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Comparative Study of Amidase Production by Free and Immobilized *Escherichia coli* Cells

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Abstract

Escherichia coli NCIM 2569 was evaluated for its potential for amidase production under submerged fermentation. Among the various amide compounds screened, maximum substrate specificity and enzyme yield (8.1 U/mL) were obtained by using 1% acetamide. Fermentation was carried out at 30°C in shake-flask culture under optimized process conditions. A maximum of 0.52 U/mL of intracellular amidase activity was also obtained from cells incubated for 24 h. Studies were also performed to elucidate the optimal conditions (gel concentration, initial biomass, curing period of beads, and calcium ion concentration in the production medium) for immobilization of whole cells. By using *E. coli* cells entrapped in alginate, a maximum of 6.2 U/mL of enzyme activity was obtained after 12 h of incubation under optimized conditions. Using the immobilized cells, three repeated batches were carried out successfully, and 85% of the initial enzyme activity was retained in the second and third batches. The study indicated that the immobilized *E. coli* cells offered certain advantages such as less time for maximum enzyme production, more stability in the enzyme production rate, and repeated use of the biocatalyst.

Index Entries: Amidase; acetamide; *Escherichia coli*; immobilization; fermentation.

Introduction

The enzyme amidase falls under the group of hydrolases that cleave the carbon-nitrogen (amide) bond to provide the acid product with the release of ammonia. Certain bacteria and fungi are known to produce different types of amidases *(1)*. Many Gram-negative and Gram-positive bacteria

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reported to produce amidases belong to the genera *Pseudomonas (2)*, *Brevibacterium (3–5)*, *Rhodococcus (6)*, *Methylophilus (7)*, *Alcaligenes (8)*, *Arthrobacter (9)*, *Escherichia (10)*, and *Bacillus (11)*. Among fungi, *Aspergillus nidulans (12)* was reported to produce four different aliphatic amidases, and *Aspergillus candidus* was reported as one of the best acetamide producers *(1)*. The ability of amidase to convert the cyanofunctionality into either an amide or an acid, together with regio- and stereoselectivity, makes it a good biocatalyst in biotransformation processes. Apart from amide hydrolysis activity, some amidases also exhibit acyl transferase activity *(13)*. The enzyme catalyzes the conversion of high molecular weight compounds such as polyacrylamide into low molecular weight compounds, which makes it ecofriendly. This enzyme finds application as industrial catalyst in organic synthesis, in the treatment of industrial effluents containing toxic amides, and also as a therapeutic agent. Because of the potential industrial applications of amidase, there is a need for the enzyme to be studied in detail. Immobilization has been considered a useful technique for production of microbial products *(14)*. Its application has been highlighted from the viewpoint of long-term utilization of biocatalyst and continuous operation stabilized systems *(15)*. In the present study, the bacterial strain *Escherichia coli* NCIM 2569 was used for amidase production, and enzyme production by free and immobilized cells under independently optimized conditions was evaluated.

Materials and Methods

Microorganism and Maintenance

The bacterial strain *E. coli* NCIM 2569 was grown at 30°C in peptone-agar slants containing 10.0 g/L of beef extract, 10.0 g/L of peptone, 5.0 g/L of NaCl, and 2.0 g/L of agar. It was subcultured fortnightly and stored on agar slants at 4°C.

Production Medium

The optimized medium used for amidase production contained 10.0 g/L of glucose, 5.0 g/L of $NaNO_3$, 1.01 g/L of KH_2PO_4, 1.63 g/L of Na_2HPO_4, 0.82 g/L of K_2HPO_4, 0.012 g/L of $CaCl_2{\cdot}2H_2O$, 0.5 g/L of $MgSO_4{\cdot}7H_2O$, 0.002 g/L of biotin, 0.0012 g/L of $ZnCl_2$, 0.0012 g/L of $FeSO_4{\cdot}7H_2O$, 0.0012 g/L of $MnSO_4{\cdot}7H_2O$, 2.5 g/L of yeast extract, and 10.0 g/L of acetamide (pH 7. 0). Glucose and acetamide were sterilized by microfiltration. The rest of the medium was autoclaved at 121°C for 15 min.

Inoculum and Shake-Flask Culture

Inoculum was prepared from the freshly grown culture slants by transferring a loopful of cells to 20 mL of peptone-agar seed culture medium placed in a 250-mL Erlenmeyer flask. The flask was incubated on a rotary shaker at 30°C and 180 rpm for 24 h. One milliliter (2.5% [v/v]) of the cell suspension was used as the inoculum.

Forty milliliters of the production medium placed in a 250-mL Erlenmeyer flask was inoculated with 24-h-old inoculum and was incubated on a rotary shaker (180 rpm) for a desired period of time. Samples were withdrawn at regular time intervals and centrifuged at 8000 rpm (15 min, 4°C) to remove the cell biomass. The supernatant obtained was analyzed for extracellular amidase activity. The cells were used to determine the intracellular amidase as described later.

Screening of Substrates

Four different compounds were screened to determine the best inducer or the substrate specificity of the amidase enzyme produced by *E. coli*: acetamide (C_2H_5NO), nicotinamide ($C_6H_6N_2O_3$), acrylamide (C_3H_5NO), and glutamine ($C_5H_{10}N_2O_3$). Production medium was supplemented individually with 1.0% (w/v) of each of these amides (filter sterilized). One milliliter of cell suspension from 24-h-old seed culture broth was used as inoculum. Fermentation was carried out for 48 h, and samples were withdrawn at regular intervals to check the amidase activity.

Optimization of Process Parameters for Amidase Production Using Free Cells

Experiments were carried out to optimize various physicochemical and biologic parameters for amidase production. To determine the required incubation time for maximum enzyme production, flasks were incubated for different time intervals (0–48 h). Samples were withdrawn at regular time intervals and centrifuged (8000 rpm, 15 min, 4°C) to obtain the supernatant. To study the effect of inoculum size, a known volume of the production medium was inoculated with varying volumes (0.5, 1.0, 2.0, and 4.0 mL) of 24-h-old seed culture, and flasks were incubated at defined conditions. The pH optimum for amidase production was determined by using production medium of varying initial pH values (5.0, 6.0, 7.0, 8.0, and 9.0). The effect of the amide concentrations on enzyme production was studied by varying the acetamide concentration (0.5–3% [w/v]) in the production medium.

The production medium (devoid of glucose) was supplemented with 1.0% (w/v) of various carbon sources, which were glucose, sucrose, lactose, sodium acetate, and glycerol, to determine their effect on amidase production. Fermentation was carried out as described in the previous section. The production medium (devoid of $NaNO_3$) was also supplemented with various nitrogen sources (1% [w/v]) sodium nitrate, peptone, urea (filter sterilized), ammonium nitrate, and ammonium chloride, to determine their effect on amidase production. Fermentation was carried out according to the defined conditions.

Immobilization of E. coli *Cells*

E. coli cells (0.2–1.0 g wet wt) were mixed thoroughly at 37°C with 10 mL of sterile sodium alginate solution (2–5% [w/v]). The mixture was extruded

as drops into a solution of 0.5 *M* $CaCl_2$. The gage number of the hypodermic needle used during extrusion controlled the bead size of 3 mm. The beads were cured in the same solution at room temperature (28–30°C) for 1 h and stored in a freshly prepared 0.5 *M* $CaCl_2$ solution at 4°C. The cured beads (biocatalyst) were rinsed with fresh culture medium before transferring them to the production medium. Studies were also carried out on the effect of storage periods on the enzyme production by the biocatalyst.

Shake Flask Using Immobilized Cells

The total beads obtained from 10 mL of alginate-cell mixture were transferred to a 250-mL Erlenmeyer flask containing 40 mL of production medium. The flask was incubated at 30°C on a rotary shaker (80 rpm). Samples were withdrawn at regular time intervals, centrifuged, and checked for amidase activity.

Repeated Batch Fermentation

Fermentation was carried out as described under Screening of Substrates, and after completion of one batch (48 h), the beads were aseptically removed from the production medium, washed three to four times with sterile distilled water, and transferred again to a new flask containing fresh production medium. The cycle was repeated unless the beads lost their shape or the leakage of cells occurred.

Optimization of Immobilization Parameters for Amidase Production

Studies were carried out for the optimization of various immobilization parameters for effective amidase production using *E. coli* cells. Various concentrations (1, 2, 3, and 5% [w/v]) of sodium alginate were used to prepare the beads. Immobilization followed by fermentation was carried out as described above. Two different curing periods of Ca-alginate beads (immobilized with *E. coli* cells) were evaluated (4 and 24 h) in 0.5 *M* $CaCl_2$ solutions at 4°C. After curing they were transferred into the production medium, and their stability and enzyme production were studied.

To optimize the initial biomass requirement for immobilization, the cells were harvested after 24 h from the production medium, and the wet weight of the biomass (after centrifugation) was determined. Various wet biomass concentrations (0.2, 0.4, 0.6, 0.8, and 1.0 g) were used for immobilization using a constant volume of sodium alginate (10 mL). Fermentation was carried out separately for each one as described earlier.

To optimize the $CaCl_2$ concentration, the cells were immobilized as described above and transferred into the production medium, which lacked $CaCl_2$, and was kept as the control flask. $CaCl_2$ solution of varying molarity (0.02, 0.04, 0.06, 0.08, and 0.1 *M*) was then supplemented to the production medium separately. The effect of calcium ions on the performance of immobilized cell beads was monitored at different time intervals.

Comparison of Free Cells and Immobilized Cells

Fifty milliliters of production medium placed in a 250-mL Erlenmeyer flask was inoculated with 2.5% (v/v) inoculum from 24-h-old seed culture. It was incubated at 30°C on an orbital rotary shaker at 180 rpm. Samples were withdrawn at regular intervals and the enzyme production was analyzed. Similarly, *E. coli* cells entrapped in alginate beads (2% alginate; 1 g initial wet biomass in 10 mL of alginate solution; beads cured for 24 h) were also kept (at 60 rpm) for comparison with respect to amidase production at different time intervals.

Intracellular Amidase Production

To check intracellular amidase production, 100 mL of production medium was prepared in 500-mL Erlenmeyer flasks. Two milliliters of inoculum was added to each flask, and they were kept for incubation for different time intervals (12–72 h). The cells were harvested at each time interval and the wet weight of the cells was determined. The cells were resuspended in 10 mL of phosphate buffer supplemented with 50 µL of 2-mercaptoethanol and 50 µL of phenylmethylsulfonyl fluoride and mixed well until an emulsion was formed. An ultrasonicator was used to disrupt the cells at an amplitude of 40% for 30 s. Cells were subjected to eight such cycles. Then, the tubes containing disrupted cells were centrifuged at 8000 rpm at 4°C for 15 min. The supernatant was checked for intracellular enzyme activity.

Analytical Methods

Cell growth was monitored by determining the optical density of the culture medium, diluted if necessary, in an ultraviolet-visible spectrophotometer (Shimadzu, Japan) at 600 nm. Amidase activity was determined by measuring the ammonia released (Nesslerization reaction) spectrophotometrically at 450 nm *(16)*. One unit of amidase activity is expressed as the amount of enzyme required to release 1 µmol of ammonia/min under the assay conditions. Soluble proteins were estimated using the method described by Lowry et al. *(17)*.

Results

All the experiments were conducted in three independent sets, and then the average value was calculated for representation. Among the various substrates screened, the extracellular enzyme activity (5.84 U/mL) obtained with 1.0% (w/v) acetamide after 24 h was the best (as shown in Fig. 1).

Hence, for further experiments acetamide was used as the inducer for amidase production. To optimize the initial concentration of acetamide, various concentrations were tested and the maximum titer (5.84 U/mL) was obtained with 1.0% (w/v) acetamide. With a concentration of 0.5% aceta-

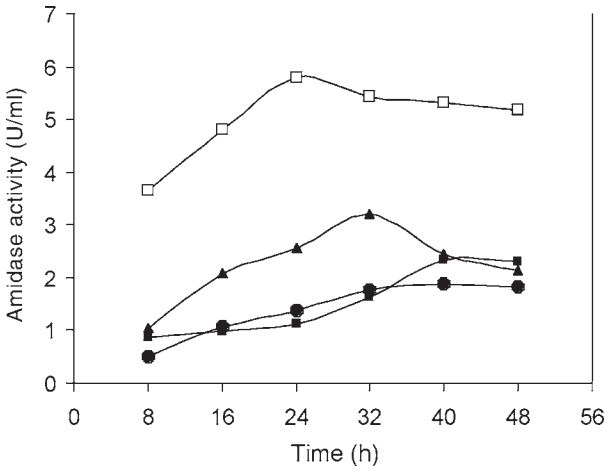

Fig. 1. Effect of various compounds on amidase production by *E. coli* NCIM 2569 cells.

mide, the enzyme activity (5.54 U/mL) was comparable with that obtained with 1.0%. However, an increase in the concentration beyond 1.0% of the substrate inhibited enzyme synthesis (i.e., 3.73 U/mL was obtained with 3% acetamide).

Figure 2 shows the growth and amidase production (both extracellular and intracellular) over a period of time. From Fig. 2 it can be seen that there was a gradual increase in the enzyme production in relation to the incubation time. A maximum activity of 5.85 U/mL was obtained after 24 h of incubation.

Intracellular accumulation of amidase was also checked in the *E. coli* cells. A maximum activity of 0.52 U/mL was obtained after 24 h of incubation. Accumulation of intracellular enzyme coincided with the maximum extracellular enzyme production (5.85 U/mL), which was also observed after 24 h of incubation.

Among the various amounts of inoculum used, 1.0 mL (2.5% [v/v]) of inoculum was proved to be the most suitable, and the activity obtained was 5.85 U/mL. An increase in inoculum size had a negative influence on amidase synthesis, with 4.0 mL (10.0%) of inoculum resulting in the lowest activity, 2.98 U/mL.

On the other hand, enzyme production was at its best when the production medium with an initial pH of 7.0 was employed (5.79 U/mL). However, the enzyme production was observed over a wide pH range, 5.0 (4.36 U/mL)–9.0 (4.7 U/mL).

Among the carbon sources supplemented with the production medium, the maximum activity was obtained with glucose (5.93 U/mL). With all the other carbon sources—sucrose, lactose, glycerol, and sodium acetate—the production was reduced to a range of 3.0–3.5 U/mL (detailed data not shown).

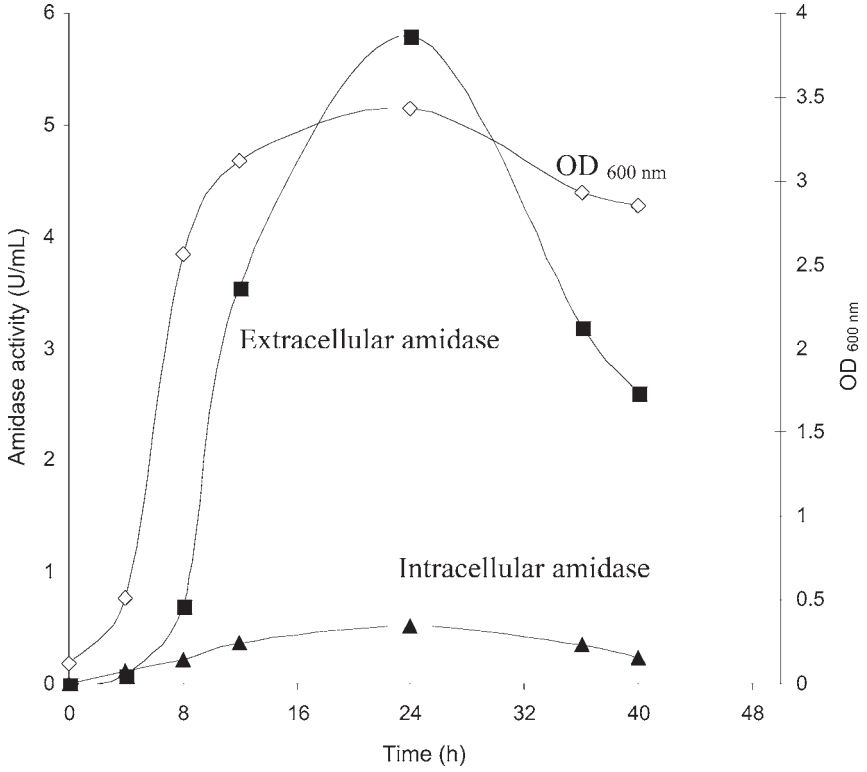

Fig. 2. Effect of incubation time on growth and amidase production by *E. coli* NCIM 2569 cells with 1% (w/v) acetamide.

The impact of supplementation of the production medium with various nitrogen sources is shown in Fig. 3. Supplementation with urea had a profound influence on amidase production, resulting in amidase activity of 8.1 U/mL. The control (sodium nitrate) showed an activity of 5.86 U/mL. The addition of ammonium chloride considerably reduced the enzyme activity (2.63 U/mL).

Four concentrations (1, 2, 3, and 5% [w/v]) of sodium alginate were used to prepare the beads. Figure 4 shows the effect of alginate concentration on amidase production. A concentration of 2% alginate was the best for immobilization for these experiments. The maximum production of enzyme obtained was 5.75 U/mL after 12 h of incubation using 2% alginate.

After the alginate beads were made, they were cured in 0.5 *M* $CaCl_2$ solution at 4°C for two different time intervals, 4 and 24 h. It is evident from Fig. 5 that a prolonged storage period of beads (24 h) led to significantly higher amidase activity.

Initial biomass concentrations varying from 0.2 to 1.0 g of cells/10 mL of gel were used. As shown in Table 1, 1.0 g of cells/10 mL of gel was the most suitable. In this case, a maximum activity of 6.1 U/mL was obtained after 12 h. However, further increase in the initial biomass concentration led to cell leakage (visual observation).

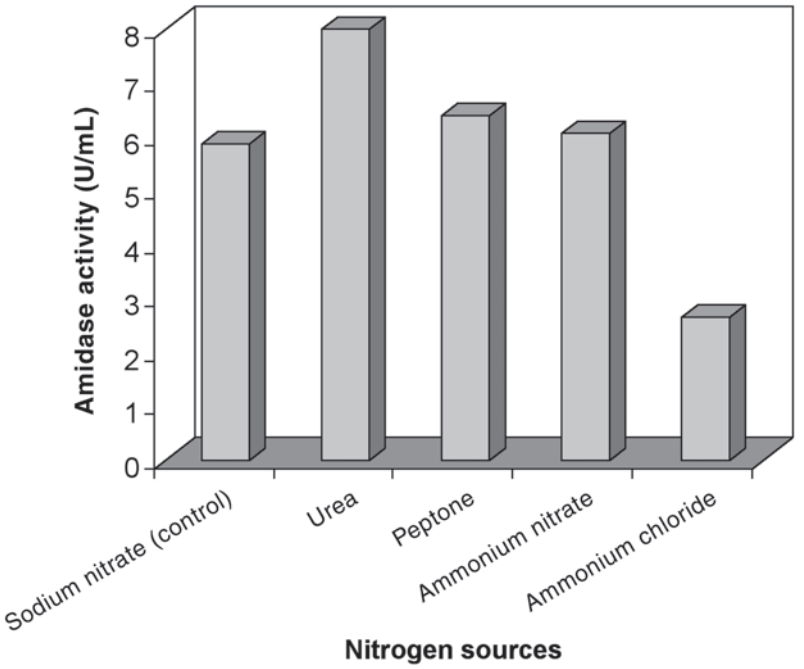

Fig. 3. Effect of supplementation of various nitrogen sources on amidase production by *E. coli* NCIM 2569 cells.

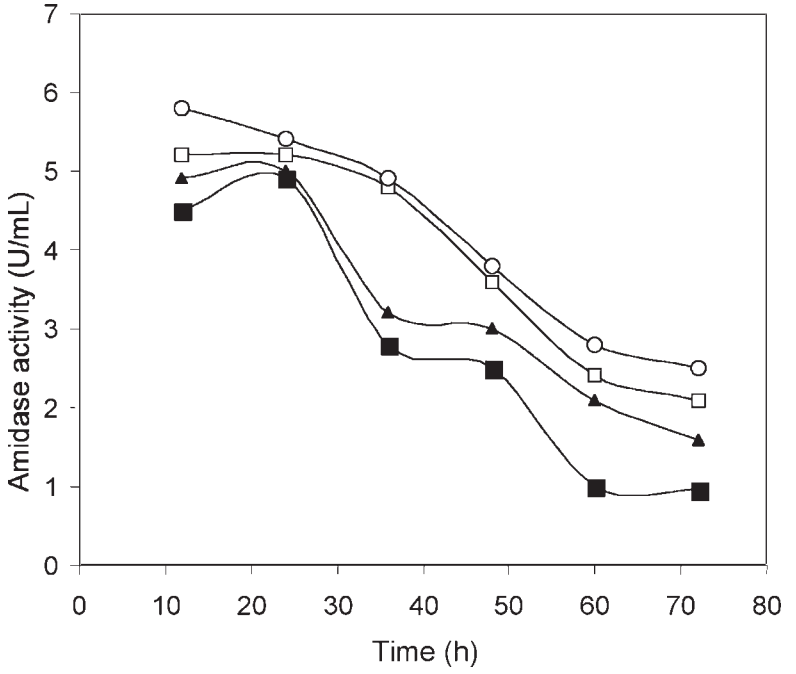

Fig. 4. Effect of alginate concentration for immobilization of *E. coli* NCIM 2569 cells on amidase activity.

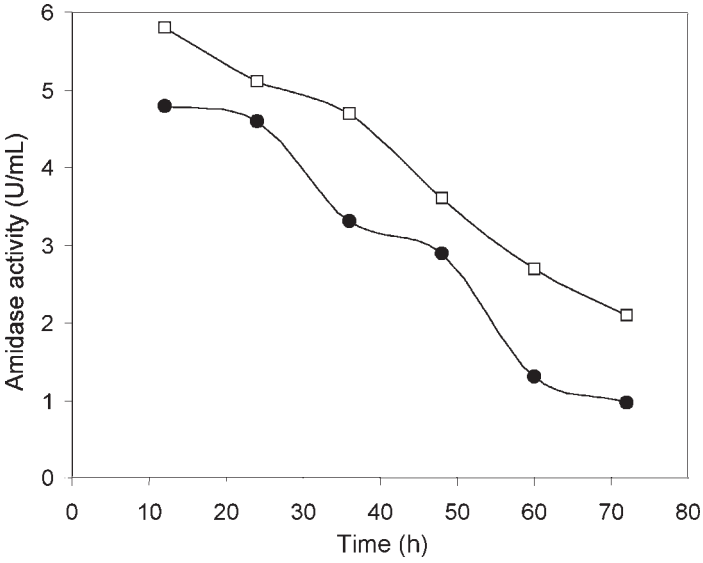

Fig. 5. Effect of curing time of immobilized beads (in 0.5 *M* $CaCl_2$) entrapped with *E. coli* NCIM 2569 cells on amidase production.

Table 1
Effect of Initial Biomass (*E. coli*)
in Immobilized Beads on Amidase Production

Time (h)	Enzyme activity (U/mL)				
	Biomass concentration (g)				
	0.2	0.4	0.6	0.8	1.0
12	2.9	3.0	4.9	5.4	6.1
24	2.3	3.1	2.9	4.8	5.1
36	2.3	1.9	2.5	3.4	3.6
48	1.8	1.3	2.0	2.1	2.5
60	1.6	1.2	1.0	1.6	1.8
72	1.3	0.9	0.58	1.0	0.98

The effect of calcium ions, which could affect enzyme production, was examined. The results indicated that the addition of suitable concentrations of $CaCl_2$ considerably increased enzyme yields. The addition of 0.02 *M* $CaCl_2$ to the medium resulted in 6.1 U/mL of enzyme activity, which was 5.84 U/mL with the control (without $CaCl_2$ supplementation). With 0.04 *M* $CaCl_2$ in the medium, the activity decreased to 4.9 U/mL (detailed data not shown).

The immobilized cells could be recycled successfully for three batches. It can be seen from Fig. 6 that a maximum of 5.84 U/mL of enzyme activity was obtained after 12 h of incubation in the first batch.

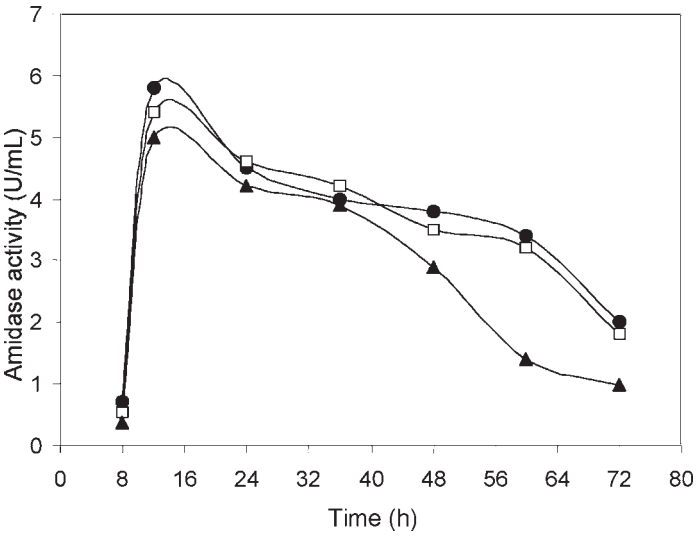

Fig. 6. Batch fermentation for production of amidase using immobilized *E. coli* NCIM 2569 cells.

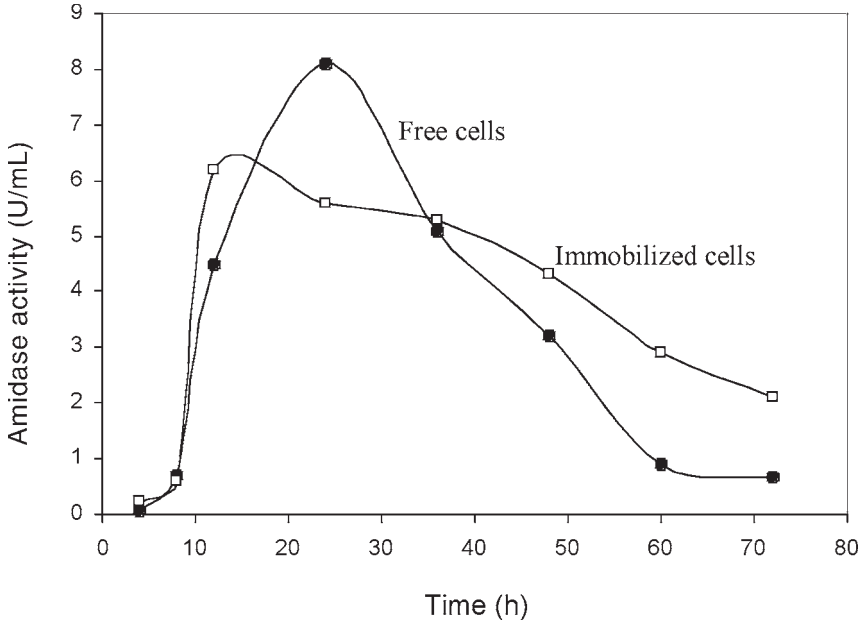

Fig. 7. Comparison of amidase production using free and immobilized *E. coli* NCIM 2569 cells under optimized conditions.

Thus, using immobilized cells, maximum enzyme production was 6.1 U/mL, whereas in the case of free cells, it was 8.1 U/mL (Fig. 7). However, the incubation period for maximum production in the case of immobilized cells was 12 h, whereas it was 24 h in the case of free cells.

Discussion

As shown in Fig. 1, even though better induction was noted with acetamide, the *E. coli* NCIM 2569 cells were capable of producing a wide spectrum of amidase. A minimum concentration of the substrate was required to induce the activity and a greater concentration adversely affects enzyme production probably owing to a high concentration of end product. Single-parameter optimization was carried out to optimize various process parameters essential for any fermentation, such as inoculum size, pH, and supplementation of the production medium with vital nutrients such as carbon and nitrogen sources.

The entrapment of cells in Ca-alginate gel beads is considered a useful procedure because of the simplicity of the method, low price, and nontoxicity of the gel. A higher concentration of alginate (5% [w/v]) resulted in harder beads, and, hence, production was decreased. In addition, from Fig. 4 it can be deduced that with an increase in gel concentration, the incubation time for maximum enzyme production shifted from 12 to 24 h. In fact, Gosmann and Rehm *(18)* have reported that the specific oxygen uptake rate of immobilized microorganisms decreases with increasing alginate concentration.

The increase in amidase production with 24-h cured beads was probably owing to improvement in the gel solidity attained with the consequent decrease in the release of cells from the beads. Alginic acid contains polymannuronic and polyguluronic acid, whose composition is dependent on the source of brown algae. The gelling is a result of the formation of an "egg-box" structure consisting of chains of either guluronic acid or mannuronic acid units in parallel being held together (crosslinked) by divalent cations such as calcium *(19)*. According to Lu and Chen *(20)*, the cells encapsulated in properly solidified beads had better storage stability than the free cells.

An increase in the initial biomass concentration led to cell leakage. The specific oxygen uptake rate of the entrapped microorganism was dependent on the biomass concentration in the gel. With increasing cell concentration in the gel, oxygen was consumed faster than it could diffuse into the beads. At this point, the cells had to compete for oxygen uptake. For this reason, the specific respiration decreased and, finally, the absolute oxygen uptake rate of the gel beads remained constant, though the cell concentration increased *(21)*.

The addition of calcium ions to the production medium containing immobilized cells enhanced enzyme production. It has been reported that for immobilized cells, growth medium must be supplemented with $CaCl_2$ to ensure mechanical stability of gel beads *(22)*. Ca-alginate gel is unstable in the presence of phosphate and certain cations such as Mg^{++} or K^+, which are the major nutrients of living cells *(20)*. The solubilizing effect of these agents can be overcome by supplementing the growth medium with $CaCl_2$.

In the case of recycling the immobilized cells, it was interesting to note that in the second and third batch, 85% of the enzyme activity was obtained in comparison with the first batch. In the fourth batch, the beads began to disintegrate.

Immobilized *E. coli* cells gave a maximum enzyme yield with a shorter incubation time than the free cells. The difference in incubation period for maximum yield may be owing to the time required by the *E. coli* to grow and proliferate. By contrast, when the cells were immobilized and put into the medium, less time was required to produce the enzyme. Immobilized cells could be used successfully for three batches, which could be considered an added advantage over that of the fermentation of free cells. Immobilization of cells also offers the possibilities of stable enzyme production rate within a short time owing to high cell concentrations, continuous operation of the biocatalyst, and less chance of contamination.

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