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Synthesis of Cyclodextrin Glucosyl Transferase by *Bacillus cereus* for the Production of Cyclodextrins

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ABSTRACT

A potent indigenous bacillus isolate identified as *Bacillus cereus* (RJ-30) was found to produce Cyclodextrin Glucosyl Transferase (CGTase) extracellularly. Process optimization of various fermentation parameters has been established for optimal growth of bacillus and the maximum enzyme synthesis. The organism had the highest specific growth rate (0.7μ) with a generation time of 1 h in glucose containing medium at the conditions of pH 7.0, 37°C at 300 rpm, 1.5 vvm of agitation, and aeration. At these conditions, it exhibited the maximum activity of 54 U/mL at the synthesis rate of 2.7 U/L/h. CGTase was produced from the early exponential growth and peaked during the midsporulating stage of about 16 h thereafter maintained at the same level of 50 U/mL. Saccharides containing media were better inducers than starch, and the influence of carbohydrate substrates has shown that enzyme synthesis is promoted by xylose (65 U/mL) and, more remarkably, by the supplementation of wheat bran extract in glucose medium (106 U/mL). This organism produced CGTase stably in a chemostat culturing over a period of 400 h with a maximum productivity of 5.4 kU/L/h (threefold higher than obtained in batch culturing [1.75 kU/L/h]). Comparatively, CGTase was produced by immobilized cells in a continuous fluidized bed reactor for over approx 360 h, at a relatively high dilution rate of 0.88 h^{-1} resulting in the productivity of 23.0 kU/L/h.

Index Entries: Bacillus cereus; CGTase; cyclodextrins.

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INTRODUCTION

Cyclodextrin Glucosyl Transferase (CGTase) (EC 2.4.1.19) is a unique transglucosidase catalyzing the reversible intermolecular and intramolecular 1-4 transglycosylation of starch, performing cyclizing, coupling, and disproportionation of glucose residues, resulting in formation of cyclic molecules called cyclodextrins (CDs). CGTase is produced extracellularly by number of microorganisms, notably *B. circulans, B. stearothermophilus, B. megaterium, B. licheniformis, B. ohbensis,* and *Klebsiella pneumoniae.* CGTase hydrolyzes starch into cyclodextrins (CDS), which are cyclic, nonreducing maltooligosaccharides possessing a hydrophilic outside and hydrophobic central cavity (*1*) to encapsulate small molecules of industrial application.

The main cyclic compounds are α, β, and γ CDS consisting of six, seven, and eight glucose units, respectively. CGTases with different properties have been found in several microorganisms (*2,3*). In most organisms, the enzymes are extracellular, and may differ in properties and in the amounts or types of CD produced. However, most of the CGTases isolated so far produce mainly α/β-CD with trace amounts of γ-CD. *B. macerans* has been widely used for a large-scale production of CDS. The extracellular CGTase produced by *B. macerans* is of α-CD-specific enzyme, and in order to obtain β-CD, the effect of different cultural conditions for CGTase production has been studied (*4*). The cultural characteristics of CGTase producing alkalophilic *Bacillus circulans* in a standard shake flask cultivation have been reported (*5*).

The most extensively studied are the CGTase from bacillus species (*6*). For the overproduction of the enzyme, CGT genes from various bacteria have been cloned (*7,8*), but the protein yields reported for the recombinant *E. coli* or *B. subtilis* strains are not significantly high enough. However, information pertaining to the optimization of fermentation parameters for the maximization of enzyme synthesis is very scarce.

Our aim is to develop a strain of industrial CGTase production by using process optimization and by immobilization technicalities. In the present investigations, we have obtained an indigenous bacillus strain producing CGTase in higher amounts when compared to *B. circulans* (ATCC 21783). The enzyme synthesis during growth and differentiation has been optimized for maximal activity, and the productivity of the enzyme was enhanced using the combination of immobilization and continuous systems.

MATERIALS AND METHODS

Microorganism

The bacterial strain used was isolated from decayed potatoes and soil samples, and characterized as *Bacillus cereus* RJ 30 (NCIMB 13123) identified by NCIMB, Aberdeen. The culture was maintained on agar slants at 4°C.

Media and Growth Conditions

The medium used for growth studies was comprised of g/L: glucose 15, peptone 5, $K_2HPO_4$1; $MgSO_4 \cdot 7H_2O$ 0.2. The pH was adjusted to 7.0 prior to sterilization.

Shake Flask Cultures

One hundred milliliters of the medium were inoculated from a overnight culture and grown in a rotary shaker, agitated at 200 rpm and maintained at 37°C. The growth was followed for 72 h by measuring the optical density. Aliquots were withdrawn at regular intervals of time and analyzed for enzyme activity.

Batch Fermentation in a Fermentor

The batch studies were conducted in a laboratory fermentor of 7-L capacity (Chemap, AG, Switzerland) with a working volume of 5 L. It was equipped with all controlled systems for temperature, pH, aeration, and agitation. The medium was sterilized *in situ* for 20 min. The fermentor medium was inoculated with a preinoculum grown in conical flasks in a rotary shaker. The pH was maintained at 6.6–7.0, and the temperature at 37°C. Samples were withdrawn every hour. The enzyme activity was measured in the supernatant. Cell pellet was used for biomass measurement.

Continuous Fermentation for CGTase Production

The experiment was performed in 1.25-L capacity fermentor (Bioflow III, New Brunswick Scientific Company Inc.) with a working volume of 1 L. The fermentor vessel containing the medium was sterilized in a laboratory autoclave at 121°C, 15 psi for 20 min. The pH of the medium was adjusted prior to sterilization.

The temperature was maintained at 37°C, and the pH was monitored. The medium was inoculated with a preinoculum grown in a orbitary shaker and cultivated for 16 h in a batch mode. Thereafter the system was converted to continuous mode by supplying fresh nutrient medium by supplying fresh nutrient medium by means of a peristaltic pump, and the fermented culture was withdrawn through the outlet provided at the top section of the fermentor. The agitation, aeration, and temperature were maintained at 300 rpm, 1 vvm, and 37°C, respectively. The flow rates were altered, and at each dilution rate, the system was allowed to attain steady state, during which samples were withdrawn and analyzed for enzyme activity, biomass, and residual reducing sugars. After the evaluation at different dilution rates, cultivation was continued further at a dilution rate of 0.06 h for 400 h.

Immobilization of *B. cereus* for CGTase Production

Immobilized Bead Preparation

The bacterial cell pellet (67.5 g/L) suspended in 100 mL of 0.85% saline was mixed with 4% sodium alginate, and the slurry was made into spherical beads (*9*).

Batch Fermentation

One hundred milliliters of medium in 500-ml conical flasks were inoculated with 5% beads comprising a cell loading of 16.5 g/L. These flasks were incubated at 37°C in a water bath shaker with mild shaking. Samples were withdrawn periodically and analyzed for CGTase activity.

For repeated batches, beads were removed, reintroduced into fresh medium, and incubated as before for 24 h. The used culture medium was analyzed for the enzyme activity.

Continuous CGTase Synthesis in a Fluidized Bioreactor

The bioreactor is a jacketed glass column of 2.5 cm diameter and 36 cm long, provided with a aluminum mesh bottom for uniform distribution of air in the reactor. Temperature was maintained at 37°C by circulating water in the outer jacket through a thermostat-controlled water bath. The reactor was filled with 30 g of freshly prepared immobilized beads, and the sterile medium was introduced from the bottom of the reactor. Air was admitted in through a rotameter and sterile air filter from the bottom of the reactor. The air flow was maintained at 3–5 mL/min, which was sufficient to fluidize the beads. The eluent from the reactor was collected continuously in a sterile container. The whole reactor assembly was sterilized prior to fermentation and maintained sterile throughout. Samples were collected every 24 h and analyzed for the CGTase activity.

Analytical Methods

Optical density of the culture was measured at 620 nm in Spectrophotometer (Shimadzu UV2100). Biomass was determined gravimetrically in the cell pellet after removing the culture supernatant by centrifugation.

Protein in the culture supernatant was estimated by Lowry's method (*10*). Reducing sugar was estimated by DNS method (*11*).

Enzyme Assays

CGTase activity was measured by phenolphathelin CD complexation using 1% soluble starch at the assay conditions of 40°C at pH 5 for 20 min. The activity of the enzyme was calculated as μg of CD formed and expressed as U/mL. One unit is defined as the μg of CD produced/mL of the culture/min under the assay conditions.

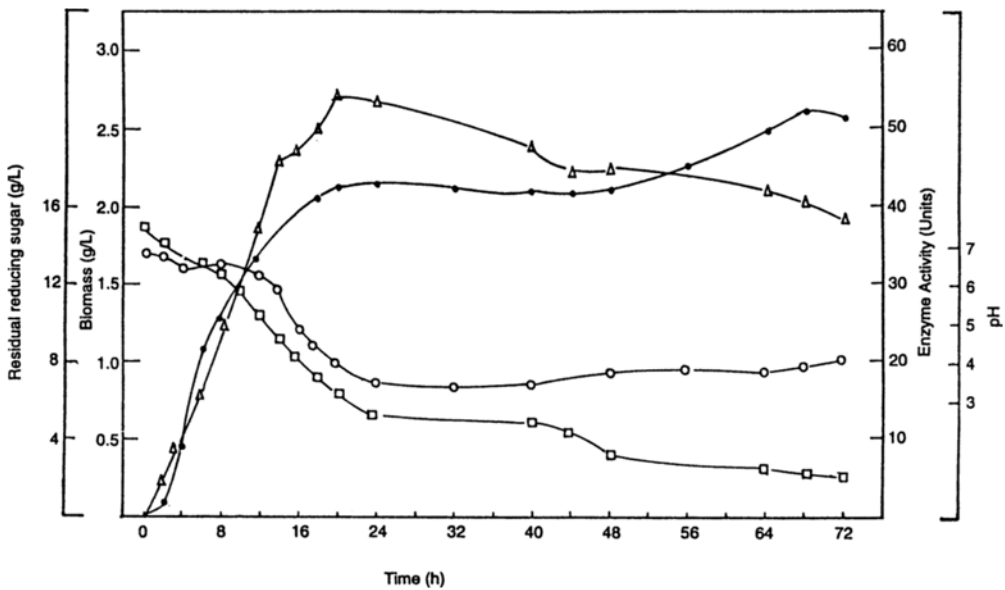

Fig. 1. Growth and CGTase synthesis of *B. cereus* in a batch culture. Symbols: ●—● biomass; □—□ residual reducing sugars; ○—○ pH; △—△ CGTase activity.

Assay for CD by HPLC

The hydrolyzates were analyzed by high-performance liquid chromatography (HPLC) (Shimadzu Corp) using a CD assay column (Astec Inc., Advanced Separation Technologies Inc.) with 20-μL portion of the filtrate. Elution was carried out with double-distilled degassed water at 1.5 mL/min flow rate. Detection was made by refractive index.

RESULTS AND DISCUSSION

Growth and CGTase Synthesis

The strain RJ-30 was selected as the best CGTase producer from a large number of bacterial isolates. It was identified as *Bacillus cereus* producing CGTase that forms both α- and β-CD. The organism was grown in the medium containing starch and glucose for 72 h. The culture characteristics, such as temperature, agitation, aeration, and pH, were varied to arrive at optimized conditions for maximal enzyme synthesis. Figure 1 shows the time-course of growth parameters of *B. cereus* and the enzyme synthesis. The lag period was followed by the logarithmic period coincident with the changes in the measured parameters. The log growth was short, lasting

for about 10–12 h. The enzyme synthesis begins from the early exponential phase, and the maximum CGTase activity as measured by dextrinizing and CD-forming activities was obtained at 16–20 h of cultivation and then on maintaining a considerably higher activity. *B. cereus* has high specific growth rate (0.7 μ) with a generation time of 1 h and appeared to synthesize CGTase at a very early period. This is unlike the reported observations with other bacillus species. *B. circulans* alkalophilic sp. exhibits cyclizing activity after 40 h of growth with a long lag period (*5*). In the case of *B. macerans,* CGTase was produced in response to exhaustion of the carbon source and continued to increase after reaching the stationary phase between 168–188 h (*12*).

Coinciding with the initiation of the substantial cell division, the pH of the culture broth decreased by 3 U. The biomass increased up to almost 2–2.5 g/L during stationary phase and remained the same thereafter. It has also been found that the cultures grown with the higher inoculum (5%) attained the peak enzyme activity much earlier in 7–9 h of growth than with that of one grown with 1%, which exhibited the maximum activity at 16–20 h. This appeared to be different from the culture characteristics of *B. circulans,* which exhibits the maximum activity at 48–50 h of growth. These results indicated that the CGTase synthesis in *B. cereus* is more growth associated, and although the change in enzyme activity was very negligible during the stationary phase, sporulation did not specifically appear to trigger the enzyme synthesis for higher activity, unlike the case of *B. circulans* (*5,13*).

The growth kinetics of *B. cereus* evaluated under various fermentation conditions in terms of maximum specific growth rate (μ max) and generation time (t_g) in correlation to enzyme synthesis is presented in Table 1. CGTase production was much lesser using starch in the growth medium grown at 300 rpm at different temperatures showing 20–30 U/mL, and the synthesis was more affected at 42°C. In the same starch medium, by altering the agitational speed in the increasing order (300–700), the enzyme activity enhanced by twofold [50 U]. The enzyme synthesis was more consistent in glucose containing medium in the range of 40–47 U. Moreover, starch medium gave problems in growth measurements in terms of optical density and biomass. Hence, further studies for CGTase synthesis have been established in glucose containing medium as a function of agitational speed, and aeration rate was established. In this case, maximum activity of 47 U/mL was obtained at 300 rpm at 1.0 vvm with 0.7 μmax. Strikingly, with the increase in the rate of aeration (0.25–2.0 vvm), the μmax of the organism gradually increased from 0.45 to 0.88. Concomitantly, the enzyme activity has also shown a corresponding increase from 42.3 to 54 U, and the highest activity was attained at 300 rpm, 1.5 vvm 37°C. (Table 1). It can be seen that 37°C was found to be the best temperature for the growth of the organism. The effect of agitational speed on CGTase synthesis has demonstrated that at 500 rpm the enzyme activity was about 20% higher than in other cases. Nevertheless, the trend of the

Table 1
Optimization of Fermentation Parameters for Growth and Enzyme Synthesis of *Bacillus cereus* RJ-30

Medium/ carbon source	Agitation, rpm	Aeration, vvm	Temperature, °C	Specific growth rate, μ	Generation time, tg	Enzyme max., U/mL	Rate of enzyme synthesis, U/mL/h
Starch	300	1.0	30	0.5	1.39	19	0.34
	300	1.0	37	0.29	2.4	30	0.58
	300	1.0	42	0.1	6.9	11	0.39
Starch	300	1.0	37	0.29	2.4	30	0.34
	500	1.0	37	0.32	2.2	50	3.1
	700	1.0	37	0.19	0.19	42	2.6
Glucose	200	1.0	37	0.11	6.6	43	2.15
	300	1.0	37	0.7	1.0	47	2.61
	500	1.0	37	0.63	1.1	42	2.62
Glucose	300	0.25	37	0.45	1.54	42.3	1.0
	300	0.5	37	0.75	0.92	42.6	2.13
	300	1.0	37	0.68	1.02	47	2.61
	300	1.5	37	0.88	0.79	53.9	2.69
	300	2.0	37	0.87	0.80	43.7	3.64

Table 2
Influence of Various Sugars on CGTase Synthesis

Sugars	CGTase activity, U/mL
Glucose	48
Fructose	47
Xylose	55
Lactose	37
Sucrose	36
Maltose	39
Galactose	41
Sorbitol	30
Manitol	28.6
Dextrin	17
Maltodextrin	26

enzyme profile was similar in all cases, and the peak of activity attained was at 16–20 h.

CGTase enzyme was found to be produced with starch as well as in glucose. It appears that the formation of CGTase does not require the presence of a specific inducer, since it occurs in the absence of starch cleavage products and in the presence of glucose and related sugar moities. However, it has been found that pentoses like xylose seem to promote the enzyme synthesis. The influence of various sugars or mono- and disaccharides on the induction of CGTase was evaluated. Table 2 shows the enzyme activity at 24 h of growth with various sugars in the medium. It is

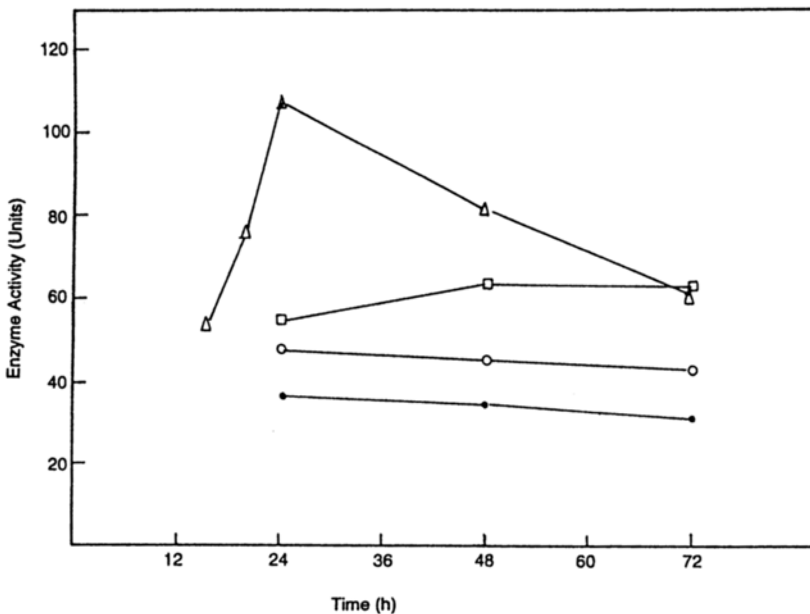

Fig. 2. Influence of carbon sources on CGTase enzyme profile. Symbols: ●—● starch; ○—○ glucose; □—□ xylose; △—△ wheat bran extract.

clearly shown that maximum activity was obtained with xylose, which is about 27% higher than that of glucose. In another set of experiments, the use of wheat bran extract (WBE) has resulted in a remarkably high activity (106 U/mL). Wheat bran extract is known to contain certain sugars, including xylose, and with the results obtained in Table 2, it can be stated that xylose could possibly be an inducer for the CGTase synthesis, which was also reflected using wheat bran extract (WBE), although the activity obtained using wheat bran extract was almost double. This remarkable increase in activity using WBE could be attributed to the presence of other trace essential amino acids and vitamins and certain other chemicals that could have stimulated the synthesis of the enzyme. Figure 2 represents the enhanced activity profile using saccharides and WBE in comparison to the starch medium. These results are in contradiction to the reported observations on the effect of various carbohydrates on CGTase production in *B. lentus* (*13*), where the liquefying and CD-forming activities were nil using sugars.

Continuous CGTase Synthesis in a Stirred Tank Reactor

To ascertain further the growth behavior and enzyme synthesis pattern of the culture, continuous fermentation in stirred tank reactor has been attempted. The 16-h batch culture in a laboratory fermentor has been switched over to continuous mode by introducing fresh medium at a low dilution rate of 0.06 h, operated continuously for over 400 h. The optical density of the culture was maintained between 2-2.5 g/L. The pH of the

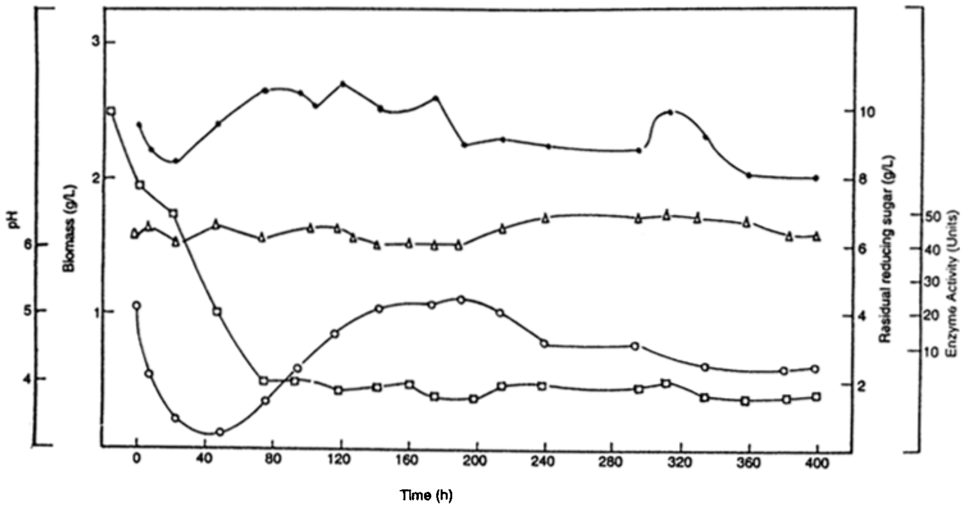

Fig. 3. Continuous synthesis of CGTase by free cells of *B. cereus* in stirred tank reactor. Symbols: ●—● biomass; ○—○ pH; □—□ residual reducing sugar; △—△ CGTase activity.

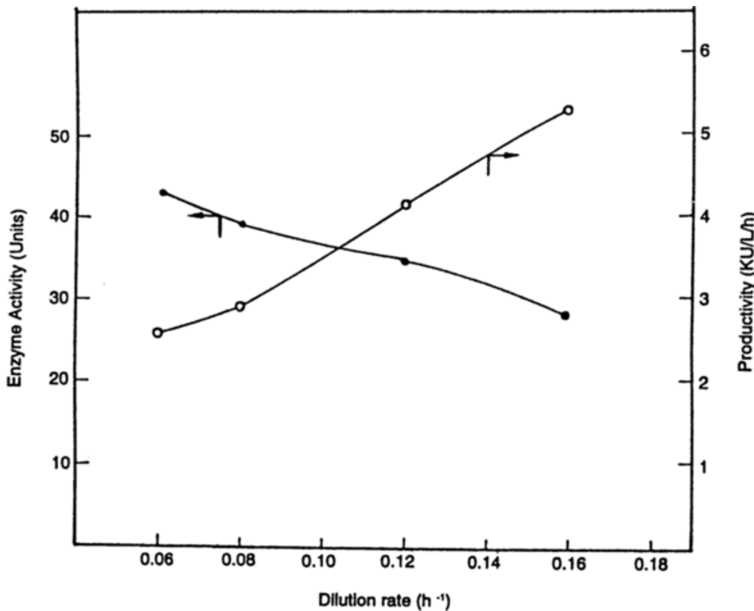

Fig. 4. CGTase productivity and enzyme activity at different dilution rates. Symbols: ●—● enzyme activity; ○—○ enzyme productivity.

culture broth showed a variation by more than 1 U. Apparently, the CGTase activity has been stably maintained ranging between 42–49 U/mL throughout the operation. CGTase synthesis in a continuous culturing and the concomitant changes in the growth parameters are presented in Fig. 3. Figure 4 represents the enzyme activity and the productivity in relation to the dilution rate. It can be seen that the enzyme activity decreases from 45

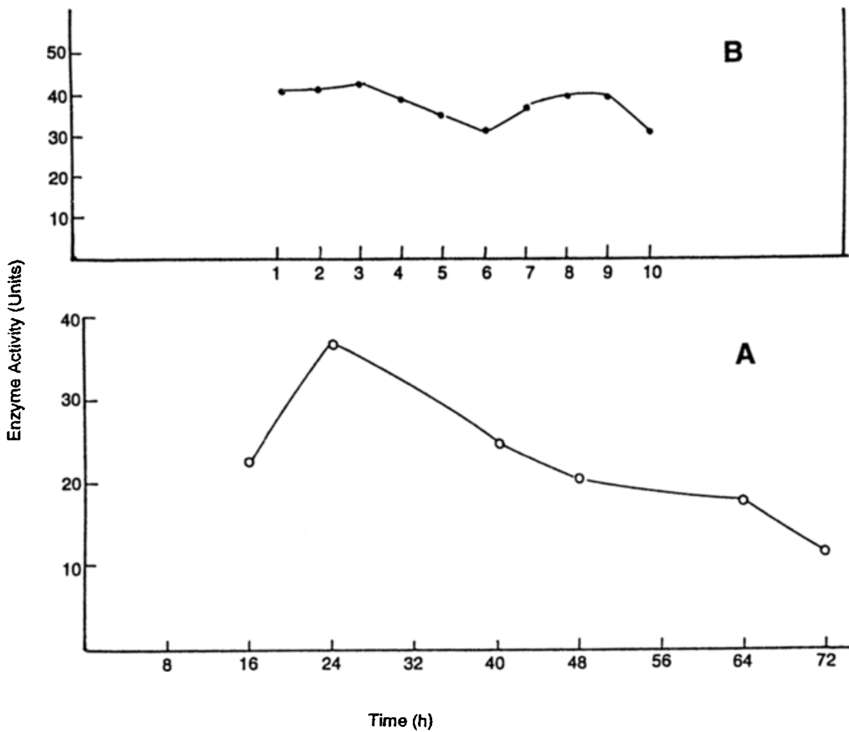

Fig. 5. Synthesis of CGTase enzyme by immobilized cells of *B. cereus*. (A) enzyme activity at different periods of growth. (B) CGTase activity during batch repeated cycles.

to 25 as the dilution rate increased from 0.06 to 0.16 h. On the contrary, the productivity increased linearly with increase in dilution rate. From the curve (Fig. 4), maximum productivity was found to be 5.4 kU/L/h, at which the enzyme activity was 27.5 U/mL, although the optimum productivity was about 3.6 kU/l/h with 35 U/mL of enzyme activity. Investigations on the continuous cultivation for CGTase synthesis are not reported elsewhere so far, but the continuous cultivation for a similar extracellular enzyme, α-amylase, reported elsewhere (*14*) indicates that the volumetric productivity achieved was in the range of 7 kU/l/h and appeared to be correlated to our present values, although our reported volumetric productivity for α-amylase was 20 kU/l/h (*9*).

CGTase Synthesis by Immobilized *B. cereus*

Batch Fermentation Studies

The log phase culture was immobilized in alginate matrix and prepared into spherical beads. The cell concentration in the beads and inoculum size were optimized for the maximal CGTase synthesis. Figure 5 shows

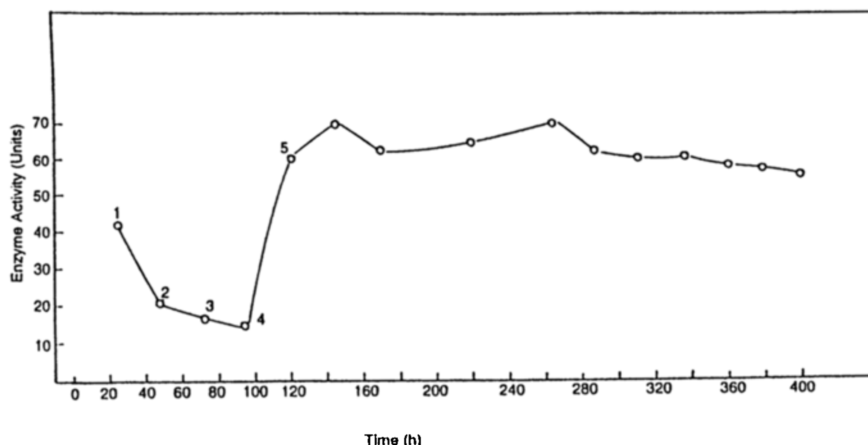

Fig. 6. Continuous synthesis of CGTase by immobilized cells of *B. cereus* in a fluidized bed reactor. The reactor was operated at different dilution rates for the initial 120 h. The numbers 1–5 denote the change in the dilution rates. 1-D 0.49; 2-D 0.61; 3-D 0.80; 4-D 1.0; 5-D 0.32.

the enzyme activity profile using immobilized beads. The activity appeared to peak up to 40 U at 24 h, after which it dropped sharply. This sharp decrease after 24 h was observed at higher cell concentrations (16 g/L), whereas at low cell concentrations (4.0 and 8.0 g/L), the activity was more stable, although a slight decline in 72 h was noticed. The sharp decline in activity after 24 h may be owing to faster depletion of nutritional components because of high cell density in beads.

In order to substantiate the fact that the stringent nutrient supply leads to the lowering of the enzyme synthesis, using the beads repeatedly with the replacement of fresh medium every 24 h has been attempted. The enzyme activity remained between 30–40 U/mL, and the results of 10 repeated cycles are shown in Fig. 5B. It has been shown that the immobilized cells were able to synthesize CGTase consistently and could be used for continuous fermentation. There are not any reports available on the aspect of immobilized whole cells for CGTase synthesis, and most of the methods for industrial production of cyclodextrins involve batch-type processes.

Continuous Synthesis of CGTase in a Fluidized Bed Bioreactor

Continuous synthesis of CGTase was accomplished in a fluidized bed reactor comprised of 30 g of freshly prepared cell immobilized beads. Fresh medium was fed into the reactor at different dilution rates of 0.4–0.93 h^{-1}. At each flow rate, the reactor was operated for 6–12 h to ensure steady state. The bioreactor operated with gel beads exhibited the activity between 60–68 U/mL with a maximum productivity of 23 kU/L/h. Figure 6 shows

Table 3
Effect of Different Dilution Rates on the CGTase Synthesis in a Fluidized Bed Reactor Using Cell Immobilized Beads

Dilution rate, h^{-1}	Enzyme activity, U/mL	Enzyme productivity, kU/L/h
0.4	56	19.2
0.5	46	22.4
0.6	20	12
0.8	14.8	8.9
0.9	10	9.0

the continuous CGTase synthesis in a fluidized bed reactor using immobilized cells for about 360 h with a productivity of 23 kU/L/h. In the initial 120 h, the reactor was operated with different dilution rates and then continued on at the dilution rate of 0.32 h^{-1}. The productivities obtained at different dilution rates are presented in Table 3. The activity remained in the range of 60 U/mL throughout the operation of the reactor, apparently indicating that the half-lifes of the immobilized cells were higher than 15 d.

Although there is no system reported for immobilized whole cells for continuous CGTase synthesis, a similar system using immobilized CGTase on a silica-based support has been used for continuous production of CD (*15*). It has been observed that the actual half-lives are likely in excess of the approx 50-d duration. However, <50% of the activity had been lost by the termination of the study. Immobilization of CGTase by an ion-exchange resin (*16*) and on synthetic adsorption resin DIAION HP-20 has also been reported (*17*).

These results have demonstrated the feasibility of continuous synthesis of CGTase by free cells in a chemostat culture or by immobilized cells in a fluidized bed reactor. Table 4 represents productivity of enzyme achieved in various systems using free as well as immobilized cells. In terms of enzyme activity, the highest was achieved using immobilized cells in continuous mode, which is about 10–40% higher than that of free cells in batch and in continuous, respectively. Nevertheless, in terms of productivity, immobilized beads under continuous operation resulted in 23 kU/L/h, which is 5–13 times higher than the other systems.

Cyclodextrin Formation

CGTase from *B. cereus* has the temperature optima and pH optima at 40°C and 5.0, respectively. The enzyme reaction under the assay conditions resulted in 90% of starch conversion (1% starch) in which 52% was α and 38% was β. As the starch concentration increased to 5%, the yield of total CD reduced 45%, in which 22% were α and 23% were β, indicating the formation of the product more toward β.

Table 4
Comparison of the CGTase Activity
and the Productivity in Various Systems

Systems	Condition mode	CGTase activity, U/mL	Enzyme productivity, kU/L/h	Fold increase productivity
Free cells	Batch	41.6	1.72	–
	Continuous	28	5.3	3.1
Immobilized cells	Batch	47	1.3	–
	Repeated batch	41	1.1	–
	Continuous	46	23	13.4

CONCLUSIONS

The results have shown the optimized parameters for the maximization of CGTase enzyme from *B. cereus* and the ability of the organism to synthesize CGTase continuously both with free cells and with immobilized cells in a fluidized bed reactor. The CGTase formed converted 90% of starch to cyclodextrin molecules as measured by high-performance liquid chromatography.

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