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Advances in Applied Science Research, 2012, 3 (4):2288-2298

ISSN: 0976-8610
CODEN (USA): AASRFC

Immobilization of cyclodextrin glycosyltransferase from newly isolated, mutated Bacillus sp. TPR71HNA6 by Entrapment technique

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ABSTRACT

The present investigation was carried out to find the suitable matrix for immobilization of Cyclodextrin glycosyltransferase (CGTase) producing strain of newly isolated and mutated Bacillus sp. TPR71HNA6. In this study entrapment technique was employed and alginate, k-carrageenan, agar agar and gelatin were used as supporting matrices. The results indicated that the cells immobilized in calcium alginate were more efficient for CGTase production than the free cells (both conventional and washed). Hence, the alginate matrix was selected as a good matrix for better production of CGTase .

Key words: CGTase, Bacillus sp., Entrapment technique, alginate, k-carrageenan, agar agar.

INTRODUCTION

Cyclodextrin glucosyltransferases (CGTases) (EC 2.4.1.19) represent one of the most important group of microbial amylolytic enzymes. CGTase catalyze the formation of cyclodextrins (CDs) from starch and related α (1 ⟶4) linked glucose polymers via a transglycosylation reaction [1,2]. Bacterial CGTase is a multifunctional enzyme that in addition to formation of CDs it also catalyze other three reactions [3,4]: (a) Coupling, where the CD molecule is opened and combined with a linear oligosaccharide to produce a longer linear carbohydrate; (b) disproportionation, which is the transfer of part of a linear oligosaccharide chain to an acceptor; and (c) saccharifying or hydrolysis of starch.

As the separation of different CDs is costly and time consuming, CGTase that synthesises predominantly one type of CD are of great interest. Bacterial CGTase usually produced mixture of different types of CDs, however most CGTases from bacteria convert starch into β-CD as the predominant product, however a mixture of other CD forms are still produced in varying ratios [5,6]. There is increasing interest in developing efficient industrial process for production of cyclodextrins and oligosaccharides for addressing applications in different industries. However, possibly, the main bottleneck in the industrial application of enzymes is the price and the stability of the biocatalyst. Enzyme immobilization is one of the most useful approaches to overcome such difficulties [7]. Continuous production of CDs and oligosaccharides using immobilized CGTase would offer several advantages including allowing reuse of expensive CGTase, simplifying product purification process and providing opportunities for scaling up [7]. Different approaches have been applied for the immobilization of CGTases based on adsorption, entrapment or covalent binding [2].

In the present investigation entrapment technique was employed using alginate, k-carrageenan, agar agar and gelatin as supporting matrices to improve the yield of CGTase.

MATERIALS AND METHODS

Microorganism
A mutated strain of *Bacillus* sp. TPR71HNA6 was used for the present study.

Chemicals and media
All the chemicals used in this study were of analytical grade. Media constituents used in this study were procured from Hi-Media, Mumbai.

Preparation of cell suspension
A 50mL of cell suspension was prepared by adjusting the total number of cells to $4.2x10^7$ cfu/mL as described earlier and was used for immobilization in various matrices.

Inoculum/ Production medium
Medium for the production of cyclodextrin glycosyltransferase contains the following ingredients Soluble Starch 3%, Yeast Extract 0.5%, K_2HPO_4 0.1%, Inoculum Level 3.5%, Inoculum Age 24 h, Incubation Period 36 h, rpm 220, Incubation Temperature 32°C and the pH of 7.5.

Preparation of Production medium
1% (0.5mL) of the above inoculum medium was transferred aseptically to 49.5mL of production medium. The flasks were kept on the rotary shaker at 30ºC. The samples were withdrawn for every 12h up to 72h and centrifuged at 10,000rpm for 10min in order to remove the cells and other insoluble materials. The clear supernatant was used as a crude enzyme for estimation.

Assay of CGTase
Assay of CGTase was carried out according to the method of Kaneko *et al.,* 1987 [8]. The amount of β-cyclodextrin produced was estimated from the standard graph of 0-500µg/mL β-CD concentration against absorbance. One unit of CGTase was defined as the amount of enzyme required to produce 1µmol of β-CD/min. All the experiments were conducted in triplicate and the mean values were calculated.

The reaction mixture containing 1mL of 40mg of soluble starch in 0.1M potassium phosphate buffer (pH 6.0) and 0.1mL of the crude enzyme from the culture and the mixture was incubated in water bath at 60°C for 10 min. The reaction was stopped with 3.5mL of 30mM NaOH. Finally, 0.5mL of 0.02% (w/v) phenolphthalein in 5mM Na_2CO_3 was added and mixed well. After leaving the mixture to stand for 15min at room temperature, the reduction in colour intensity was measured at 550nm. A blank lacking the enzyme is tested simultaneously with each batch of samples.

ENTRAPMENT TECHNIQUE
Preparation of supporting matrices for entrapment technique Sodium alginate solution
Sodium alginate solution (3%) was prepared by dissolving sodium alginate in 100mL of hot distilled water. The contents were stirred vigorously for 10min to obtain thick uniform slurry without any undissolved lumps and sterilized by autoclaving.

Calcium chloride solution
A liter volume of calcium chloride in concentrations of 0.2M was prepared in distilled water. The solution was stored at room temperature.

k-Carrageenan
k-Carrageenan was completely dissolved in distilled water (3g/100mL) at the desired concentration at 50°C, then the solution was sterilized at 110°-121°C for 1h. The solution was cooled to < 38-40°C with stirring.

Entrapment of cells in calcium alginate
Entrapment of cells in alginate is one of the simplest, cheapest, less toxic and the most frequently used method of immobilization [9-11]. The cell suspension was added to prepared alginate slurry and stirred for 10min to get uniform mixture. The slurry was taken into sterile syringe, added drop wise into 0.2M calcium chloride solution from 5cm height to form calcium alginate beads. The beads were kept for curing at 4°C for 1h. The cured beads were washed with sterile water for 3-4 times. The beads were preserved in normal saline solution at 4°C. All these operations were carried out aseptically under laminar airflow unit [12].

Entrapment of cells in k-Carrageenan
The cell suspension was added to molten k-Carrageenan solution maintained at 40°C, mixed well and poured into sterile flat bottomed 10mm dia petriplates. After solidification it was cut into equal size cubes (4x4x4mm) and added to sterile 2% potassium chloride solution and kept in the refrigerator for 1h for curing. The cubes were washed 3-4 times with sterile distilled water.

Entrapment of cells in Agar-Agar
A definite quantity of agar-agar was dissolved in 18mL of 0.9% sodium chloride solution to get the final concentration of 2% w/v and sterilized by autoclaving. Then 2mL of cell suspension was added to the molten agar-agar maintained at 40°C, shaken well for few seconds, poured into sterile flat bottomed 10mm dia petriplates and allowed to solidify. The solidified agar block was cut into equal size cubes (4x4x4mm) added to the sterile 0.1M disodium phosphate buffer (pH 6.5) and kept in refrigerator 1h for curing. After curing, phosphate buffer was decanted and the cubes were washed with sterile distilled water for 3-4 times.

Entrapment of cells in Gelatin
2mL of the cell suspension was added to 15mL of 15% sterile gelatin solution maintained at 45°C and poured into sterile petriplate. The gel was over layered with 10 mL of glutaraldehyde for hardening at 30°C. The resulted blocks was cut into small cubes (4x4x4mm) and the cubes were washed thoroughly with sterile distilled water for complete removal of excess glutaraldehyde.

Production of CGTase by batch process with immobilized cells
The immobilized beads/ cubes were transferred into 50mL selected production medium contained in 250mL Ehrlenmeyer flasks. The flasks were incubated at 30°C for 72h. Samples were withdrawn at regular intervals of 12h and assayed as described earlier.

Production of CGTase by repeated batches
The fermentation production medium with immobilized beads/cubes was conducted for 36h for the succeeding batches, where maximum productivity was attained. The medium was replaced with fresh production medium and the process was repeated for several batches until the beads/ cubes started disintegrating. The CGTase titres for each cycle were evaluated as described earlier.

RESULTS AND DISCUSSION

Immobilization in calcium alginate
CGTase production of immobilized cells of mutated *Bacillus* sp. TPR71HNA6 in calcium alginate was investigated and the results are presented in Table 1 and Figure 1. The data indicates that the CGTase production was started at 24h with immobilized cells and reached a maximum level of 68.50U/mL at 36h. Little change in CGTase titre was observed after 60h.

Immobilization in K-Carrageenan
The application of K-Carrageenan as entrapment matrix was reported for the production of tyrosine and many antibiotics and drugs [13]. For the present study the fermentation was conducted with immobilized cubes and the results were presented in Table 2 and Figure 2. The results indicated that there was no CGTase production at 12h. It was recorded that CGTase production started at 24h and a high yield (33.62U/mL) was observed at 36h. A slight decrease in the yield was observed on further incubation. The CGTase titre obtained with this carrier was less than that of free or immobilized cells with alginate.

Immobilization in Agar-Agar
CGTase production pattern with immobilized cells in agar-agar was also conducted in the present study. Agar-Agar (2% w/v) was used for immobilization and the fermentation was conducted for 72h. The results were presented in Table 3 and Figure 3. It was observed that the CGTase production with immobilized cells in agar-agar was less than the immobilized cells with other two matrices ie., calcium alginate and K-Carrageenan. Here also the same trend was observed. The data indicates that the CGTase production was started at 24h with immobilized cells and reached a maximum level of 29.69U/mL by 36h. Little change in CGTase titre was observed after 36h (up to 60h) whereas maximum CGTase titre was observed by 48h in the case of free cells.

Immobilization in Gelatin
The CGTase production with immobilized cells in gelatin was evaluated and the results were shown in Table 4 and Figure 4. The results indicated that there was no CGTase production at 12h. It was recorded that CGTase production started at 24h and a maximum level of 25.39U/mL CGTase titre was observed up to 36h. A slight decrease in the

yield was observed on further incubation. The CGTase titre obtained with this carrier was very less when compared with the titres of free cells and immobilized cells of other carriers.

Table 1. CGTase production by immobilized cells of mutated *Bacillus* sp. TPR71HNA6 in calcium alginate

Fermentation Time (h)	CGTase Activity (U/mL)
12	Nil
24	14.83±1.1
36	**68.50**±1.0
48	68.02±1.3
60	57.84±1.6
72	22.20±1.1

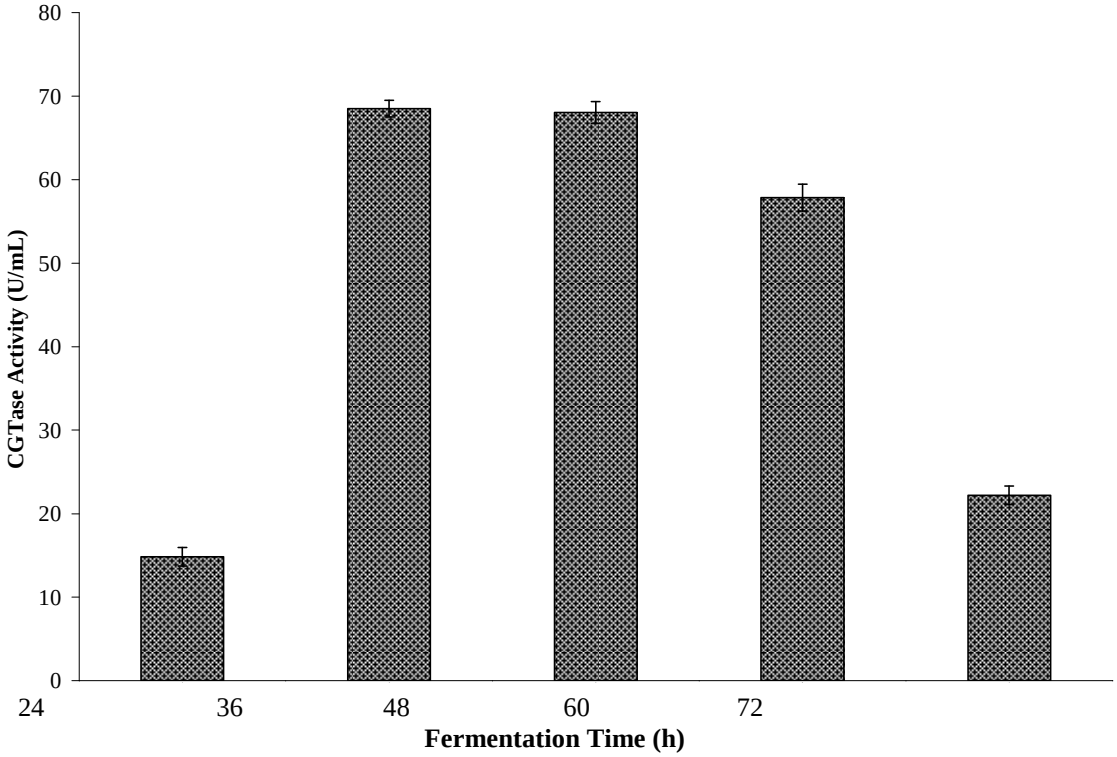

Fig 1. CGTase production by immobilized cells of mutated *Bacillus* sp. TPR71HNA6 in calcium alginate

Repeated batch fermentation with free cells and immobilized cells

Repeated batch fermentations with immobilized cells in alginate, K-Carrageenan, Agar-Agar and gelatin matrices were carried out to evaluate the CGTase production capacity and the longevity of the biocatalysts. In these experiments, the fermentation medium was decanted at every 36h incubation, and the beads/ cubes were washed twice with 50 mL of sterile saline solution. Fresh production medium (50mL) was added to each flask and incubated for 36h. The above process was repeated for several batches and CGTase content was determined as described earlier. The results were presented in Table 5-8 and Figure 5-8.

In the repeated batch fermentation, the amount of CGTase production with alginate beads was increased gradually from first batch to 4th batch followed by a gradual decrease in CGTase titre with subsequent batches. The beads started disintegrating during 8th batch operation. Thus the repeated batch fermentation with alginate beads was successfully run for seven batches. Lower CGTase production in the first cycle may be probably due to the time required for the cells to adapt to the new physical environment [14].

The immobilized cells entrapped in K-Carrageenan were also used for repeated batch fermentation, where relatively low CGTase titre was observed when compared to alginate immobilized cells. The cubes were disintegrated after four batches.

Comparison of CGTase with immobilized cells in alginate and K-Carrageenan by repeated batch fermentation

The comparative data on total CGTase production with free cells and immobilized cells in alginate and K-Carrageenan was presented in the Table 9 and in Figure 9. The data shows that the average CGTase titre per day with calcium alginate immobilized cells was 28.86U/mL and for free cells (conventional) 29.09U/mL. The yield of CGTase with washed free cells was found to be 19.93U/mL. Among all the immobilization techniques used, it was found that the average CGTase production per day with carrageenan immobilized cells was very low.

Table 2. CGTase production by immobilized cells of mutated *Bacillus* sp. TPR71HNA6 in K-Carrageenan

Fermentation Time (h)	CGTase Activity (U/mL)
12	Nil
24	18.00±0.8
36	**33.62**±0.6
48	29.04±0.7
60	16.32±0.9
72	10.96±0.6

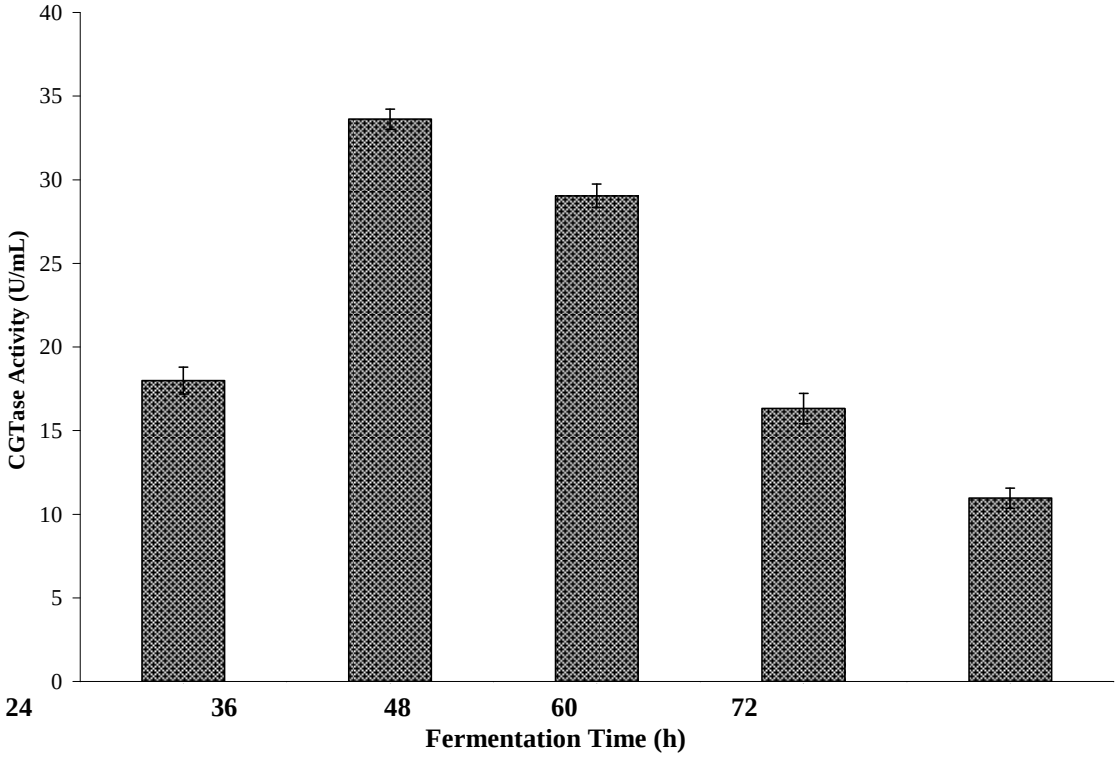

Fig 2. CGTase production by immobilized cells of mutated *Bacillus* sp. TPR71HNA6 in K-Carrageenan

Table 3. CGTase production by immobilized cells of mutated *Bacillus* sp. TPR71HNA6 in Agar-Agar

Fermentation Time (h)	CGTase Activity (U/mL)
12	Nil
24	12.33±0.7
36	**29.69**±0.9
48	18.02±0.5
60	15.46±0.6
72	10.42±0.7

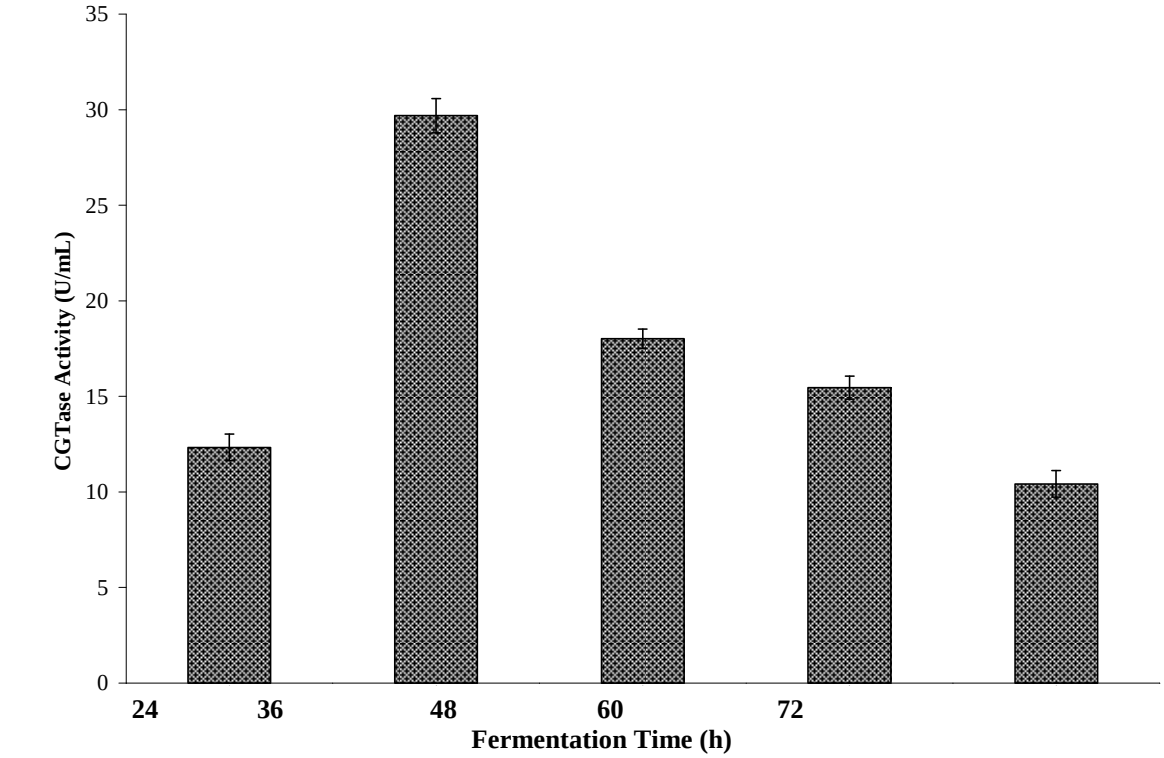

Fig 3. CGTase production by immobilized cells of mutated *Bacillus* sp. TPR71HNA6 in Agar-Agar

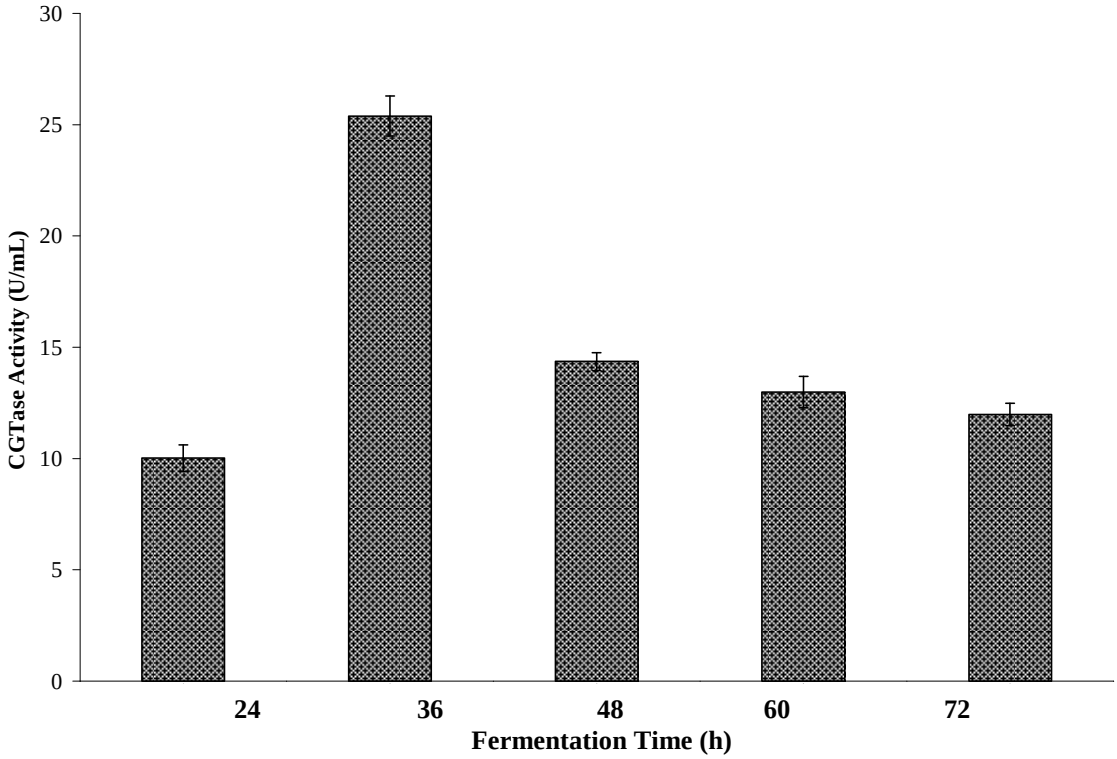

Fig 4. CGTase production by immobilized cells of mutated *Bacillus* sp. TPR71HNA6 in Gelatin

Table 4. CGTase production by immobilized cells of mutated *Bacillus* sp. TPR71HNA6 in Gelatin

Fermentation Time (h)	CGTase Activity (U/mL)
12	Nil
24	10.02±0.6
36	**25.39**±0.9
48	14.36±0.4
60	12.99±0.7
72	11.98±0.5

Table 5. CGTase production by repeated batch fermentation with immobilized cells of mutated *Bacillus* sp. TPR71HNA6 in calcium alginate

Cycle No.	CGTase Activity (U/mL)
1	68.50±1.5
2	68.93±1.1
3	70.02±1.0
4	**75.80**±1.4
5	69.72±1.4
6	66.30±1.2
7	42.90±1.1

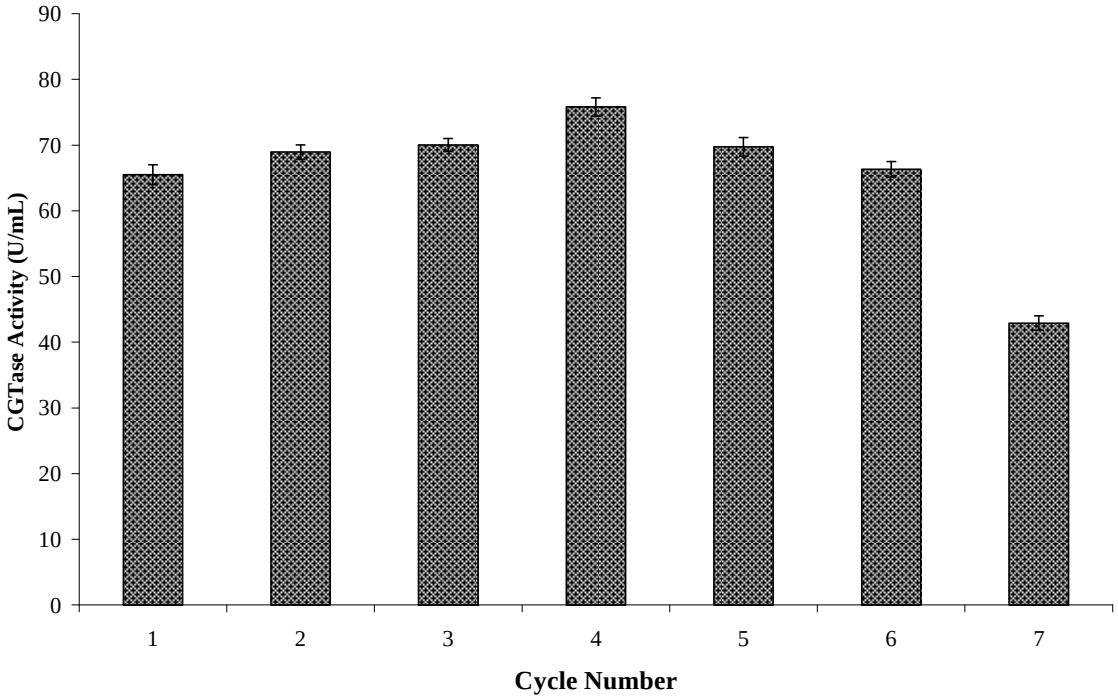

Fig 5. CGTase production by repeated batch fermentation with immobilized cells of mutated *Bacillus* sp. TPR71HNA6 in calcium alginate

Table 6. CGTase production by repeated batch fermentation with immobilized cells of mutated *Bacillus* sp. TPR71HNA6 in K-Carrageenan

Cycle No.	CGTase Activity (U/mL)
1	33.62±0.8
2	**45.32**±0.7
3	34.06±0.4
4	27.42±0.6

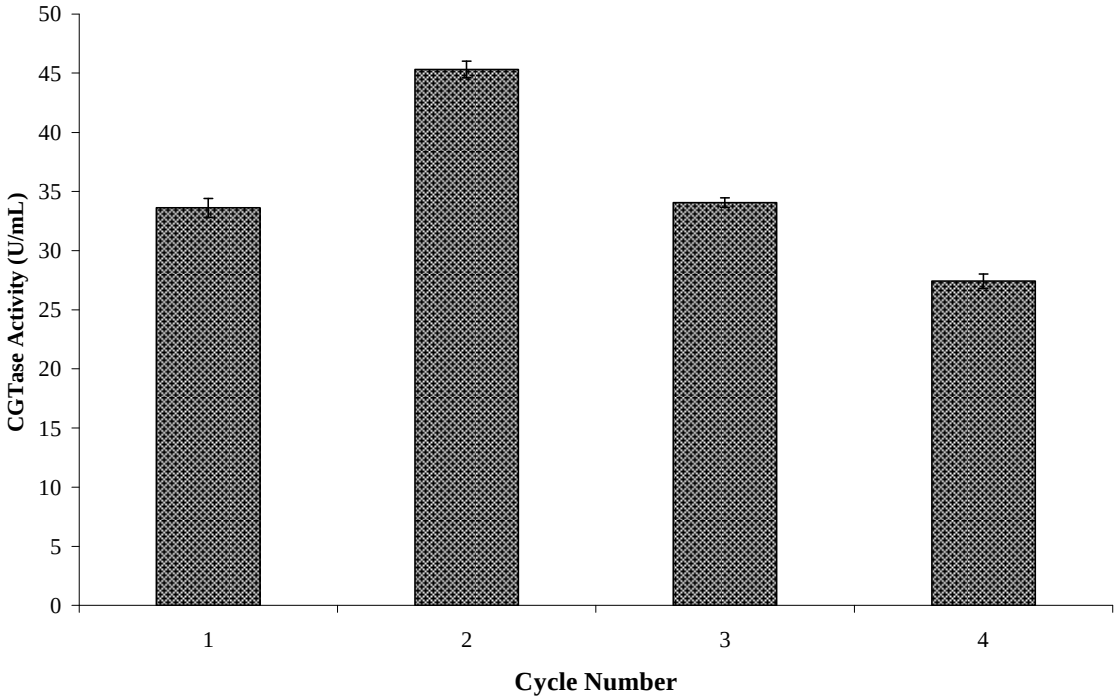

Fig 6. CGTase production by repeated batch fermentation with immobilized cells of mutated *Bacillus* sp. TPR71HNA6 in K-Carrageenan

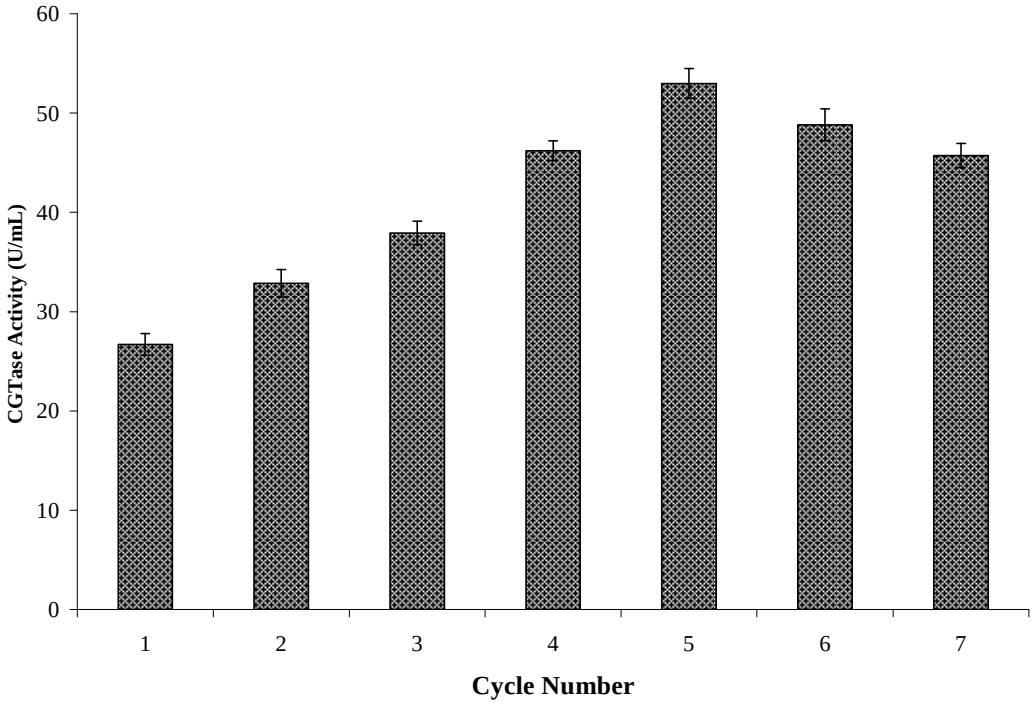

Fig 7. CGTase production by repeated batch fermentation with immobilized cells of mutated *Bacillus* sp. TPR71HNA6 in Agar-Agar

Table 7. CGTase production by repeated batch fermentation with immobilized cells of mutated *Bacillus* sp. TPR71HNA6 in Agar-Agar

Cycle No.	CGTase Activity (U/mL)
1	29.69±1.1
2	32.86±1.4
3	37.91±1.2
4	46.20±1.0
5	**52.99**±1.5
6	48.81±1.6
7	45.73±1.2

Table 8. CGTase production by repeated batch fermentation with immobilized cells of mutated *Bacillus* sp. TPR71HNA6 in Gelatin

Cycle No.	CGTase Activity (U/mL)
1	25.92±1.4
2	34.32±1.6
3	**46.48**±1.0
4	40.26±1.2
5	29.23±1.1

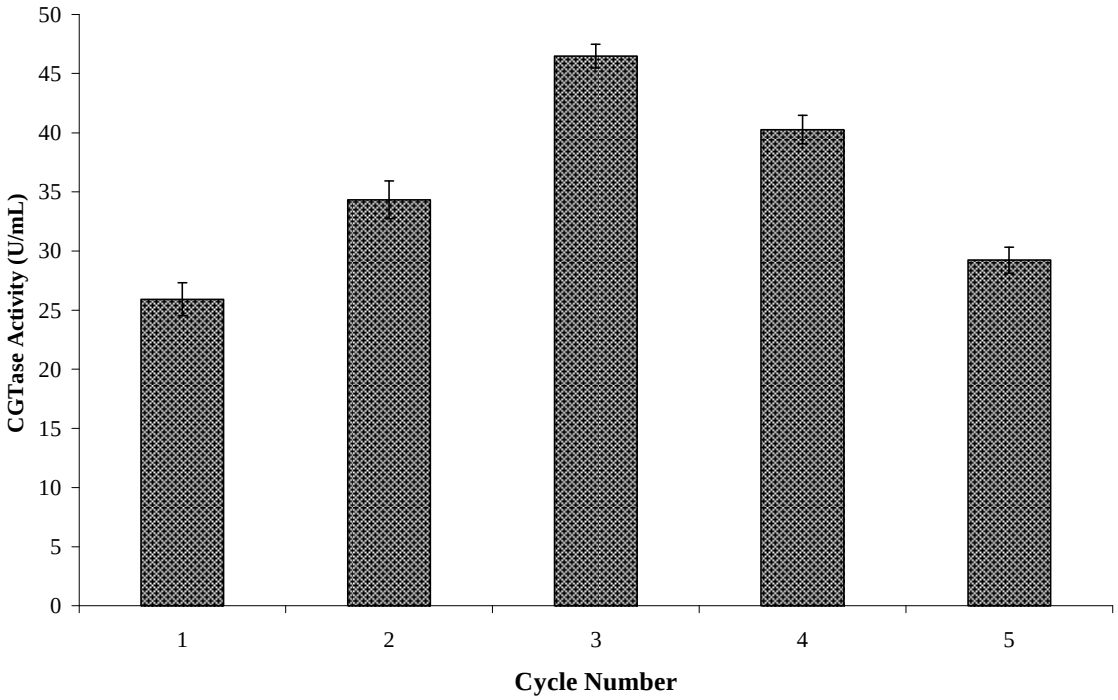

Fig 8. CGTase production by repeated batch fermentation with immobilized cells of mutated *Bacillus* sp. TPR71HNA6 in Gelatin

Table 9. Comparison of CGTase production with immobilized cells of mutated *Bacillus* sp. TPR71HNA6 in different matrices by repeated batch cultures

Matrix	No. of batches	Total fermentation time (Days)	Total CGTase Production (U/mL)	Average CGTase titre per batch (U/mL)	Average CGTase titre per day (U/mL)
Ca. Alginate	7	10.5	462.17	66.02	44.01
Agar Agar	7	10.5	294.19	42.03	28.02
Gelatin	5	7.5	176.21	35.24	23.49
K-Carrageenan	4	2.7	140.42	35.11	23.41
Free cells (washed)	4	2.7	226.85	56.71	37.81
Free cells (conventional)	1	2	58.18	58.18	29.09

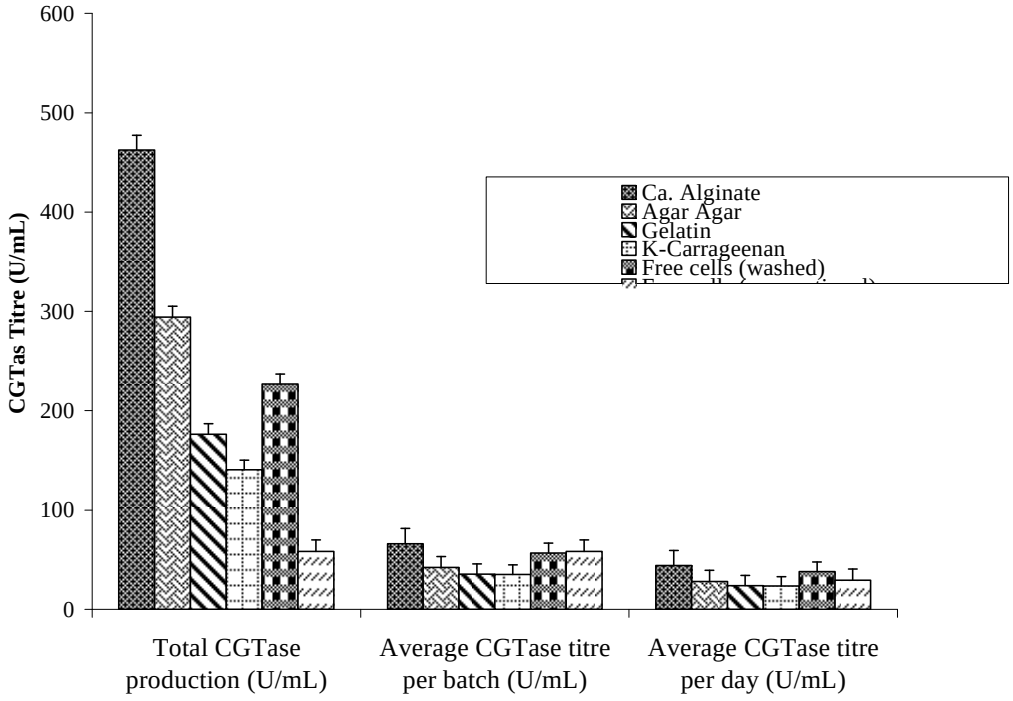

Fig 9. Comparison of CGTase production with immobilized cells of mutated *Bacillus* sp TPR71HNA6 in alginate and K-Carrageenan by repeated batch cultures

CONCLUSION

From the results it is clear that the cells immobilized in calcium alginate were more efficient for CGTase production than the free cells (both conventional and washed). Finally, it is concluded that among various immobilization techniques, some adsorption techniques as well as entrapment techniques are good for the CGTase production. Even though the production was equally good with immobilized cells by adsorption techniques, the cell leakage is more and employment of such materials in reactors is rather difficult. But in case of gel matrices, the immobilized biocatalysts (cells) occur in a spherical shape and are suitable for use in bioreactors. Hence, the alginate matrix was selected to optimize for better production of CGTase with increased half life of the biocatalyst.

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