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PURIFICATION AND CHARACTERIZATION OF CYCLODEXTRIN GLYCOSYLTRANSFERASE FROM ALKALIPHILIC *BACILLUS OSHIMENSIS*

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ABSTRACT

Alkaliphilic cyclodextrin glycosyltransferase (CGTase) producer was isolated from the rice fields of Maharashtra, India. 16S rRNA sequence analysis identified the isolate as *Bacillus oshimensis.* CGTase enzyme was purified by ammonium sulphate precipitation along with starch adsorption, which is a simple and efficient method followed by ultra filtration. The purity was found to increase by 2.5 fold with 11% yield. The molecular weight of partially purified enzyme was found to be 66kDa respectively, as determined by Native poly-acrylamide gel electrophoresis. The characterization of the enzyme was carried out for pH and temperature and was found to be optimum at pH 8 and 37^{o}C. Km was found to be 1.66 g% and Vmax as 19.38 U/ml.

KEYWORDS: Cyclodextrin glycosyltransferase, purification, characterization.

INTRODUCTION

Cyclodextrins are macrocyclic, non-reducing maltooligosaccharides composed of glucose units linked by α-1,4-glycosidic bonds. CDs are commonly found to contain six (α-CD), seven (β-CD), and eight (γ-CD) glucose molecules in the ring. CDs possess a unique torus shape and the polar hydroxyl groups are oriented toward the outside, keeping the interior cavity relatively hydrophobic.[1] Therefore, CDs are soluble in water and the hydrophobic environment of the cavity enables them to form inclusion complexes with many organic and inorganic molecules, thereby changing the physical and chemical properties of the included compounds. The global market demand for CD's has increased to 354 metric tons in the past

decade. Thus, CD's have are highly modified valuable starches that have applications in various industries like food, pharmaceutical, dairy and cosmetic, agriculture and textile.

Cyclodextrin glycosyltransferase (CGTase, E.C. 2.4.1.19) synthesizes cyclodextrins (CDs) from starch and related carbohydrates.[2] It is an extracellular bacterial enzyme and is classified under the alpha amylase family or the glycosyl hydrolase family 13.[3] The purpose of the present study was to isolate, partially purify and characterize an alkaliphilic β-CGTase producing bacteria from agricultural soil.

MATERIALS AND METHODS

Screening of CGTase producer

Alkaliphilic CGTase producing bacteria was isolated from soil samples obtained from rice fields in Navi-Mumbai, Maharashtra. The screening of CGTase producer was done using the supernatant collected from 1gm of soil sample suspended in water. The screening of CGTase producer was done according to Park *et. al.*[4] on a medium containing on alkaline Horikoshi (II), pH 10.5 containing 1% soluble starch, 0.5 % peptone, 0.5% yeast extract, 0.1% KH_2PO_4, 0.02% $MgSO_4.7H_2O$, 2% agar, 0.03% phenolphthalein and 1% Na_2CO_3. Formation of yellowish halo zones around the colonies was considered as an indication of CGTase activity. The bacterial colony with biggest halo zone was selected and streaked on Horikoshi (II) medium for several times until uniform colonies were formed. The isolates were cultivated in alkaline liquid medium and CGTase activity was measured in the culture supernatants. The phylogenetical analysis was done by 16S rRNA analysis and the isolate was identified as *Bacillus oshimensis*.

CGTase assay

CGTase activity was determined using phenolphthalein assay method [4,5] with slight modifications. Reaction mixture containing 1% soluble starch in 50mM Tris HCl (pH 10), and 1ml of crude enzyme was incubated at 37^{o}C for 15 min. The reaction was stopped by incubating this reaction mixture in boiling water bath for 3 min. To this 4 ml of 0.04 mM phenolphthalein dissolved in 125 mM $Na_2CO_3.NaHCO_3$ buffer solution was added. The absorbance was measured at 550 nm. The amount of β-cyclodextrin produced was estimated from the standard graph of 0-500 µg/mL β-CD concentration against absorbance. One unit (U) of enzyme activity was defined as the amount of enzyme that produced 1µmol of β CD per minute under standard conditions.[5, 6]

Partial Purification of CGTase

CGTase from *Bacillus oshimensis* was purified by adsorption of the crude enzyme. The cell free supernatant was used as the crude enzyme preparation and was purified by starch adsorption. Insoluble corn starch and ammonium sulfate were added to one liter of to concentration of 5 % (w/v) and 1M (80%) and kept at 4^{o}C with continuous agitation for 1h to allow enzyme adsorption.[7] The crude enzyme was eluted using 5mM β-CD for 30 mins at 37 ^{o}C. The supernatant was pooled and dialyzed against 50 mM Tris HCl buffer (pH 8.0) at 4^{o}C with continuous stirring and occasional changes of the buffer. The purity of the partially purified enzyme was determined using Native PAGE according to Laemmli.[8] Protein bands were visualized using coomassie blue staining on 8% PAGE.

Kinetic parameters

The Kinetic constants, Km and Vmax values, were determined for the partially purified CGTase by measuring initial rates of the reaction at different concentrations of soluble starch (0 to 50 mg/ml) as a substrate in Tris HCl buffer solution (50 mM, pH 8.0) at 37^{o}C. The kinetic constants Km and V max were determined from Lineweaver-Burk plot.

Effect of pH

Optimum pH for the activity of partially purified CGTase were determined by measuring the cyclization activity using soluble starch with designated buffers of 50 mM sodium phosphate (pH 7.0, 7.5), 50 mM Tris HCl buffer (pH 8.0, 8.5), 50 mM glycine NaOH buffer (pH 9.0, 9.5 and 10.0) and 50 mM sodium carbonate – bicarbonate buffer (pH 10.5 to 12.0) respectively. Separate blanks were run for each of the pH with inactivated enzyme. Incubations were carried out at 37^{o}C for 10min.

Effect of temperature

Optimum temperature were determined by measuring the CGTase activity at varying temperatures of 30^{o}C, 37^{o}C, 45^{o}C, 60^{o}C, 75^{o}C and 90^{o}C respectively in the presence of soluble starch dissolved in 50mM Tris-HCl buffer at pH 8.0. Separate blanks were run for each of the temperatures with inactivated enzyme.

Effect of metals

Effect of metal ions on CGTase activity were investigated by incubating the partially purified enzyme with 50mM of Tris HCl buffer pH 8.0 containing 0.1% of potassium chloride, ferrous chloride, maganese sulphate, zinc sulphate, ferrous sulphate, magnesium sulphate,

calcium carbonate and sodium chloride. After the incubation for 30 min at 37°C, CGTase activity was measured. Separate blanks were run for each of the metal ions with inactivated enzyme.

Effect of concentration of Magnesium sulphate

Magnesium sulphate facilitates the growth of *Bacillus species*, hence variations in concentrations of magnesium sulphate (0 - 0.2%) were studied. After 24 h of incubation the supernatant was analyzed for CGTase activity.

RESULTS AND DISCUSSION

The strain was characterized for various physiological properties. The isolate was found to be Gram positive rods with slight motility. Based on the molecular characterization the strain was identified as *Bacillus oshimensis*. The sequence of 16S rRNA was submitted to the GenBank database and can be accessed under Genebank accession number KF415293. The partial purification of CGTase was carried out by using the cell free supernatant from a 36 h old culture of *Bacillus oshimensis*. The cell free supernatant was concentrated with 1M ammonium sulphate and 5% corn starch. Starch adsorption resulted with a yield of 49 % and specific enzymatic activity of 1.501 U/mg. The specific activity was found to increase to 2.174 U/mg after ultrafiltration with 11 % yield. The purification fold obtained was about 2.5 fold. The activity of the partially purified CGTase was measured using the standard assay method. Table 1 shows the fold purification and yield values of CGTase.

Table 1: Partial Purification of CGTase from *Bacillus oshimensis*

	Protein Concentration (ug/ml)	CGTase Activity (U/ml)	Specific Activity (U/mg)	Purification Fold	Yield (%)
Crude Enzyme	19.6	17.5	0.892	1	100
Starch Adsorption	5.68	8.53	1.501	1.682	49
Dialysis	3.02	5.2	1.721	1.929	30
Ultrafiltration	0.87	1.89	2.174	2.437	11

Similar results were reported by Martins and Hatti-Kaul[9] for *Bacillus agaradherens* LS-3C wherein CGTase was purified by starch adsorption as the sole purification step with recovery of 50%. Fujita *et al.*[10] and More *et al.*[11] also reported 55 % and 49.44 % yield on starch adsorption. The CGTase enzyme was purified by one step purification by starch adsorption. The partially purified enzyme was subjected to dialysis and ultrafiltration to remove any impurities. In order to find out the approximate molecular weight of partially purified

CGTase enzyme, Native poly-acrylamide gel electrophoresis (PAGE) was performed and compared with protein marker. The protein fraction obtained by starch adsorption, dialysis and ultrafiltration were run on the 8% polyacrlamide gel. The fraction obtained by ultrafiltration is marked as indicated in Figure 1.

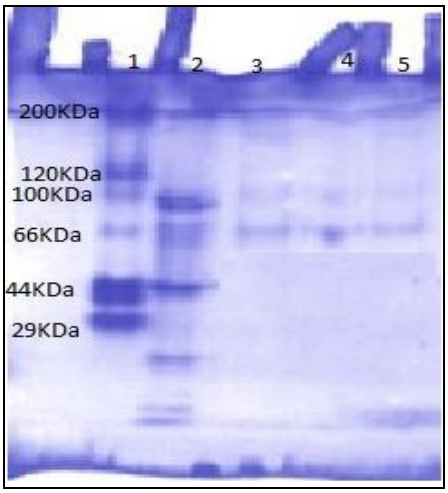

Figure 1: Native-PAGE analysis of purification steps of *Bacillus oshimensis* CGTase on 8 % poly-acrylamide gel.

Lane 1: Protein marker; Lane 2: Crude enzyme (cell free supernatant); Lane 3: Corn starch eluate; Lane 4: Dialyzed fraction; Lane 5: Ultra filtered fraction.

It was seen that the approximate molecular weight of the partially purified CGTase enzyme obtained by starch adsorption and dialyzate showed the presence of two bands (lane3 and 4) with molecular weight of 100 KDa and 66 KDa whereas, the process of ultrafiltration shows the presence of a specific band (lane 5) with approximate molecular weight of 66 KDa. Sian *et al.*[12] reported the molecular weight of 79 KDa from *Bacillus spp*. G1. Most of the previously purified CGTase from various *Bacillus spps* had a molecular weight in the range of 68 - 88 KDa.[7] CGTase from *Bacillus firmus* had a molecular weight of 76 KDa[13]; while Tomita *et al.*,[14] reported it to be 70 KDa in *Bacillus autolyticus* 11149. Martins and Hatti Kaul[9] reported high molecular weight CGTase enzyme of 103 and 110 KDa for *Bacillus circulans* and *Bacillus agaradherens* while Wang *et al.*[15]; Moriwaki *et al.*,[16] and More[11] reported a low molecular weight CGTase of 38KDa from *Bacillus spps*, 59 kDa from *Bacillus sphaericus* and 28 KDa from *Bacillus alcalophilus* AFO78812.

Characterization of partially purified enzyme

The effect of soluble starch concentration on the catalytic rate was measured at different substrate concentration and the kinetic constants of CGTase isolated from *Bacillus oshimensis* determined. The enzymatic activity was measured at soluble starch concentrations of 0.5 to 5 g% at pH 8.0 and 37°C. Km and Vmax were determined using the linearized Lineweaver – Burk plot as indicated in Figure 2.

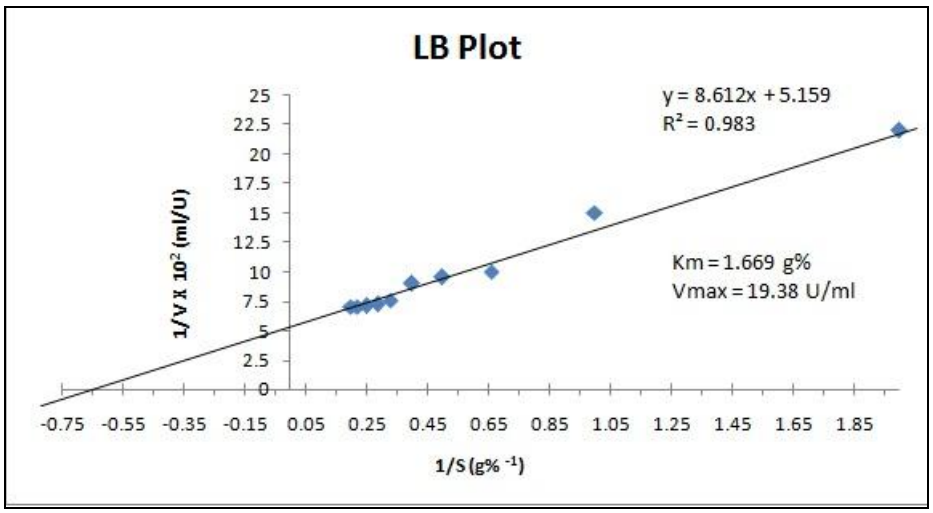

Figure 2: Lineweaver Burk plot for the determination of Km and Vmax for partially purified CGTase enzyme

The Km and Vmax values obtained with soluble starch as the substrate was 1.66 g% and 19.38 U/ml. Martins and Hatti Kaul[9] reported Km value of 2.12 g% while Wang *et. al.*[17] reported it to be 3.39 g% with cassava starch. The activity of partially purified CGTase was measured using the standard assay method as described earlier, by varying the pH values from 7 to 11 at 37°C in buffers with the same ionic concentrations.

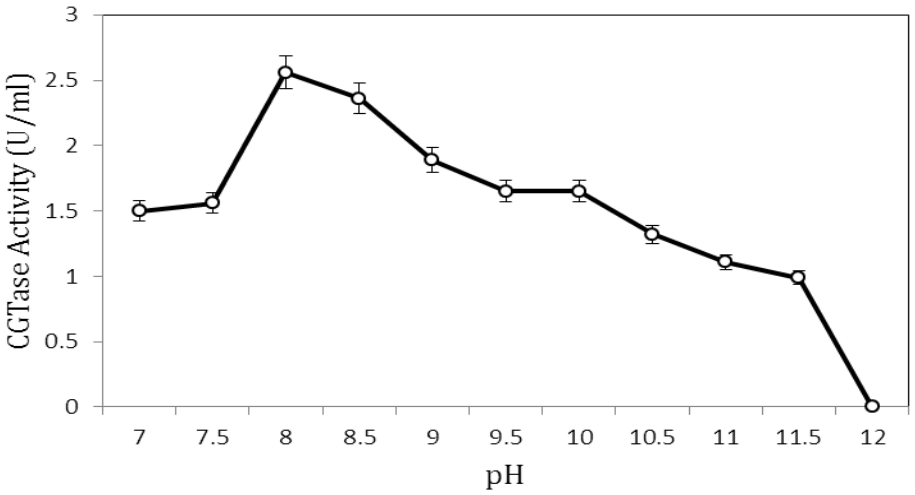

Figure 3: Effect of pH on the activity of partially purified CGTase enzyme isolated from *Bacillus oshimensis*

Figure 3 indicates that the CGTase activity increased as the pH increases up to pH 8 and decreased after pH 8.0. The partially purified CGTase was characterized for pH and temperature optimum. The optimum pH was determined as pH 8.0. Mori *et al.* reported the optimum pH to be 10.0 in *Brevibacterium sp*. No. 9605.[18] pH 7.8 was found to be suitable for the CGTase production by *Bacillus sp*.G1[6] and *Bacillus firmus.*[19] The effect of temperature was studied and the enzyme activity was measured at different temperatures at pH 8.0, the optimum temperature was determined to be 37°C as shown in Figure 4.

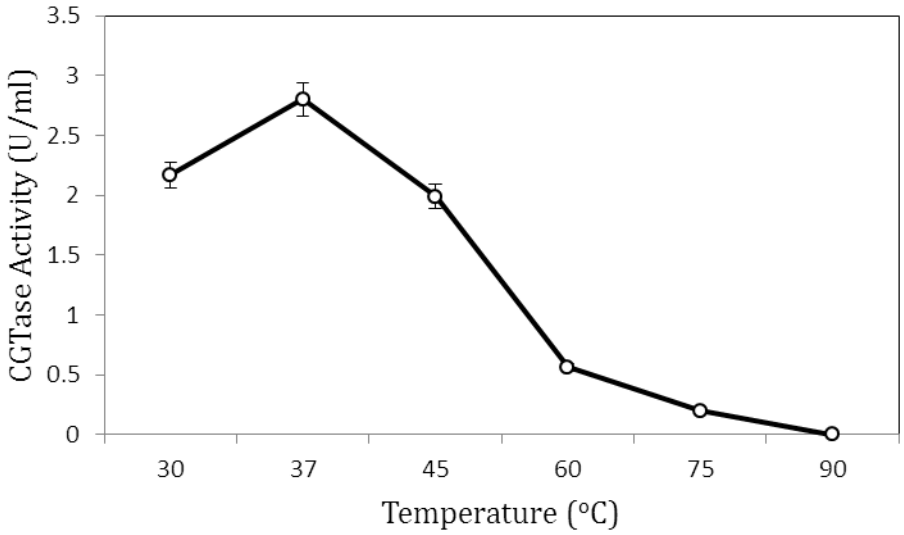

Figure 4: Effect of Temperature on the activity for partially purified CGTase enzyme isolated from *Bacillus oshimensis*

The CGTase enzyme lost its activity as the temperature increases above 37°C. The bacterial culture was isolated from soil sample collected from agricultural fields because of which it would not resist the high temperature and its activity was found to decrease. The enzyme activity was measured at different temperatures and the optimum temperature was found to be 37°C. Temperature has a vital role in the growth and productivity of microbes. The optimum temperature reported for *Bacillus cereus* N1[20] was 40°C and 45°C for *Bacillus alcalophilus* AFO78812.[11] The temperature optima in the range of 55°C - 65°C have been reported previously for CGTase from various alkaliphiles.[12,21,22] Rosso *et al.*,[23] observed that pH 8.3 and incubation temperature of 37°C is suitable for the CGTase production from *Bacillus circulans* DF 9R.

The effect of mineral sources on CGTase production is indicated in Figure 5. The order of CGTase activity was found to be $MgSO_4$ > $CaCO_3$ > $ZnSO_4$ > $FeSO_4$. Magnesium sulphate

was found to be a good source of trace elements for CGTase. Metal ions are necessary for cell growth and maintenance of active conformation of enzymes.[22]

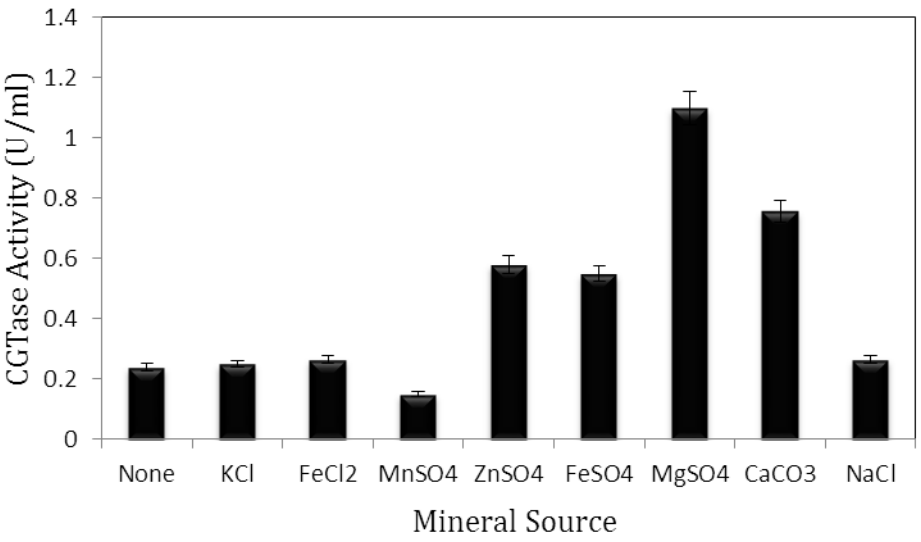

Figure 5: Effect of different mineral sources on CGTase production by *Bacillus oshimensis*

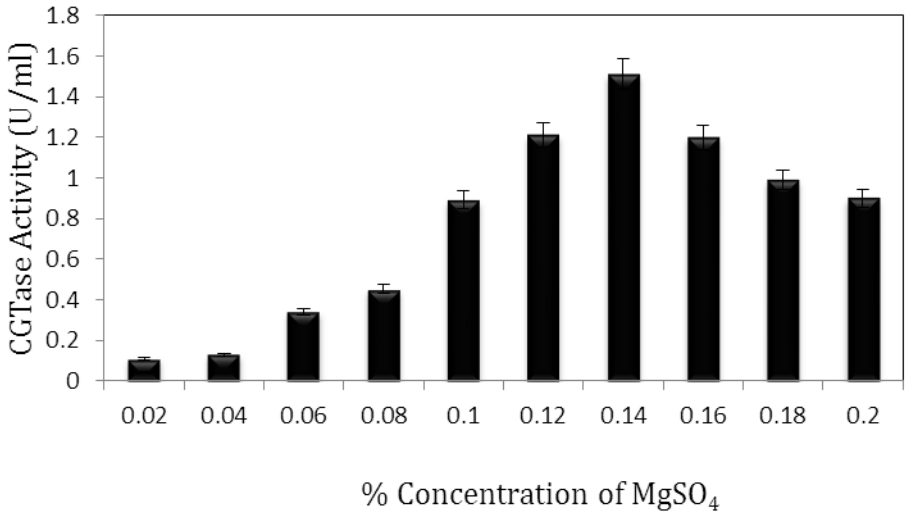

Figure 6: Effect of Different Concentration of Magnesium Sulphate on CGTase production by *Bacillus oshimensis*

Magnesium sulphate at 0.14% concentration showed highest CGTase activity as indicated in Figure 6. The literature survey reports $MgSO_4$ as the best mineral source for CGTase production. *Bacillus oshimensis* showed highest CGTase activity with 0.14% $MgSO_4$.

CONCLUSION

A novel strain was isolated from the soil sample of rice fields at Navi- Mumbai, Maharashtra which showed good alkaline CGTase activity. Based on the morphological, physiological and molecular studies the selected strain was identified as *Bacillus oshimensis*. The partial purification of CGTase enzyme was carried out. One step purification technique was administered using starch adsorption. The partially purified enzyme was subjected to dialysis and ultrafiltration to remove any impurities. The molecular weight of 66 KDa was confirmed with Native PAGE. After purification the specific activity of the CGTase increased from 0.892 U/mg to 2.174 U/mg with 11% yield and 2.5 fold purification. The partially purified enzyme was characterized for optimum pH and temperature. The activity was found to be optimum at pH 8.0 and 37^{o}C. Km was found to be 1.66 g% and Vmax as 19.38 U/ml.

REFERENCES

1. Gawande BN, Patkar AY. Purification and properties of a novel raw starch degrading-cyclodextrin glycosyltransferase from *Klebsiella pneumoniae* AS- 22. Enzyme and Microbial Technology, 2001; 28(9): 735-743.
2. Gawande BN, Sonawane AM, Jogdand VV and Patkar AY. Optimization of cyclodextrin glycosyltransferase production from *Klebsiella pneumonia*e AS-22 in batch, fed-batch and continuous cultures. Biotechnology Progress, 2003; 19: 1697-1702.
3. Park JK, Chang HN. Microencapsulation of microbial cells. Biotechnology Advances, 2000; 18: 303-319.
4. Park SC, Park HH, Kim SH. A rapid screening method for alkaline β cyclodextrin glucanotransferase using phenolphthalein-methyl orange containing solid media. Agricultural Biological Chemistry, 1989; 53: 1167- 1169.
5. Kashipeta R, Tadimalla P, Katikala P, Guruju V, Phani B and Nalli V. Screening, Isolation and Characterization of cyclodextrin glycosyltransferase producing bacteria from soil samples. International Journal of Pharmacy and Pharmaceutical Sciences, 2012; 4(3):408- 414.
6. Ibrahim ASS, Al - Salamah AA, El – Tayeb MA, El – Badawi YB, Antranikian G. A Novel Cyclodextrin glycosyltransferase from alkaliphilic *Amphibacillus sp*. NPST – 10: Purification and Properties. International Journal of Molecular Science, 2012; 13: 10505 – 10522.
7. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 1970; 227: 680-685.

8. Martins RF, and Hatti-Kaul R. A new cyclodextrin glycosyltransferase from an alkaliphilic *Bacillus agaradhaerens* isolate: Purification and characterization. Enzyme Microbial Technology, 2002; 30: 116-124.
9. Fujita Y, Tsubouchi H, Inagi Y, Tomita K, Ozaki A, Nakanishi K. Purification and properties of cyclodextrin glycosyltransferase from *Bacillus sp.* AL-6. Journal of Fermentation and Bioengineering, 1990; 70: 150-154.
10. Geetha R and More SS, Isolation and Characterization of Cyclodextrin glucanotransferase from soil bacterium. Research Journal of Biological Science, 2010; 5(10): 699 – 707.
11. Sian HK, Said M, Hassan O, Kamaruddin K, Ismail AF, Rahman RA, Mahmood NN, Illias RM. Purification and Characterization of Cyclodextrin Glucanotransferase from Alkalophilic *Bacillus Sp.* G1. Process Biochemistry, 2005; 40(3): 1101-1111.
12. Savergave LS, Dhule SS, Jogdand VV, Nene SN, Gadre RV. Production and single step purification of cyclodextrin glycosyltransferase from alkalophilic *Bacillus firmus* by ion exchange chromatography. Biochemical Engineering Journal, 2008; 39: 510 - 515.
13. Tomita K, Kaneda M, Kawamura K, Nakanishi K. Purification and properties of a cylcodextrin glucanotransferase from *Bacillus autolyticus* 11149 and selective formation of ß-cyclodextrin. Journal of Fermentation and Bioengineering, 1993; 75(2): 89- 92.
14. Wang J, Cao Y, Sun B, Wag C. Physicochemical and release characterization of garlic oil – β- cyclodextrin inclusion complexes. Food Chemistry, 2011; 127: 1680 – 1685
15. Moriwaki C, Ferreira LR, Rodella JRT, Matioli G. A novel cyclodextrin glycosyltransferase from *Bacillus sphaericus* strain 41: Production, characterization and catalytic properties. Biochemical Engineering Journal, 2009; 48: 124–131.
16. Wang GS, Chen PL, Liu YT, Wang LH. Purification of cyclodextrin glycosyl transferase from a mutant of *Bacillus species.* Report on Taiwan Sugar Research Institute, 1995; 150:53-66.
17. Mori S, Hirose S, Oya T, Kitahata S. Purification and properties of cyclodextrin glucanotransferase from *Brevibacterium sp.* no 9605. Bioscience Biotechnology Biochemistry, 1994; 58(11): 1968-1972.
18. Gawande BN, Singh RK, Chauhan AK, Goel A, and Patkar AY. Optimization of cyclomaltodextrin glucanotransferase production from *Bacillus firmus*. Enzyme Microbial Technology, 1998; 22: 288-291.
19. Ismail SAM, El-Sayed OH, Helmy SA, Mohammad Y, Mohammad NR. Production, Purification and Biochemical Characteriztion of Cyclodextrin Glucanotransferase from

Bacillus Cereus N1. Research Journal of Agriculture and Biological Sciences, 2010; 6(4): 370-377.

20. Cao X, Jin Z, Wang X, Chen F. A novel cyclodextrin glycosyl transferase from an alkalophilic *Bacillus species*: Purification and characterization. Food Research International, 2005; 38: 309–314.
21. Alves Prado HF, Carneiro AJ, Pavezzi FC, Gomes E, Boscolo M, Franco CL. Production of cyclodextrins by CGTase from *Bacillus clausii* using different starches as substrates. Applied Biochemistry and Biotechnology, 2008; 146: 3–13.
22. More SS, Niraja R, Evelyn C, Byadgi AM, Shweta V and Mangaraj SD: Isolation, Purification and Biochemical characterization of CGTase from *Bacillus halodurans*. Croatian Journal of Food Technology, Biotechnology and Nutrition, 2012; 7(1-2): 90 – 97.
23. Rosso AM, Ferrarotti1 SA, Krymkiewicz N, and Nudel BC. Optimization of batch culture conditions for cyclodextrin glucanotransferase production from *Bacillus circulans* DF 9R. Microbial Cell Factories, 2002; 1(3): 1-9.