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Abstract

The immobilized cells of five bacterial cultures on different carriers were investigated for the production of cyclodextrin glucosyltransferase (CGTase). The entrapped cells of *Bacillus amyloliquefaciens* in calcium alginate showed the highest enzyme activity (70.8 U ml^{-1}). The enzyme production with respect to alginate concentration, bead diameter, and maximal cell loading in the immobilization matrix was optimized. In repeated batch fermentation, the immobilized cells retained their ability to produce CGTase consistently over 14 cycles and the activity remain between 70 and 88 U ml^{-1} throughout the cycles. Continuous culture was investigated in packed-bed and fluidized-bed reactors. In packed-bed reactor, maximal productivity (23 KU l^{-1} h^{-1}) with enzyme concentration of 48 U ml^{-1} and specific productivity of 141.8 U g wet $cells^{-1}$ h^{-1} was attained at a dilution of 0.48 h^{-1}. Continuous production in fluidized-bed reactor showed maximal productivity (30.4 KU l^{-1} h^{-1}) with enzyme concentration of 53.0 U ml^{-1} and specific productivity of 230.9 U g wet $cells^{-1}$ h^{-1} at a relatively high dilution rate of 0.57 h^{-1}. © 2000 Elsevier Science S.A. All rights reserved.

Keywords: Cyclodextrin glucosyltransferase; Production; Immobilized cells; Continuous

1. Introduction

Bacterial cyclodextrin glucosyltransferase (CGTase) (EC 2.4.1.19) represent one of the most important groups of microbial amylolytic enzymes [1]. They are widely known to catalyze the formation of cyclic, non-reducing malto-oligosaccharides from starch consisting of six (α-cyclodextrin, α-CD), seven (β-CD), and eight (γ-CD) 1,4-α-D-linked glucopyranose residues. These cyclic products can form versatile inclusion complexes with many organic and inorganic compounds, of which property is of paramount importance, especially concerning the widespread applications of CDs in the pharmaceutical, food, and chemical industry [2,3].

Immobilized cell systems have been applied for many biochemical processes and have been reviewed several times [4,5]. The application of the immobilized whole cells for biochemical processes offers many advantages, such as the ability to separate cell mass from the bulk liquid for possible reuse, facilitating continuous operation over a prolonged period and enhanced reactor productivity.

The production of amylolytic enzymes by immobilized cells have been reported by many investigators [6–9] However, such reports on the production of CGTase by immobilized cells are rather low [10].

This paper reports the experimental results for CGTase production by immobilized *Bacillus amyloliquefaciens*. We have also evaluated the using of the immobilized cells of *Bacillus amyloliquefaciens* for CGTase production in repeated batch and continuous culture with respect to the yield, productivity, and long-time operational stability.

2. Materials and methods

2.1. Microorganisms

The bacterial strains used in the present work were obtained from the Center of Cultures of the National Research Center, Cairo, Egypt. They were maintained on potato dextrose agar slants at 4°C.

2.2. Culture media and growth conditions

The medium used for the cell biomass production for the cell immobilization was composed of (g l^{-1}) glucose, 10; starch, 10; yeast extract, 3.0; peptone, 6.0; meat extract, 1.5. The pH was adjusted to 6.5 prior to the sterilization. The

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medium was inoculated from 24 h old slants and was grown at 37°C. The cultures were grown in 250 ml Erlenmeyer flasks each containing 50 ml of sterile medium. Aliquots were drawn periodically to assess the growth. The cells obtained from the logarithmic phase of growth were used for the immobilization experiments.

Potato dextrose (PD) medium was used for enzyme production in both batch and continuous cultures. The medium had the following composition ($g\,l^{-1}$): potato slices, 400; glucose, 20. The pH was adjusted to 6.5.

2.3. Immobilization procedures

All the immobilization processes were performed under aseptic conditions. In separate experiments, the cell pellets obtained from each culture (in the logarithmic phase of growth) were collected by centrifugation (5000 rpm, 15 min) in a refrigerated centrifuge. Then, the wet cell pellets were suspended in 0.85% sterile saline and used for the cell immobilization experiments.

2.3.1. Immobilization in Ca-alginate

Unless otherwise stated, the wet cell pellets obtained from 50 ml culture of each organism were mixed with 10 ml of sodium alginate solution (BDH, 30105), in separate experiments. The final sodium alginate concentration was kept at 4% (w/v). The beads (the mean diameter 3 mm) were obtained by dropping mixtures into sterile $CaCl_2$ (0.05 M). The beads obtained from 10 ml gel were used for inoculation of 50 ml of the PD medium.

2.3.2. Immobilization in agar

The wet cell pellets obtained from 50 ml culture of each organism were mixed with 10 ml of 3% (w/v) agar solution at 45°C. The mixture was quickly cooled to 4°C, cut into 2 mm × 2 mm × 2 mm fragments, and transferred to 50 ml of PD medium [11].

2.3.3. Immobilization in polyacrylamide

The wet cell pellets obtained from 50 ml culture of each organism were mixed with 10 ml of 5% (w/v) acrylamide solution with 3% (w/w) cross-linker (N,N-methylene bisacrylamide) concentration [12]. The gel obtained was cut into 2 mm × 2 mm × 2 mm fragments and transferred to 50 ml of PD medium.

2.4. Batch experiments

Unless otherwise indicated, the batch experiments were performed in 250 ml Erlenmeyer flasks each containing 50 ml of PD medium. The flasks were inoculated with the beads obtained from 10 ml gel with the calculated amounts of the immobilized cells. Parallel experiments were carried out with equal amounts of free cells. The cultures were incubated for 48 h in a rotary shaker (120 rpm) at 37°C. All the experiments were carried out in triplicates.

2.5. Repeated batch experiments

This was done in 250 ml Erlenmeyer flasks each containing 50 ml of PD medium. Each flask was inoculated with the beads obtained from 10 ml alginate gel comprising a cell loading of 1.08 g wet cells $50\,ml^{-1}$ culture. Fermentation was conducted at 37°C for 24 h under shaking conditions (120 rpm). At the end of each run, the gel particles were filtered and washed with 25 ml of 0.05 M $CaCl_2$ and distilled water and transferred to 50 ml of fresh medium. All the experiments were carried out in triplicates.

2.6. Continuous fermentation

2.6.1. In packed-bed reactor

A glass column 30 cm in length and 1.5 cm in diameter was used for continuous production of CGTase The bioreactor was incubated at 30°C. The column was packed with 56 g of cell-immobilized beads (average diameter 3 mm) comprising about 20.6 g of wet weight cells (viable cell count = 7.13×10^9 per g wet cells). The void volume was 125 ml. The medium was fed through the bottom by a peristaltic pump with a flow rate regulator. Aeration was provided by means of an air filter. The air flow was optimized at 0.3 v/v/min. The effluent was collected in a holding tank. The schematic representation of the bioreactor is illustrated in Fig. 1.

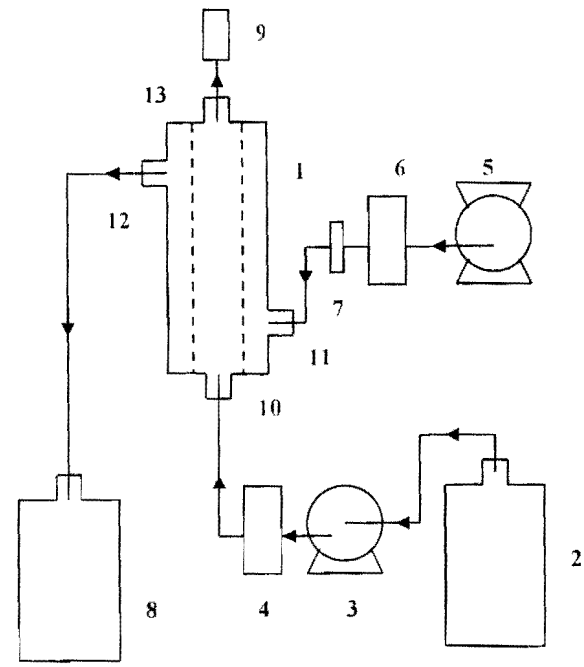

Fig. 1. Schematic diagram of the continuous fermentation system. (1) Bioreactor with the immobilized beads, (2) medium feed reservoir, (3) peristaltic pump, (4) flow rate regulator, (5) air pump, (6) rotameter, (7) air filter, (8) product collection vessel, (9) outlet air filter (10) medium inlet, (11) air inlet, (12) product outlet, (13) air outlet.

2.6.2. In fluidized-bed bioreactor

The bioreactor is a glass column of 2.0 cm diameter and 32 cm long. The bioreactor was incubated at 30°C. The reactor was filled with 115 g of freshly prepared immobilized beads comprising 32.34 g wet weight cells (viable cell count = 7.13×10^9 per g wet cells). The fresh 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 optimized at 0.33 v/v/min, which was sufficient to fluidize the bed. The effluent from the reactor was collected in a holding tank. The schematic representation of the bioreactor is similar to that of packed-bed which illustrated in Fig. 1.

2.7. Analytical methods

Optical density of cell growth was measured using spectrophotometer (Spectronic 2000, Bausch and Lomb) at 620 nm. Biomass was determined gravimetrically in the cell pellets after removing the culture supernatants by centrifugation. Viable-cell number was counted on 1.5% nutrient agar plates. Reducing sugars were determined using the method of Somogyi [13]. Protein was estimated by the method of Lowry et al [14].

2.8. Enzyme assays

CGTase was determined by the method reported by Nogrady et al [15]. The reaction mixture containing 40 mg of water-soluble starch (Sigma) in 1.0 ml 50 mM phosphate buffer (pH 6.5) and 0.1 ml of the enzyme solution was incubated at 60°C for 20 min. The reaction was stopped by the addition of 3.5 ml of 30 mM NaOH solution, and then 0.5 ml 0.02% (w/v) phenolphthalein solution prepared in 5 mM Na_2CO_3 was added. After incubating for 15 min at room temperature, the abasorbance at 550 nm was read. One unit of the enzyme activity (U) is defined as the amount of the enzyme forming one μg of CD per min under the assay conditions.

Amylase activity was determined according to the Bergmann et al. [16] by estimating the released reducing sugars from 1.0% saline starch in 0.2 M phosphate buffer (pH 6.5). One unit of the enzyme activity (U) is defined as the amount of the enzyme forming 1 μmol of reducing sugars (as glucose) per min under the assay conditions.

3. Results and discussion

3.1. Screening of immobilized microorganisms suitable for CGTase production

The suitability for production of CGTase activity by the immobilized cells of five bacterial cultures in different immobilization matrices was investigated in submerged culture. In another set of experiments, the same amount of free cells from each culture was inoculated along with the same amount of the immobilized cells. The results (Table 1) indicated that, in all cases the activity of the immobilized cells was lower than the corresponding amount of free cells. The effectiveness factor of the immobilized cells, which is the ratio of the enzyme activity of the immobilized cells to

Table 1
Production of CGTsae enzymes by free and immobilized bacterial cultures

Bacterial strain	Matrix of immobilization	Biomass loaded (g wet cells 50 ml culture^{-1})	Protein content (mg ml^{-1})	CGTase activity (U ml^{-1})	Specific productivity (U g wet cells^{-1} h^{-1})	Effectiveness factor[a]
Bacillus amyloliquefaciens 312	free cells	0.77	3.56	88.50	119.70	–
	agar	0.77	1.37	67.06	90.70	0.74
	Ca-alginate	0.77	1.63	70.80	95.78	0.80
	polyacrylamide	0.77	1.72	67.80	91.72	0.76
Bacillus macerans 314	free cells	0.92	3.29	79.38	89.87	–
	agar	0.92	2.02	60.74	68.77	0.50
	Ca-alginate	0.92	1.16	63.76	72.19	0.77
	polyacrylamide	0.92	1.71	67.75	76.71	0.77
Bacillus macerans 3185	free cells	0.95	4.40	86.49	94.83	–
	agar	0.95	2.37	60.74	66.60	0.44
	Ca-alginate	0.95	1.41	65.75	72.10	0.76
	polyacrylamide	0.95	1.82	63.21	69.30	0.73
Bacillus macerans 3168	free cells	1.23	3.75	78.40	66.39	–
	agar	1.23	1.98	61.32	51.93	0.56
	Ca-alginate	1.23	0.87	60.56	51.28	0.77
	polyacrylamide	1.23	1.69	60.19	50.97	0.76
Bacillus megaterium NRC 4	free cells	0.9	3.05	78.61	91.00	–
	agar	0.9	1.88	55.27	63.97	0.42
	Ca-alginate	0.9	1.78	58.70	67.94	0.74
	polyacrylamide	0.9	1.90	59.23	68.55	0.75

[a] The ratio of the enzyme activity of the immobilized cells to that of the same amount of free cells.

that of the same amount of free cells under identical conditions, was in the order of 0.5–0.8. These values are similar to those reported elsewhere for other immobilized bacterial amylases [6,7]. The effectiveness factor of the immobilized cells would always be less than one because the immobilized cells represent a heterogeneous catalysis fermentation in which the activity, or rather synthesis, of primary or secondary metabolites is dependent upon the external and internal mass transport and adequate oxygen supply [7].

Of all preparations, the cells of *Bacillus amyloliquefaciens* entrapped in Ca-alginate showed the highest CGTase activity (70.8 U ml^{-1}) and the highest specific productivity (95.78 U g wet cells h^{-1}). This strain produces CGTase that forms β-CD. Thus, it was used throughout this study.

3.2. Optimum conditions for immobilization of Bacillus amyloliquefaciens in Ca-alginate

Different concentrations of sodium alginate (2–5%, w/v) were investigated for the immobilization process. In all cases, a constant amount of cells was used (equivalent to 0.77 g wet cell pellets per 10 ml gel, contained viable cell count of 5.5×10^{10} cells). Inoculation of 50 ml PD medium was performed with the beads resulted from 10 ml Na-alginate solution. The results recorded in Table 2 indicated that beads prepared from 2% (w/v) alginate concentration were much softer and showed the highest number of leaked cells (11.3%) and therefore, they were excluded. On the other hand the enzyme yield of the beads made of 5% (w/v) was 84.56% of that recorded for the beads prepared from 3% (w/v) alginate concentration. This is most likely because the resulting beads had a lower diffusion efficiency. Maximal enzyme yield (78.4 U ml^{-1}) was obtained at 3% (w/v) alginate concentration. This concentration was recommended for the production of α-amylase [7] and CGTase [10] by immobilized bacterial cells.

Using the same conditions from the previous experiment, the effect of bead diameter was investigated using 3% (w/v) alginate concentration. The alginate solution was made into beads of different diameters (2–6 mm). The results (Table 3) indicated that the beads of smaller diameter (2 mm) showed the highest number of leaked cells (11.3%). In fact, the bacterial cells grown preferentially near the bead surface, are continuously released out in the culture medium. It is worthy to note that the surface area of the beads resulted from certain volume of alginate gel increased with the decrease of the beads diameter. Therefore, the number of the leaked cells increased with the decrease of beads diameter. Similar observations were previously reported for the immobilized cells of *Lactococcus lactis* in alginate beads [18]. On the other hand, the beads of diameter higher than 5–6 mm showed a lower CGTase yield (63.28 U ml^{-1}). The highest enzyme yield (78.18 U ml^{-1}) was obtained with bead diameter of 3–4 mm.

The effect of cell loading on CGTase production was investigated. In separate experiments, 10 ml of alginate solutions (3%, w/v) with different cell contents (0.77–1.4 g wet cell pellets) was made into beads (3–4 mm in diameter) and inoculated into 50 ml of PD medium. The results (Table 4) indicated that there was a marginal increase in the activity with the increase of cell loading up to 1.08 g wet cell pellets, whereby maximal enzyme yield was attained (109 U ml^{-1}). Further increase of cell loading, however, did not significantly increase the enzyme yield. On

Table 2
Effect of alginate concentration on the production of CGTase by the immobilize cells of *B. amyloliquefaciens*

Alginate concentration (%)	Protein content of culture filtrate (mg ml^{-1})	CGTase activity (U ml^{-1})	Amylase activity (U ml^{-1})	Leaked cells (%)[a]	Specific CGTase productivity (U g wet $cells^{-1}$ h^{-1})
2	1.94	79.8	8.4	11.0	103.63
3	1.72	78.4	5.6	3.5	101.80
4	1.37	70.8	4.5	2.0	91.94
5	0.84	66.3	3.85	2.0	86.10

[a] This was calculated from the original concentration of cells in the beads resulted from 10 ml alginate gel (0.77 g wet cells, 5.5×10^{10} cells).

Table 3
Effect of bead diameter on the productivity of CGTase by the immobilized cells of *B. amyloliquefaciens*

Bead diameter (mm)	Protein content of culture filtrate (mg ml^{-1})	CGTase activity (U ml^{-1})	Amylase activity (U ml^{-1})	Leaked cells (%)[a]	Specific CGTase productivity (U g wet $cells^{-1}$ h^{-1})
2	2.10	80.00	6.91	11.3	103.90
3–4	1.72	78.18	5.60	3.60	101.53
5–6	1.33	63.28	4.37	2.50	82.18

[a] This was calculated from the original concentration of cells in the beads.

Table 4
Effect of cell loading on the production of CGTase by the immobilized cells of *B. amyloliquefaciens*

Biomass concentration		Protein content of culture filtrate (mg ml^{-1})	CGTase activity (U ml^{-1})	Amylase activity (U mg^{-1})	Specific CGTase productivity (U g wet $cells^{-1}$ h^{-1})
(g wet cells 10 ml of alginate gel^{-1})	Total viable cell count				
0.77	5.5×10^{10}	1.72	78.46	5.50	101.90
0.92	6.6×10^{10}	1.77	92.30	6.20	100.32
1.08	7.7×10^{10}	1.90	109.0	6.94	100.0
1.23	8.8×10^{10}	2.10	110.0	7.24	81.30
1.40	9.9×10^{10}	2.50	109.0	7.70	77.85

the other hand, the specific CGTase productivity was constant at a level of 101.9–100 U g wet $cells^{-1}$ h^{-1} up to a cell loading of 1.08 g wet cells 10 ml alginate gel^{-1} but decreased to 81% of this activity at a loading level of 1.23 g wet cells 10 ml gel^{-1} (Table 4). Similar results were reported for other entrapped cells in Ca-alginate [7,11]. Cheetham et al. [11] reported that at very high cell concentration, the beads were actually less active because the porous structure of the beads was lost.

3.3. Culture conditions for maximization of CGTase production by Bacillus amyloliquefaciens

Using the optimum conditions reached from the previous experiments (3%,w/v, alginate concentration, 3–4 mm bead diameter, and cell loading of 1.08 g wet cell pellets per 10 ml gel), the effect of some cultural conditions for CGTase production was investigated. The effect of pH of the culture medium was investigated for the production of CGTase over pH range 5.0–7.5. Maximal enzyme activity (108 U ml^{-1}) was attained at pH 6.5. This results are similar to that reported by Lee et al. [17].

On equivalent carbon basis, the glucose in the PD medium was substituted with different carbon sources (i.e., galactose, maltose, fructose, lactose, sucrose, sorbose and xylose). The results (Table 5) indicated that CGTase occurred with all the investigated sugars and the highest activity was obtained with glucose (110 U ml^{-1}). Similar observations were explicitly reported by Ismail et al. [20] and Nogrady et al. [15] using the free cells of *B. macerans*. Jamuna et al. [10] reported that the formation of CGTase did not require the presence of specific inducer, since it occurred in absence of starch and starch cleavage products, and in the presence of glucose and related sugar moieties. The effect of glucose concentration was also investigated and the results indicated that maximal CGTase activity was attained at 2% (w/v).

On equivalent nitrogen basis (1.0 g N l^{-1}), addition of organic nitrogen sources (i.e., casein, peptone, yeast extract, soybean, meat extract, milk whey) to the culture medium (Table 6) did not improve the enzyme yield (78–99.0 U ml^{-1}). Allison and Macfarlane [19] reported on the fall in the enzyme yield with the use of some organic complex nitrogen sources. They referred that to the feed back inhibition mechanism resulting form the presence of certain amino acids repressed the enzyme biosynthesis. On the other hand the use of inorganic nitrogen source (i.e., $(NH4)_2SO_4$, $NaNO_3$) showed the lowest levels of CGTase yield (56.9–70.2 U ml^{-1}).

Addition of wheat bran (5–10 g l^{-1}, w/v) or its extract had no effect on the enzyme yield (108 U ml^{-1}). These results are in contradiction to those reported by Jamuna et al.

Table 5
Influence of various sugars on CGTase biosynthesis

Sugars	Final pH of the culture medium	Protein content (mg ml^{-1})	CGTase activity (U ml^{-1})	Amylase activity (U ml^{-1})
None	6.29	1.04	53.35	1.66
Glucose (%, w/v)				
0.5	7.37	1.16	87.70	2.25
1.0	6.85	1.44	96.70	2.92
1.5	6.59	1.55	102.20	4.33
2.0	6.31	1.63	110.13	5.61
2.5	6.17	1.69	106.30	8.97
Galactose	5.04	1.20	83.90	3.82
Fructose	5.07	1.68	64.44	3.73
Maltose	5.41	1.23	63.00	4.77
Lactose	5.70	1.62	67.50	4.93
Sucrose	5.73	1.38	65.50	3.97
Sorbose	5.76	1.40	87.40	3.65
Xylose	5.90	1.54	80.43	2.88

Table 6
Influences of various nitrogen sources on CGTase biosynthesis

Nitrogen source added[a]	Final pH of the culture	Protein content of culture filtrate (mg ml^{-1})	CGTase activity (U ml^{-1})	Amylase activity (U ml^{-1})
None	6.31	1.63	110.13	5.61
Casein	7.86	3.25	96.09	3.77
Peptone	3.51	3.51	99.80	2.05
Yeast extract	8.21	4.13	90.26	4.42
Soybean	7.94	3.85	89.40	3.00
Meat extract	7.70	4.70	78.00	4.39
Milk whey	7.28	3.51	87.27	4.10
$NaNO_3$	7.51	3.80	70.38	4.00
$(NH_4)_2SO_4$	7.72	3.18	56.9	4.50

[a] Final nitrogen concentration 1.0 g N/l.

[10] concerning on the stimulating effect of wheat bran for CGTase biosynthesis. The aforenamed author attributed the positive action of wheat bran for CGTase formation to the presence of other trace essential amino acids and vitamins.

The addition of $CoCl_2$, $ZnSO_4$ and $MgCl_2$ (0.1, w/v) to the culture medium showed about 20, 27, and 5% drop on CGTase activity, respectively. On the other hand, addition of $CaCl_2$ at 0.15% (w/v) showed about 9% increase of enzyme yield (119 U ml^{-1}). Higher level (0.4%, w/v), however, showed about 11.8% drop in the enzyme activity. The positive effect of Ca^{2+} ions may be due to the stabilizing effect of the alginate beads. These results do coincide with those reported by Ismail et al. [20] and Jamuna et al. [10] for CGTase production by free and immobilized cells, respectively.

3.4. Repeated batch operation with immobilized cells

The activity of immobilized *Bacillus amyloliquefaciens* cells for the production of CGTase continuously was explored by using the cell-immobilized beads, respectively, for several batches. The medium was replaced every 24 h and the beads were washed thoroughly with 0.05 M $CaCl_2$, and distilled water at the end of each cycle before reuse. The results (Fig. 2) show the activity of 14 cycles. The activity

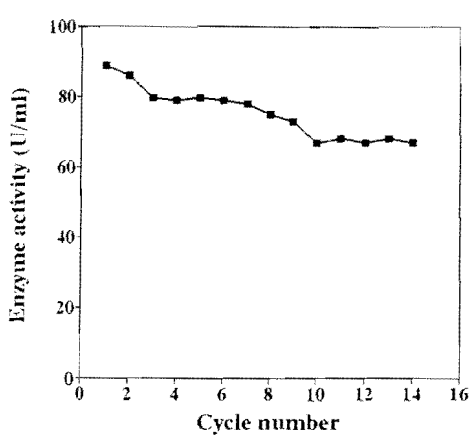

Fig. 2. Repeated use of the immobilized cells of *Bacillus amyloliquefaciens* for CGTase production.

of the immobilized cells remain between 69.4 and 88.8 U ml^{-1} throughout the cycles. These results are similar to those reported by Jamuna and Ramakrishna [7] for the production of extracellular α-amylase by the immobilized cells of *Bacillus* sp. in repeated batch fermentation. On the other hand, the enzyme activity of alginate-entrapped cells of *Bacillus amyloliquefaciens* (69.4–88.8 U ml^{-1}) was higher than those reported by Jamuna et al. [10] for *Bacillus cereus* for CGTase production in repeated batch fermentation (30–40 U ml^{-1}). Furthermore, the specific CGTase biosynthesis in terms of U g wet $cells^{-1}$ h^{-1} of *B. amyloliquefaciens* was about 133.8–171.3 (U g wet $cells^{-1}$ h^{-1}), which is about 64.7–71.5% higher than those reported by Jamuna et al. [10] for *Bacillus cereus* (78–104 U g wet $cells^{-1}$ h^{-1}).

3.5. Continuous production of CGTase in packed-bed bioreactor

The continuous production of CGTase was accomplished in a packed-bed reactor (Fig. 1), using the optimized medium. The fermentation was carried out in a batch operation, initially for 24 h, then continuous operation was started. The flow rate was varied between 6.25 and 90.0 ml h^{-1}. The dilution rate was found to be in the range of 0.05–0.72 h^{-1}, corresponding to 20–1.39 h residence time (inverse of the dilution rate). The aeration rate was investigated and maximal enzyme yield was obtained at the rate of 0.33 (v/v/min). Higher rates, however, did not significantly affect the enzyme yield. The system was considered to be in a steady state only, after at least five replacement volumes (residence times). The samples were collected at each dilution rate at the steady state conditions. The results of CGTase activity, and reactor productivity at different dilution rates are shown in Fig. 3. After scanning the whole range of the dilution rate, it was noticed that maximum activity occurred at low dilution rate (or higher residence time) and gradually decreased as the dilution rate increased. This trend was expected, since the contact time between the medium and the immobilized cells decreased as the dilution rate increased. Maximal enzyme activity in the effluent (92.1 U ml^{-1}) was attained at a dilution rate of

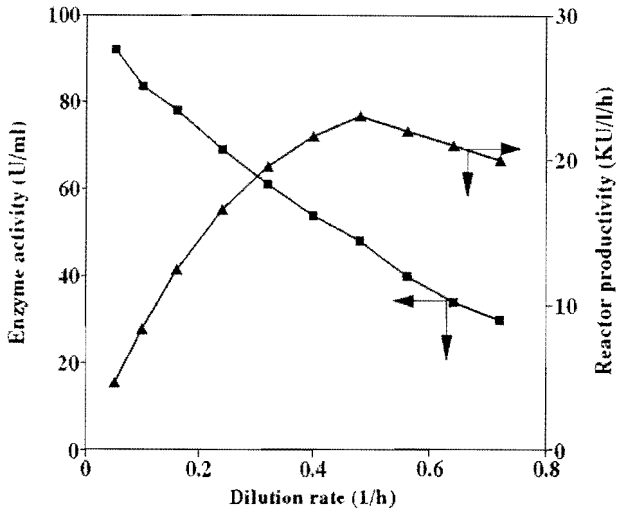

Fig. 3. Enzyme activity and CGTase productivity at different dilution rates in a packed-bed reactor.

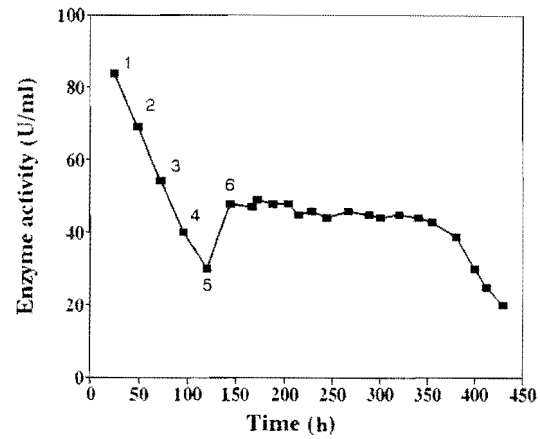

Fig. 4. Continuous synthesis of CGTase by immobilized cells of *Bacillus amyloliquefaciens* in a packed-bed reactor. The reactor was operated at different dilution rates for initial 144 h. The numbers 1–6 denote the changes on the dilution rates (D). 1-D = 0.1, 2-D = 0.24, 3-D = 0.4, 4-D = 0.56, 5-D = 0.72 and 6-D = 0.48.

0.052 h^{-1}. The enzyme productivity (calculated by multiplying the dilution rate × enzyme activity) which represents that efficiency of the system was increased with increasing of the dilution rate up to 0.48 h^{-1} and decreased thereafter. This is due to the fact that the total amount of the enzyme output from the reactor increases although the activity is lower, because of the high flow rate. This implies that in operating a continuous reactor, there may be an optimum dilution rate at which maximum enzyme productivity was attained. In general, a similar behavior was reported for continuous production of α-amylase [7] and isomalto-oligosaccharides [21] by immobilized cells in packed-bed reactors. Maximal productivity (23 KU l^{-1} h^{-1}) with enzyme concentration of 48 ml^{-1} and specific productivity of 141.8 U g wet $cells^{-1}$ h^{-1} was attained at a dilution of 0.48 h^{-1}. In the initial 144 h the reactor was operated with different dilution rates and then switched on at the dilution rate which showed maximal productivity (0.48 h^{-1}). The reactor was able to keep producing CGTase activity, in the effluent of 48 U ml^{-1}, for about 10 days at the same level. After that, the activity started to decline gradually to the level of 20 U ml^{-1} after 18 days of the operation (Fig. 4).

3.6. Continuous production of CGTase in fluidized-bed bioreactor

Continuous production of CGTase from *Bacillus amyloliquefaciens* was also studied in fluidized bed reactor. The fermentation was carried out in a batch operation, initially for 24 h, then the fresh medium was fed into the reactor at different dilution rates (0.1–0.7 h^{-1}, representing 10–1.42 h residence time). The aeration rate was optimized at 0.33 (v/v h^{-1}), which was sufficient to fluidize the bed. Like the packed-bed reactor, the system was considered to be in a steady state only, after at least five residence times and the samples were collected at each dilution rate at the steady state conditions. The enzyme activity at different dilution rates is plotted in Fig. 5. It can be seen that the reduction in the enzyme activity was found to be 57%, when the residence time was lowered from 10 to 1.42 h. Maximal enzyme productivity (30.44 U ml^{-1} h^{-1}) with enzyme concentration of 53.4 U ml^{-1} was attained at the dilution rate of 0.57 h^{-1}. This value is about 32% higher than that obtained for the packed-bed reactor. The increase of enzyme productivity in fluidized bed reactor may be ascribed to the reduction of diffusional limitation by the effect of agitation in the fluidized bed. However, this mechanical agitation normally reduce the operational stability of the alginate beads and consequently prohibited further use [22]. In the initial 144 h the reactor was operated with different dilution rates and then switched on at the dilution rate which showed maximal productivity (0.57 h^{-1}). The reactor was able to keep producing nearly CGTase activity of 53 U ml^{-1} at a dilution rate of 0.57 h^{-1} for 10 days. After that, the activity

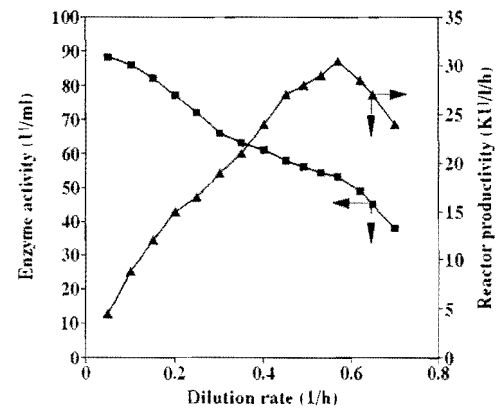

Fig. 5. Enzyme activity and CGTase productivity at different dilution rates in a fluidized-bed reactor.

Table 7
Comparison of CGTase activity and productivity in various systems

System	Maximal enzyme concentration (KU l^{-1})	Productivity of the system (KU l^{-1} h^{-1})	Specific productivity (U g wet cells^{-1} h^{-1})	Relative productivity (fold)
Free cells				
Batch[a]	88.5	1.85	119.72	1
Immobilized cells				
Batch (basal medium)[b]	70.8	1.47	94.7	0.8
Batch (at the optimized conditions)[c]	110	2.3	106.5	1.24
Repeated batch[d]	75.0	3.13	156.25	1.67
Continuous (packed-bed)[e]	48.0	23.04	141.87	12.45
Continuous (fluidized-bed)[f]	53.4	30.44	230.90	16.45

[a] Data from Table 1.
[b] Data from Table 1.
[c] 3% Na-alginate, 3 mm bead diameter, and 1.08 g wet cells 10 ml alginate gel^{-1}.
[d] Average of 14 cycle.
[e] At the dilution rate of 0.48 h^{-1}, which showed the maximal productivity.
[f] At the dilution rate of 0.57 h^{-1}, which showed the maximal productivity.

started to decline gradually over extended periods of times to reach 35 U ml^{-1} after 20 days of the operation (Fig. 6). Maximal specific CGTase productivity of immobilized cells of *Bacillus amyloliquefaciens* (230.9 U g wet cells^{-1} h^{-1}) which attained at dilution rate of 0.57 h^{-1} is favorably comparable to that reported for CGTase production from *Bacillus cereus* (220 U g wet cells^{-1} h^{-1}) in fluidized-bed reactor [10].

3.7. Comparison of CGTase activity and productivity in various systems

The results in Table 7 compares the activity and productivity of free and immobilized cells of *Bacillus amyloliquefaciens* in batch and continuous cultures. In terms of enzyme activity, the highest was achieved in free cells which is 7.5, 12.5, 65, and 85% higher than the immobilized cells in, repeated batch, batch, continuous in fluidized bed, and continuous in packed bed, respectively. On the other hand, in terms of productivity, the immobilized beads under continuous operation in fluidized bed resulted in 30.44 KU l^{-1} h^{-1}, which is 1.32–16.45 times higher than the other systems. In addition, the long term viability and continued metabolic activity is one of the most important advantages when working with the immobilized system, this is particularly so with the continuous fermentation. Rychtera et al. [23] reported that the long viability of the immobilized cells may be due to the different composition of proteins, nucleic acids, and inorganic substances, in comparison to the free cells, but definite evidence in this regard is lacking.

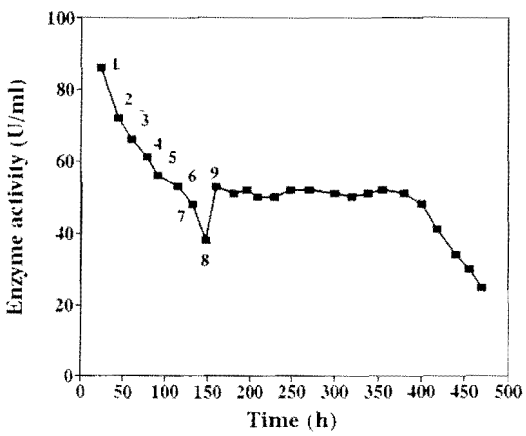

Fig. 6. Continuous synthesis of CGTase by immobilized cells of *Bacillus amyloliquefaciens* in a fluidized-bed reactor. The reactor was operated at different dilution rates for initial 144 h. The numbers 1–9 denote the changes on the dilution rates (D). 1-D = 0.1, 2-D = 0.2, 3-D = 0.3, 4-D = 0.4, 5-D = 0.5, 6-D = 0.57, 7-D = 0.62, 8-D = 0.7 and 9-D = 0.57.

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