococcus*, Lyngb, *Lyngbya*; S. acid, *Sulfolobus acidocaldarius*; S. sulf., *Sulfolobus solfataricus*; P95869, M. hako, *Metallosphaera hakonensis*; Q6SZP7, C. glut, *Corynebacterium glutamicum*; M. smeg, *Mycobacterium smegmatis*; Rhiz., *Rhizobium*; X. oryz, *Xanthomonas oryzae*; B. helv, *Brevibacterium helvolum*, and Arthr, *Arthrobacter*.

sites (underlined) for *Nde*I and *Bam*H1 respectively, and the stop codon (bold bases). The PCR reaction mixture (100 µl) contained 25 pmol each of primer, 50 µg of genomic DNA, 0.2 mM each of 2-deoxynucleotide 5-triphosphate (dNTP) and 1 U of *Taq* polymerase. The programme included pre-denaturation at 95°C (5 min), denaturation at 95°C (1 min), annealing at 49°C (1 min) and extension at 72°C (1.3 min) for 30 cycles with a final extension at 72°C (15 min). Recombinant plasmid was ligated into pET-19b vector having His tag.

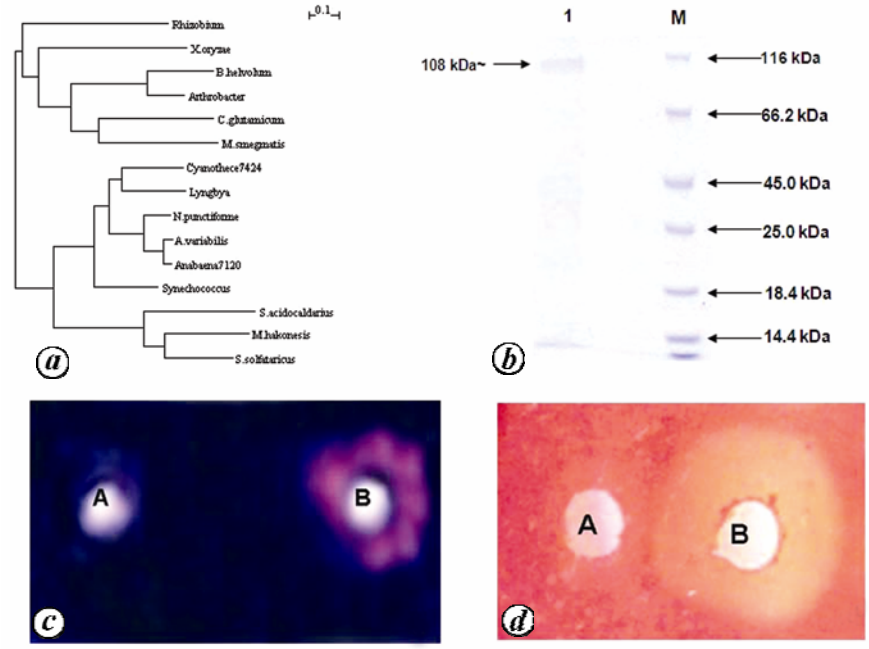
Overnight-grown culture (2 ml) was added to fresh LB medium containing ampicillin (100 µg ml^{-1}) and grown until OD_{600} reached 0.6–0.8. The culture was then induced by adding IPTG (0–2 mM) for different time durations at varying incubation temperatures. The cells were centrifuged (10,000 rpm, 15 min, 4°C) and stored at –20°C for further processing. The pellets were divided into: (i) 5X sample buffer (320 mM Tris–HCl, pH 6.8, 50% glycerol, 10% SDS, and 0.05% bromophenol blue with 100 mM DTT, and (ii) suspended in sodium phosphate buffer (20 mM, pH 7.4), broken in liquid nitrogen and centrifuged (10,000 rpm, 20 min, 4°C) for removing insoluble fractions.

Ni^{2+}-NTA (5 ml, Novagen) washed with binding buffer (20 mM sodium phosphate buffer, pH 7.4, 500 mM NaCl and 20 mM imidazole) was added to the supernatant and put on an orbital shaker (30 min). The resin was collected at 1000 rpm (2 min, 4°C), poured into a 1.5 × 12 cm polypropylene column (Bio-Rad Economopac) and washed with 10 column volume of buffer (20 mM sodium phosphate, pH 7.4, 500 mM NaCl and 30 mM imidazole). The target protein was eluted with 2-1 bed volume of elution buffer (20 mM sodium phosphate buffer; pH 7.4; 500 mM NaCl and 500 mM imidazole) and checked on SDS PAGE (10%)[15].

MTSase transglycosylation activity was assayed as described by Fang *et al.*[16] at 70°C (10 min) using maltohexaose (1.4 mM; Boehringer, Germany) as substrate in citrate buffer (50 mM, pH 6). The amount of residual reducing sugars was determined by DNS method with maltohexaose as standard[17]. The effect of pH on enzyme activity was also monitored using different buffers (50 mM) such as: citrate–phosphate (pH 4–7), Tris–HCl (pH 7–9), and $NaHCO_3$–Na_2CO_3 (pH 6–9). Temperature optima for MTSase activity were determined by varying the temperature under standard conditions. The purified enzyme in sodium phosphate buffer (pH 7.4) was spotted on agar (1%) plates with 0.5% starch. The plates were incubated at 37°C (48 h) and hydrolytic areas observed after spraying with iodine solution or in the presence of congo red (0.1%) representing the enzyme activity.

MTSase of *Anabaena* 7120 contained amino acid sequences corresponding to the four highly conserved regions in the α-amylase family along with other conserved regions and residues (Figure 1). The phylogenetic tree showed the cyanobacterial MTSase in a separate cluster (Figure 2 *a*). Multiple sequence alignment of translated amino acid sequences of MTSase from different organisms showed several conserved regions and specific amino acids such as D330, E357 and D600 residues of *Anabaena* 7120 (Figure 1) corresponding to D228, E255 and D443 residues of *Sulfolobus acidocaldarius*, as reported by Maruta *et al.*[18], indicating its importance in specificity of MTSase, as mutations in such residues led to the loss of transglycosylation and hydrolytic activity. In a mutational analysis of the MTSase enzyme in *Sulfolobus solfataricus*, alterations in F405 residue (although in the non-conserved region) resulted in higher trehalose yield from starch[16]. The F405 now corresponds to M442 of *Anabaena* 7120 after multiple sequence alignment, thus paving the way for putative change(s) that can be brought in MTSase of *Anabaena* 7120 leading to enhanced trehalose production.

The molecular weight of MTSase enzyme of *Anabaena* 7120 is 105.56 kDa (according to http://genome.kazusa.or.



Figures 2. ***a***, Phylogenetic tree showing evolutionary relatedness of MTSase among selected prokaryotes. Branch length represents calculated phylogenetic distance. ***b***, SDS–PAGE (10%) showing Ni^{+2}–NTA chromatographically purified recombinant MTSase (lane 1) along with protein markers (lane M). Activity staining of purified MTSase enzyme on agarose (1%) plates with starch (0.5%) added with iodine (***c***) and with congo red (0.1%; ***d***). A and B represent zones with enzyme extraction buffer and purified enzyme respectively.

Table 1. Comparison of maltooligosyltrehalose synthase activity in preparations from cells of *Escherichia coli* BL 21 (DE3) harbouring recombinant vector

Source	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Purification fold
Cell extract	160	40	0.25	1
Ni^{+2}-NTA column purified	1	25	25.0	100

jp/cyanobase/Anabaena). This was confirmed by cloning and expression studies along with its activity (Figure 2 *b–d* and Table 1). The optimum expression of MTSase was at 1 mM IPTG (14 h and 28°C) and activity at pH 6 and 40°C. Increase in pH and temperature caused a decrease in enzymatic activity (data not shown). In contrast, a similar enzyme from *S. solfataricus* ATCC 35092, *S. solfataricus* MT4, and *S. acidocaldarius* showed maximum transglycosylation at pH 5 and was thermostable (70–75°C)[19]. Gueguen *et al.*[20] also observed optimum expression of MTSase gene of *S. acidocaldarius* (1 mM IPTG, 16 h and 37°C); while for *Metallosphaera hakonesis*, it was 21°C (ref. 21). Similarly, Kim *et al.*[22] observed maximum enzyme expression in *Brevibacterium helvolum* at 1 mM IPTG, 4 h and 37°C. However, IPTG failed to induce MTSase gene expression in *S. solfataricus*[19]. The specific activity of MTSase from *Anabaena* 7120 was 25 units mg^{-1} protein, close to purified MTSase/TDFE (trehalosyl dextrin-forming enzyme) of 28.5 and 19 units mg^{-1} protein in *S. solfataricus* MT4 and *S. solfataricus* ATCC 35092 respectively[2]. Thus enzymes from various sources may differ in their pH and temperature optima. The hydrolytic activity of MTSase from *S. solfataricus* depended on incubation time and substrate type as the amount of glucose was just 2.5% for maltohexaose as the substrate[19], although we have not quantified the glucose formed. Therefore, the study of individual gene-producing specific osmolytes by making 'knockout' strains and complementation of such genes after cloning, would help decipher the precise role of the particular gene as well as a osmolytes in growth and development of organisms in various environmental regimes.

1. Paul, J. M., Primavesi, L. F., Jhurreea, D. and Zhang, Y., *Annu. Rev. Plant Biol.*, 2008, **59**, 417–441.
2. Schiraldi, C., Di Lernia, I. and De Rosa, M., *TIBTECH*, 2002, **20**, 420–425.
3. Wingler, A., *Phytochemistry*, 2002, **60**, 437–440.
4. Elbein, A. D., Pan, Y. T., Pastuszak, I. and Carroll, D., *Glycobiology*, 2003, **13**, 17R–27R.
5. Avonce, N., Mendoza-Vargas, A., Morett, E. and Iturriaga, G., *BMC Evol. Biol.*, 2006, **6**, 109–124.
6. Saavedra, R., Segura, E., Tenorio, E. P. and Lopez-Marin, L. M., *Microbes Infect.*, 2006, **8**, 533–540.
7. Tanaka, M., Machida, Y., Niu, S., Ikeda, T. and Jana, N. R., *Nature Med.*, 2004, **10**, 148–154.
8. Liu, R., Barkhordarian, H., Emadi, S., Park, C. B. and Sierks, M. R., *Neurobiol. Dis.*, 2005, **20**, 74–81.
9. Higo, A., Katoh, H., Ohmori, K., Ikeuchi, M. and Ohmori, M., *Microbiology*, 2006, **152**, 979–987.
10. Asthana, R. K., Nigam, S., Maurya, A., Kayastha, A. M. and Singh, S. P., *Curr. Microbiol.*, 2008, **56**, 429–435.
11. Asthana, R. K., Srivastava, S., Singh, A. P., Kayastha, A. M. and Singh, S. P., *J. Plant. Physiol.*, 2005, **162**, 1030–1037.
12. Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M. and Stanier, R. Y., *J. Gen. Microbiol.*, 1979, **111**, 1–61.
13. Larkin, M. A. *et al.*, *Bioinformatics*, 2007, **23**, 2947–2948.
14. Marmur, J., *J. Mol. Biol.*, 1961, **3**, 208–218.
15. Laemmli, U. K., *Nature*, 1970, **227**, 680–685.
16. Fang, T. Y., Tseng, W. C., Chung, Y. T. and Pan, C. H., *J. Agric. Food Chem.*, 2006, **54**, 3585–3590.
17. Miller, G. L., *Anal. Chem.*, 1959, **31**, 426–428.
18. Maruta, K., Kuboto, M., Yamashita, H., Nishimoto, T., Chaen, H. and Fukuda, S., *J. Appl. Glycosci.*, 2006, **53**, 199–203.
19. Fang, T. Y., Hung, X. G., Shih, T. Y. and Tseng, W. C., *Extremophiles*, 2004, **8**, 335–343.
20. Gueguen, Y., Rolland, J. L., Schroeck, S., Flament, D., Defretin, S., Saniez, M. H. and Dietrich, J., *FEMS Microbiol. Lett.*, 2001, **194**, 201–206.
21. Ju-Seok, S. *et al.*, *J. Microbiol. Biotechnol.*, 2007, **17**, 123–129.
22. Kim, Y. H. *et al.*, *Appl. Environ. Microbiol.*, 2000, **66**, 4620–4624.

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Dumortierite from Susunia Hill, Bankura District, West Bengal, India

Susunia Hill (442 m high), famous for its holy spring, is located about 27 km northwest of Bankura town, in the Chhotanagpur gneissic plateau of West Bengal (Figure 1). The hill is chiefly composed of sillimanite and kyanite-bearing quartzites, which at places show evidence of shearing. The bedding planes dip generally 20°–25° towards 10°. However, owing to some folding the strike varies to the east, northeast and northwest.

An outcrop of blue-coloured pegmatitic rock trending N60°W–S60°E, covering few square metres area is found within the quartzite country rock of Susunia Hill (around 23°23′39″E, 86°59′11″N). Physically, the blue-coloured mineral which gives the overall blue hue to the rock, is similar to kyanite in appearance (Figure 2 *a* and *b*). However, microscopic studies show that unlike kyanite this mineral is strongly pleochroic from colourless to azure blue (Figure 2 *c* and *d*). The mineral is euhedral to subhedral, shows one set of perfect cleavage parallel to elongation and cross fractures and straight extinction with respect to cleavage. Petrographic study also reveals the presence of tourmaline and quartz in the pegmatite. Tourmaline grains are complexly zoned at places and vary considerably both in shape and size (Figure 2 *d*).