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# Improvement of Trehalose Biosynthesis in *Corynebacterium glutamicum* by a Combination of Conventional Mutagenesis and Analysis of Enzymatic Properties

**Maohua Qu, Juan Du, Fengying Zhang, Mingfang He and Weiping Chen***

Key Lab of Agricultural Products Processing and Quality Control of Nanchang City, Jiangxi Agricultural University, Nanchang, 330045, China.

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***Corynebacterium glutamicum*** **mutant strain with higher yield of trehalose was obtained using UV-chemical mutagenesis. The yield increased to 284.4% compared to wild strain and then studied the enzymatic properties of five synthases for trehalose. The optimum pH value for trehalose synthase, MTSase, MTHase, TPS and TPP was 7.0, 7.5, 6.5, 6.5, 7.5 and the range of pH 6.5-8.0, pH 6.5-7.5, pH 6.0-7.5, pH 6.0-7.5, pH 6.5-8 with over 80% of highest activity, respectively. The optimum reaction temperature for them was 35°C, 35°C, 40°C, 35°C, 35°C with maintaining higher reactivity at the range of 30-35°C, 35-40°C, 30-45°C, 25-40°C, 30-35°C, respectively.**

**Key words:** Trehalose, mutagenesis, enzymatic property.

Trehalose, as energy substance in cell [1,2], is a naturally disaccharide which consists of two glucose molecules linked by a-1,1 glycosidic bond [3]. It is a solute in cell which has effect on resisting hostile conditions and strengthens stability of cytoarchitecture [4,5]. Trehalose is one of the most effective solute in cells under hostile conditions. It's widespread in thermophilic bacterium, yeast, insects and plants [6,7]. Under stress conditions, such as salt shock, heat, osmotic stress, trehalose accumulation in cell increased. Substrates for trehalose synthsis contain maltose, glucose-6-phosphate, UDP-glucose, maltodextrin i.e [8,9]. Enzymes for trehalose synthsis contain MTSase, MTHase, TreSÿTreTÿTrePÿTPS and TPP [10,11]. Trehalose is produced in bacteria, yeast, fungi and a few plants.

* To whom all correspondence should be addressed.
E-mail: iaochen@163.com

There are five pathways as TreYZ0TreS0OtsAB0TreP and TreT in nature [12,13]. *Corynebacterium glutamicum* contains OtsAB0TreYZ and TreS pathways [14,15]. This paper attempted to improve the trehalose production of *Crynebacterium glutamicum* by using classical mutagenesis by ultraviolet (UV) radiation and chemical mutagen and then analyzed enzymes for trehalose synthsis in the strain.

## MATERIALS AND METHODS

### Microorganism and cultural conditions

*C.glutamicum* ATCC 13032, the wild strain used in this study, was obtained from China General Microbiological Culture Collection Center (CGMCC). The DMCG I and LB medium was used to grow the bacterial strain. Trehalose production was carried out in 250-ml baffled conical flasks with a culture volume of 50ml of each medium in a rotary shaker at 200rpm and 30!. The inoculum dose in each culture was 10% (v/v) and pH was controlled at 7.0. After culturing 30h, collecting cells by centrifuging and suspended in sodium phosphate buffer at pH 7.0.

**Preparation of cell suspension**

*C.glutamicum* strain was grown firstly in LB media after overnight incubation at 30!. This strain was further cultured in 50 ml DMCG I media (containing $K_2HPO_4$(8g), $KH_2PO_4$(1g), $(NH_4)_2SO_4$ (5.0g), $MgSO_4$ (0.2g), $CuCl_2$(0.0002g), $(NH_4)_6Mo_7O_{24}$(0.0001g), $Na_2B_4O_7$(0.002 g), $CaCl_2$ (0.05g), $FeSO_4$ (0.025g), $MnSO_2$(0.002 g), sodium citrate(1.1g), $FeCl_3$(0.002g), NaCl(1g) and maltose (20g) per liter of distilled water)with pH of 7.0. Cells of the wild strain were obtained at log phase (after 15 h of incubation at 30!) by centrifuging at 10,000 g for 30 min and then resuspended in 20 ml of buffer(pH 7.0). After 20min agitation to scatter cells, calculated the cell number using blood count chamber to prepare mutagenesis.

**UV mutagenesis**

The UV lamp was about 30cm above the surface of the cell suspension. During 3min of the treatment process, 1 ml aliquots were withdrawn and then cultured 1 day in dark. In both cases, all the collected aliquots were then serially diluted up to $10^6$ and inoculated onto DMCG I media. The cultivation was carried out in a rotary shaker at 30°C and 200rpm for 48h and trehalose was determined.

**Chemical mutagenesis**

For IPTG mutagenesis, 2 ml of diluted suspension of *C.glutamicum* was treated with different concentrations of IPTG (0.01, 0.02,0.03, 0.04g IPTG in 1ml) and incubated at 30! in shaker, each for 0, 10, 20, 30 and 40 min. The potential UV-generated mutant was further treated with IPTG. Screening and selection of potential mutants were done based on their trehalose productivity. Colonies were picked from media plate on the basis of trehalose productivity. The selected colonies were further screened for higher higher production and genetic stability.

**Analysis of Enzyme properties**

**Determination of enzymes activity**

Supernatant was obtained by using centrifuge (16000×gÿ30min) method and then added 1mL 1% substrate into tube for reaction at 30! under condition of water bath with 1mL enzyme. Boiled for 5min after 30min of enzymolysis and then determined trehalose content using HPLC. In this step, only the best performing mutants were selected for further studies. Dry cell weight was determined, too. One unit (U) of enzyme was defined as the amount of the enzyme to produce 1ug substrate per minute under the assay conditions and the specific activity was defined as the number of units per mg of protein.

**Effects of pH, temperature and metal ion on activity**

Set different values to make reaction and then determine enzyme activities. These conditions include pH, temperature and different metal ions. Each experiment made in triplicates. First, optimum pH was measured. 1mL of enzyme in different buffers was added to a final pH in the range of 4.0-11.0, then mixed and incubated at 30! for 30min to determine activity. Second, the temperature on enzyme activity was determined. Mixed 1mL enzyme and 1% substrate and the mixture was incubated at each different temperatureÿ5!, 10!, 15!, 20!, 25!, 30!, 35!ÿ40!, 45!, 50!, 55!, 60! ÿ i n the range of 5!-60! for 30min to determine enzyme activity. Finally, 1mL enzyme(0.1mg/mL) containing metal ion was mixed with equal volume of 1% substrate(w/v) in deionized water at a metal ion concentration of 1mM, then incubated at 30! for 30min to determine enzyme activity.

**Calculation of kinetic parameters**

The effect of substrate concentration on enzyme reaction velocity was tested using an enzyme concentration of 0.1 mg/mL and a substrate concentration of 2mM, 4mM, 6mM, 10mM, 12mM, 16mM and 18mM at 30°C for 10 min before measuring the kinetic parameters of enzymes(TreS, MTSase, MTHase, TPS and TPP). Then Kinetic Parameters for conversation with enzymes was determined at 30°C. Using Langmuir method to determine Km and Vmax with formula: [S]/V = Km/Vm + [S]/Vm, [S] is substrate concentration, V is velocity of reaction, Vmax is the highest velocity of reaction.

## RESULTS AND DISCUSSION

**Mutagenesis and screening of mutants**

Random mutagenesis using UV irradiation and chemical agents is an easy method to achieve functional and genetic modifications of a strain. Trehalose productivity for both IPTG mutagenesis and UV mutagenesis was determined in the mutagenesis experiments. There's direct ratio between treating time and cell fatality rate, as well as time and cell mutation rate. It was found to be 0.03g/ml IPTG exposure for 40 min and 2min

exposure to far UV source from a distance of 15cm and 80s UV exposure plus 15min 0.03g/mL IPTG treatment.

Five mutants after IPTG mutagenesisÿUV mutagenesis and UV plus IPTG mutagenesis were recovered and screened on the basis of trehalose production level(Table 1). Three mutants($CG_{UV7}$, $CG_{IP5}$, $CG_{UP15}$) were selected after primary screening for further studies. Trehalose production in mutant $CG_{UP15}$ (1.28g /100 g cell dry weight·$h^{-1}$)was found to be better than other mutants (1.11g/100g cdw·$h^{-1}$ for $CG_{UV7}$, 1.25g/100g cdw·$h^{-1}$ for $CG_{IP5}$, 0.96g/100g cdw·$h^{-1}$ for $CG_{IP13}$, and 0.94 g/100g cdw·$h^{-1}$ for $CG_{UP9}$, respectively). There was 2.8-fold increase in trehalose productivity of mutant $CG_{UP15}$ over the parent strain under the same conditions.

**Genetic stability of mutants**

The genetic stability of the selected mutants($CG_{UV7}$, $CG_{IP5}$, $CG_{UP15}$)was determined by determining trehalose production for several successive generations. The $CG_{UP15}$ mutant was stable in comparison to other selected mutants (Fig. 1). Hence, $CG_{UP15}$ mutant was finally selected.

**Effect of pH on stability**

The effect of pH on the enzymes was examined at 30! setting different pH values from 4.0 to 11.0. Trehalose synthase has high stability over the range of 5.5-9.0 and the enzyme activity reached maximum when pH was 7.0 where Trehalose synthase activity decreased with decreasing or increasing pH. Although the enzyme holds a high stability from 5.5 to 9.0, it's no meaningful except pH 7.0 because of low enzyme activity in other pH values.

The maximum MTSase activity was at pH 7.5 and MTHase activity was at pH 6.5. MTSase has high stability from 6.0 to 9.0 and the enzyme activity decreased with decreasing pH below 6.5 or increasing pH over 7.5, so the optimum pH range was 6.5-7.5. MTHase hold a high stability in the range pH 5.0 to 9.5 and had high enzyme activity from 6.0- 7.5.

The stability of TPS and TPP are shown in Fig. 2c. The optimum pH range of TPS is 6.0-7.0 and maximum TPS activity was at pH 6.5. The maximum TPP activity was at pH 7.5 and hold high

**Table 1.** Trehalose production in parent strain and screened mutants

| Strain/mutant | Trehalose productivity g /100 g cell dry weight·$h^{-1}$ | Relative productivity % |
|---|---|---|
| CG(parent)without mutagenesis | 0.45 | 100 |
| $CG_{UV7}$(UV mutagenesis) | 1.11±0.11 | 246.7 |
| $CG_{IP5}$(IPTG mutagenesis) | 1.25±0.04 | 277.8 |
| $CG_{IP13}$(IPTG mutagenesis) | 0.96±0.08 | 213.3 |
| $CG_{UP9}$(IPTG mutagenesis) | 0.94±0.06 | 208.9 |
| $CG_{UP15}$(IPTG mutagenesis) | 1.28±0.06 | 284.4 |

**Table 2.** Kinetic parameters of enzymes from *C. glutamicum.*

| Enzyme | Substrate | Km (mM) | Vmax mmol/mL·$min^{-1}$ | Relative Vmax(%) |
|---|---|---|---|---|
| MTSase | Maltopentaose | 8.7 | 0.8 | 100 |
| | Maltohexaose | 1.4 | 0.38 | 45 |
| | Maltoheptaose | 0.9 | 0.46 | 58 |
| MTHase | Maltotriose trehalose | 4.6 | 1.1 | 100 |
| | Maltotetraose trehalose | 10 | 1.6 | 149 |
| | Maltopentaose trehalose | 4.2 | 1.2 | 115 |
| TPS | UDP-glucose | 0.4 | 0.2 | |
| | Glucose-6-phosphate | 2.6 | 0.2 | |
| TPP | Trehalose-6-phosphate | 0.8 | 0.1 | |
| TreS | Maltose | 1.2 | 0.12 | |
| | Trehalose | 1.3 | 0.14 | |

stability from pH5.5-9.0. TPP activity is high from 6.5-8.0 with decreasing outside of the range. The results were similar to those obtained by Stacey Klutts[1] , who obtained TPP from the cytosol of *Mycobacterium smegmatis*.

**Effect of temperature on stability**

Different enzymes have different optimum temperatures because of the different structure of enzymes. The TreS activity increased with increased temperature in the range 5-35! and the optimum temperature for the enzyme was 35!. The stability of TreS was high below 35!.

The maximum MTSase activity was at 35! with high stability. MTSase has high stability from 20!-45!. The maximum MTHase activity was at 40! and the stability of MTHase decreased rapidly over

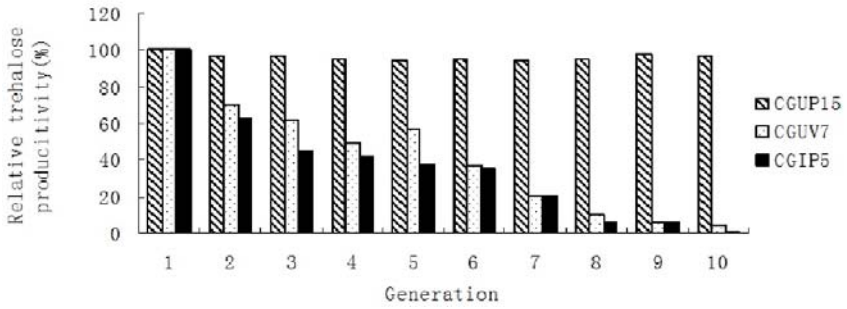


**Fig. 1.** Genetic stability evaluation for the selected mutants

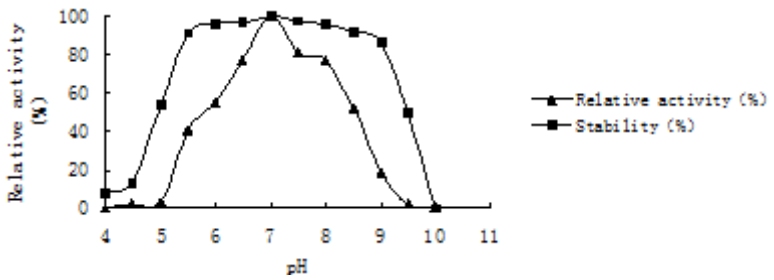


**Fig. 2a.** Effect of pH on TreS activity and stability

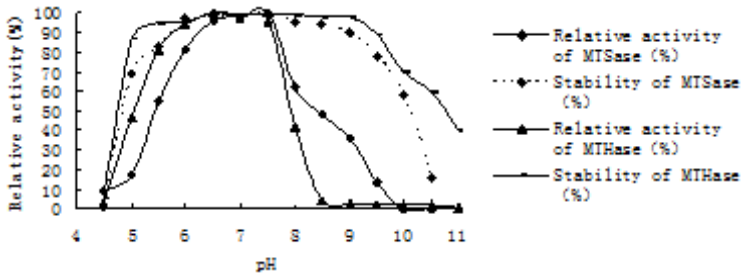


**Fig. 2b.** Effect of pH on activity and stability of MTSase and MTHase

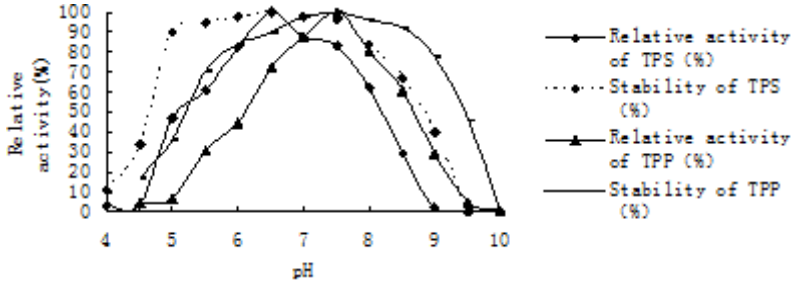


**Fig. 2c.** Effect of pH on activity and stability of TPS and TPP

45!. The optimum temperature ranges were 35-40! for MTSase and 30-45! for MTHase.

TPS and TPP activities reached the highest level at 35! and hold high stability from 30-35!. TPS activity decreased over 40!. TPP stability was high when it's at 50! with very low activity. Londesborough & Vuorio have reported that fructose 6-phosphate is a powerful activator of the Tre6P synthase activity below 35![17], especially at physiological phosphate concentration, so in the culture containing phosphate is in favour of trehalose production by *C.glutamicum*.

**Effect of metal ion on stability**

Different metal ions had different effects on enzymes activity. $Pb^{2+}$, $Ba^{2+}$, $Cu^{2+}$, $Hg^{2+}$, $Ni^{2+}$, $Zn^{2+}$ had a strong inhibitory effect on TreS , whereas $Al^{3+}$, EDTA, $Fe^{3+}$ inhibited slightly the enzyme activity. In contrast, $Mg^{2+}$, $Fe^{2+}$, $Sr^{2+}$, $Mn^{2+}$ had a slight stimulatory effect on TreS activity. These esults were similar to the report by Tae-Kyun Kim[18] , who reported that $Mg^{2+}$ and $Fe^{2+}$ promoted slightly the TreS enzyme activity.

$Al^{3+}$ and $Sr^{2+}$ had almost no effect on MTSase. $Ca^{2+}$, $Co^{2+}$, $Mn^{2+}$ and $Pb^{2+}$ had slightly inhibitory effect on MTSase activity, whereas $Cu^{2+}$ and $Hg^{2+}$ could cause the MTSase lost all activity. $Zn^{2+}0Mg^{2+}0Fe^{2+}$ also had a strong inhibitory effect on MTSase, MTSase lost half activity in these ion solutions. In contrast, $Fe^{3+}$0EDTA0$Ba^{2+}$ could promote MTSase activity.

$Mg^{2+}$, $Ca^{2+}$, $Al^{3+}$, $Ba^{2+}$ and $Mn^{2+}$had little effect on MTHase activity. $Cu^{2+}0Ni^{2+}0Hg^{2+}0Fe^{2+}0Zn^{2+}$ had a strong inhibitory effect on MTHase activity. EDTA had a slight stimulatory effect on MTHase activity. From these results, added EDTA into the TreYZ reaction

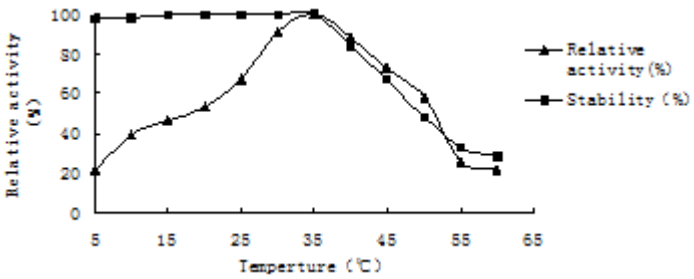


**Fig. 3a.** Effect of temperature on activity and stability of TreS

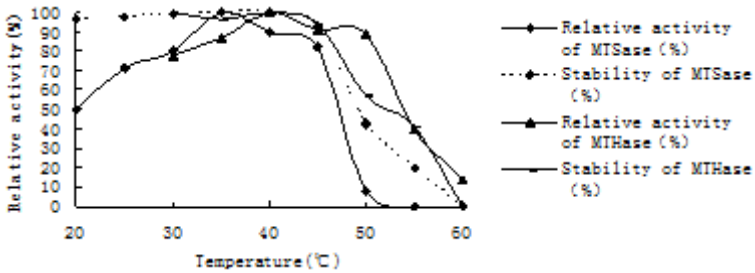


**Fig. 3b.** Effect of temperature on activityand stability of MTSase and MTHase

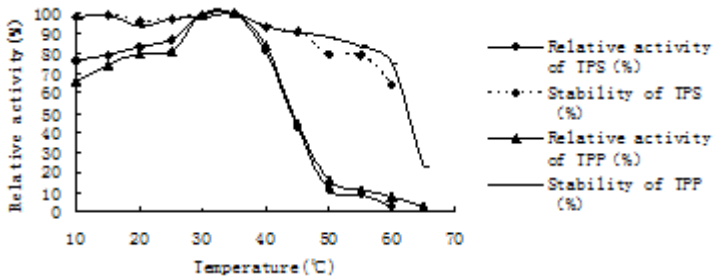


**Fig. 3c.** Effect of temperature on activity and stability of TPS and TPP

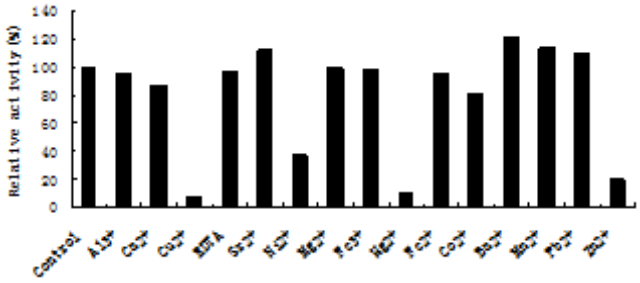


**Fig. 4a.** Effect of metal ion on activity of TreS

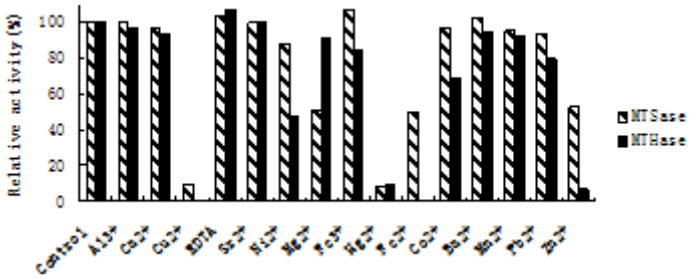


**Fig. 4b.** Effect of metal ion on activity of MTSase and MTHase

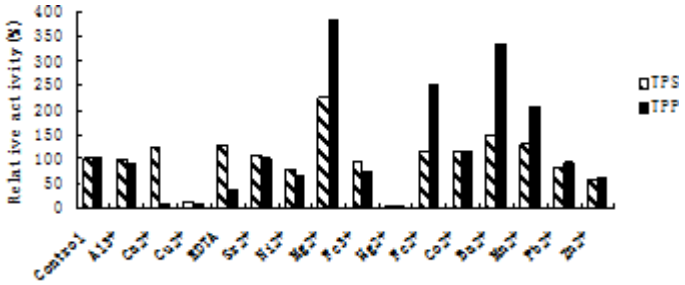


**Fig. 4c.** Effect of metal ion on activity of TPS and TPP

solution could promote trehalose productivity. Maruta has indicated that the activity level of MTHase was decreased severely when reaction was carried out in the ion liquors of $Hg^{2+}$, $Cu^{2+}$, $Fe^{2+}$ and $Zn^{2+}$ [19].

$Cu^{2+}$ had a strong inhibitory effect on all two enzymes, whereas $Al^{3+}$ had a slight inhibitory effect on TPS and TPP with over 90% enzyme activities in $Al^{3+}$ solution. In contrast, $Mg^{2+}0Fe^{2+}0Co^{2+}0Ba^{2+}$ and $Mn^{2+}$ had a strong stimulatory effect on TPS and TPP activities. $Mg^{2+}$ was the best ion in promoting activities of TPS and TPP. Pan also reported that, the acivity of TPS from *Mycobacterium tuberculosis* was increased by $Mn^{2+}$ [20].

**Km and Vmax**

The Michaelis constant for reaction with enzymes was determined at 30°C and an enzyme concentration of 0.1 mg/mL. Km is a feature constant of enzymes indicating affinity activity between enzyme and substrate. Define of Km is the substrate concentration at which the reaction velocity is half of its maximum value. Results were shown as Table 2.

## CONCLUSION

In this study, the wild *C. glutamicum* was promoted using combination of chemical and UV mutagenesis methods. The mutant obtained had a high trehalose productivity(1.28g /100 g cell dry weight·$h^{-1}$) compared with wild strain. Optimum pH for TreS, MTSase, MTHase, TPS and TPP was 7.0, 7.5, 6.5, 6.5, 7.5, respectively. Optimum temperature for TreS, MTSase, MTHase, TPS and TPP was 35!, 35!, 40!, 35!, 35!, respectively. From results of analysis of each ion effect on enzyme activity, we suggested to add $Ba^{2+}$ into reaction

solution when using TreS to produce trehalose and add EDTA when using TreYZ pathway to produce trehalose and add $Mg^{2+}$ when using OtsAB pathway to produce trehalose.

## ACKNOWLEDGEMENTS

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## REFERENCES

1. Xia A, Cheng J, Lin R, *et al*. Combination of hydrogen fermentation and methanogenesis to enhance energy conversion efficiency from trehalose. Energy, 2013, **55**: 631-637.
2. Shi Q, Chung J S. Trehalose metabolism in the blue crab Callinectes sapidus: Isolation of multiple structural cDNA isoforms of trehalose-6-phosphate synthase and their expression in muscles. Gene, 2014, **536**: 105-113.
3. Zhu Y, Wei D, Zhang J, *et al*. Overexpression and characterization of a thermostable trehalose synthase from *Meiothermus ruber*. *Extremophiles*, 2010, **14**: 1-8.
4. Jain N K, Roy I. Effect of trehalose on protein structure. *Protein Science*, 2009, **18**: 24-36.
5. Ganjalikhany M R, Ranjbar B, Hosseinkhani S, *et al*. Roles of trehalose and magnesium sulfate on structural and functional stability of firefly luciferase. *Journal of Molecular Catalysis B: Enzymatic*, 2010, **62**: 127-132.
6. Mahmud S A, Hirasawa T, Shimizu H. Differential importance of trehalose accumulation in *Saccharomyces cerevisiae* in response to various environmental stresses. *Journal of bioscience and bioengineering*, 2010, **109**: 262-266.
7. Carvalho A L, Cardoso F S, Bohn A, *et al*. Engineering trehalose synthesis in *Lactococcus lactis* for improved stress tolerance. *Applied and environmental microbiology*, 2011, **77**: 4189-4199.
8. Ngamskulrungroj P, Himmelreich U, Breger J A, *et al*. The trehalose synthesis pathway is an integral part of the virulence composite for *Cryptococcus gattii*. *Infection and immunity*, 2009, **77**: 4584-4596.
9. Sugawara M, Cytryn E J, Sadowsky M J. Functional role of *Bradyrhizobium japonicum* trehalose biosynthesis and metabolism genes during physiological stress and nodulation. *Applied and environmental microbiology*, 2010, **76**: 1071-1081.
10. Xiuli W, Hongbiao D, Ming Y, *et al*. Gene cloning, expression, and characterization of a novel trehalose synthase from *Arthrobacter aurescens*. *Applied microbiology and biotechnology*, 2009, **83**: 477-482.
11. Zhang Y, Primavesi L F, Jhurreea D, *et al*. Inhibition of SNF1-related protein kinase1 activity and regulation of metabolic pathways by trehalose-6-phosphate. *Plant Physiology*, 2009, **149**: 1860-1871.
12. Ruhal R, Kataria R, Choudhury B. Trends in bacterial trehalose metabolism and significant nodes of metabolic pathway in the direction of trehalose accumulation. *Microbial biotechnology*, 2013, **6**: 493-502.
13. Yamamoto T, Maruta K, Watanabe H, *et al*. Trehalose-producing operon treYZ from *Arthrobacter ramosus* S34. *Bioscience, biotechnology and biochemistry*, 2001, **65**: 1419-1423.
14. Carpinelli J, Krämer R, Agosin E. Metabolic engineering of *Corynebacterium glutamicum* for trehalose overproduction: role of the TreYZ trehalose biosynthetic pathway. *Applied and environmental microbiology*, 2006, **72**: 1949-1955.
15. Tzvetkov M, Klopprogge C, Zelder O, *et al*. Genetic dissection of trehalose biosynthesis in *Corynebacterium glutamicum*: inactivation of trehalose production leads to impaired growth and an altered cell wall lipid composition. *Microbiology*, 2003, **149**: 1659-1673.
16. Klutts S, Pastuszak I, Edavana V K, *et al*. Purification, cloning, expression, and properties of mycobacterial trehalose-phosphate phosphatase. *Journal of Biological Chemistry*, 2003, **278**: 2093-2100.
17. Londesborough J, Vuorio O E. Purification of trehalose synthase from baker's yeast. *European journal of biochemistry*, 1993, **216**: 841-848.
18. Kim T K, Jang J H, Cho H Y, *et al*. Gene cloning and characterization of a trehalose synthase from *Corynebacterium glutamicum* ATCC13032. Food Science and Biotechnology, 2010, **19**: 565-569.
19. Maruta T N K, Tsusaki K, Kubota M, *et al*. Puriûcation and Properties of a Novel Enzyme, Maltooligosyl Trehalose Synthase, from *Arthrobacter sp*. Q36. 1995, **59**:2215-2218.
20. Pan Y T, Carroll J D, Elbein A D. Trehalose phosphate synthase of Mycobacterium tuberculosis. *European Journal of Biochemistry*, 2002, **269**: 6091-6100.