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Production of alkaline protease by *Teredinobacter turnirae* cells immobilized in Ca-alginate beads

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The conditions for immobilizing the new alkaline protease-producing bacteria strain *Teredinobacter turnirae* by entrapment in calcium alginate gel were investigated. The influence of alginate concentration (20, 25 and 30 g/l) and initial cell loading (ICL) on enzyme production were studied. The production of alkaline protease improved significantly with increasing alginate concentration and reached a maximum enzyme yield of 8000 U/ml at 25 g/l alginate concentration. This was about 176.8% higher than that obtained by free cells (2890 U/ml). The immobilized cells produced alkaline protease consistently over 5 repeated cycles and reached a maximal value of 9000 U/ml on the third cycle. This was 311.4% (3.11-fold) as compared with the control (free cells). Simple mass balance analysis was applied to describe the growth and the protease production behaviour of both fractions the cells in free form and the entrapped in Ca-alginate beads. Scanning electron microscope studies indicated the internal distribution pattern of the cells encapsulated in Ca-alginate beads. The results presented in this paper show the potential for using immobilized *T. turnirae* cells in Ca-alginate for the production of a novel alkaline protease.

Key words: Alkaline protease, Ca-alginate, immobilization, *Teredinobacter turnirae*, repeated batch.

INTRODUCTION

Alkaline proteases have found a wide application in several industrial processes such as an additive to detergents and in bating of hides and skins in leather industries (Godfrey and Reichet, 1985). They hold more than 50% of the total enzyme market (Sachidanandham et al*.,* 1999). In this study, the protease produced has been isolated from a symbiotic bacterium found in the gland of Deshayes of the marine shipworm (Griffin et al*.,* 1992). It retains a high level of activity above 50°C. The protease is unique in that its activity is not only stable to, but also stimulated by high concentrations of oxidizing agents. Moreover, the activity of the produced protease remains unaffected by several compounds known to inhibit other proteases. These properties of the protease suggest widespread utility in detergents and other low-temperature industrial applications. The alkaline protease of *Teredinobacter turnirae* possesses unique properties in terms of a high salt tolerance. However, *T. turnirae* cultivated in submerged cultures is not an appropriate good producer of alkaline protease, since enzyme titters are relatively low (Beshay and Moreira, 2001). One of the approaches to improve its production efficiency is by long-term continuous production of alkaline protease under cell immobilization. At present, cell immobilization technology is often studied for its potential to improve fermentation processes and bioremediation (Beshay et al*.,* 2002; Abd-EL-Haleem et al., 2003).

Immobilization of whole cells for the production of extracellular enzymes 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 (Zhang et al*.,* 1989; Galazzo and Bailey, 1990). However, proper selection of immobilization techniques and supporting materials is needed to minimize the disadvantages of immobilization. One of the most suitable

Nomenclature: X, cell density (Cell dry weight) (g l^{-1}); t, time of cultivation (h); E, enzyme (protease) activity (U ml^{-1}); , specific growth rate (h^{-1}).

Abbreviations: CDW. Cell dry weight; Gl, alginate-Gel beads; M, medium; Gr, growth cells; Re, released cells; Pg, enzyme production in gel beads; Pm, enzyme production in medium.

methods for cell immobilization is entrapment in calcium alginate, because this technique is simple and cheap. Sodium alginate is readily available and it is a non-toxic biological material. Therefore, it is suitable as an immobilization matrix for bio-molecules and microorganisms (Mattiasson, 1983). Beads of calcium alginate are prepared under mild conditions and have been used extensively for microencapsulating and entrapping cells. The objective of the present study was to find the optimal conditions for Ca-alginate gel immobilization of *T. turnirae* cells and to determine the operational stability of the resulting biocatalyst in the production of alkaline protease under semicontinuous cultivation conditions. Moreover, scanning electron microscope was used to elucidate the morphology of *T. turnirae* cells entrapped in the alginate matrix.

MATERIALS AND METHODS

Bacterial strain and cultivation conditions

The bacterial strain used in this work, *T. turnirae* was kindly provided by Dr. Antonio Moreira, Department of Chemical and Biochemical Engineering, University of Maryland- Baltimore County, USA. Placket-Burman medium (PB), which was recently modified (Beshay and Moreira, 2001) was used throughout all experiments. Cultivation was carried out with 50- ml cultures in 250-ml Erlenmeyer flasks shaken at 120 rev min^{-1} and 30°C.

Calcium alginate entrapment

Very wide ranges of alginate are available with varying molecular masses, mannuronate: glucoronate ratios and distribution of units between blocks and alternating sequences. Alginate solutions with a concentration range of 0.5–10% can be used for cell immobilization. In current study, different sodium alginate concentrations (20, 25 and 30 g/l) were studied. The alginate entrapment of cells was performed according to the method previously described (Johnsen and Flink, 1986). Alginate was dissolved in boiling water and autoclaved at 121°C for 15 min. Cells were harvested during the mid-logarithmic growth phase by centrifugation (5000 g, 10 min), resuspended in 2 ml of saline and added to 100 ml of sterilized alginate solution. This alginate/cell mixture (with stirring) was extruded drop by drop into a cold, sterile 0.2 M $CaCl_2$ solution through a sterile 5 ml pipette. Gel beads of approximately 2 mm diameter were obtained. The beads were hardened by resuspending into a fresh $CaCl_2$ solution for 24 h at 4°C with gentle agitation. Finally these beads were washed with distilled water to remove excess calcium ions and unentrapped cells. Then the beads were transferred to 50 ml production medium and cultivated for the required time.

Repeated batch cultivation

One of the advantages of using immobilized biocatalysts is that they can be used repeatedly and continuously. Therefore, the reusability of *T. turnirae* cells immobilized in alginate gel was examined. This process was carried out by decanting the spent medium every 48 h and replacing it by fresh medium after washing the alginate beads with sterile saline. On the other hand, a similar experiment was carried out with free cells to compare the efficiency of free and immobilized cells for the production of alkaline protease under these conditions.

Cell growth and cell leakage

Both cell growth in freely suspended cultures and cells leaked from the gel beads were determined as cell dry weight by measuring the optical density at 600 nm. One absorbency unit was equivalent to 0.16 g/l (cell dry weight).

Enzyme assay

Proteolytic activity was determined using azocasein as substrate as described elsewhere (Greene et al*.,* 1989). The unit of activity was defined as the amount of azocasein in microgram digested per hour at pH 7.0 and 25°C.

Microscopic examinations

For morphology studies of immobilized *T. turnirae* cells in alginate beads, scanning electron microscopy (SEM) was used. The alginate beads were fixed with 2% glutaraldehyde and then dehydrated in ethanol. Finally, the samples were dried, coated with gold and observed with a Philips XL 30 microscope attached to an EDX unit.

RESULTS

Effect of alginate concentration

In order to find out the optimum alginate concentration for *T. turnirae* cell immobilization, alginate solutions of different concentrations (20, 25 and 30 g/l) were used. The results of these experiments are shown in (Figure 1). The production of alkaline protease improved with increasing alginate concentration and reached a maximum yield of 8000 U/ml at 25 g/l alginate (Figure 1A). This was about 176.8% higher than that obtained by free cells (2890 U/ml). Moreover, at low alginate concentration (20 g/l) the beads were relatively soft and showed rapid leakage of the cells from the beads compared to high alginate concentration 30 g/l as shown in (Figure 1B). However, further increase in alginate concentration beyond 25 g/l was accompanied by decreasing the enzyme yield.

Effect of initial cell loading (ICL)

By varying the number of beads from 100 to 600 beads/flask using 25 g/l alginate concentration, the influence of the initial cell loading was tested. It was assumed that, increasing ICL in the form of the number of beads could increase protease production. The results revealed that increasing the number of alginate beads/flask up to 400 was accompanied by increase the protease concentration (Figure 2). This may result from

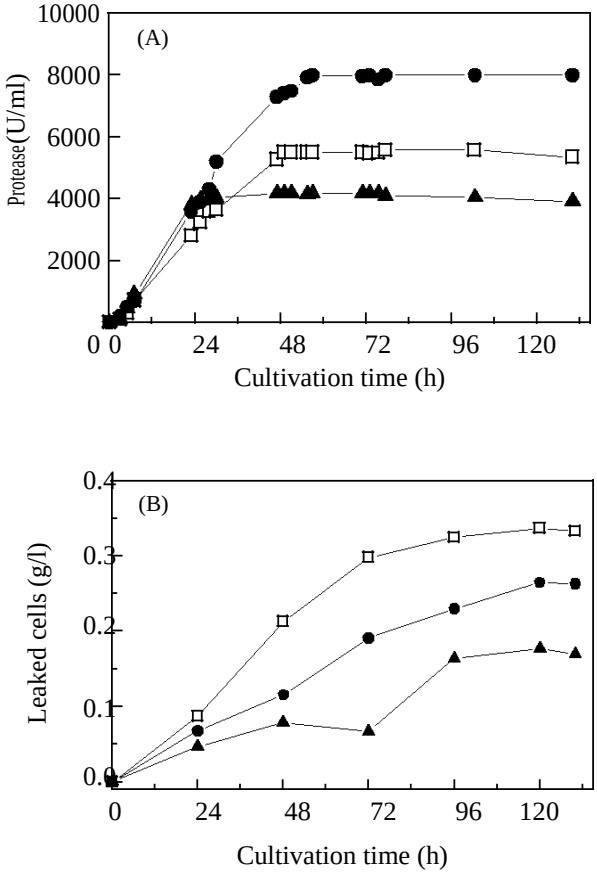

Figure 1. Effect of alginate concentration on both alkaline protease production (A) and cell leakage (B) by *T. turnirae* cells immobilized in Ca-alginate beads (Alginate concentrations (g/l): 20 ( ), 25 ( ), 30 (▲)).

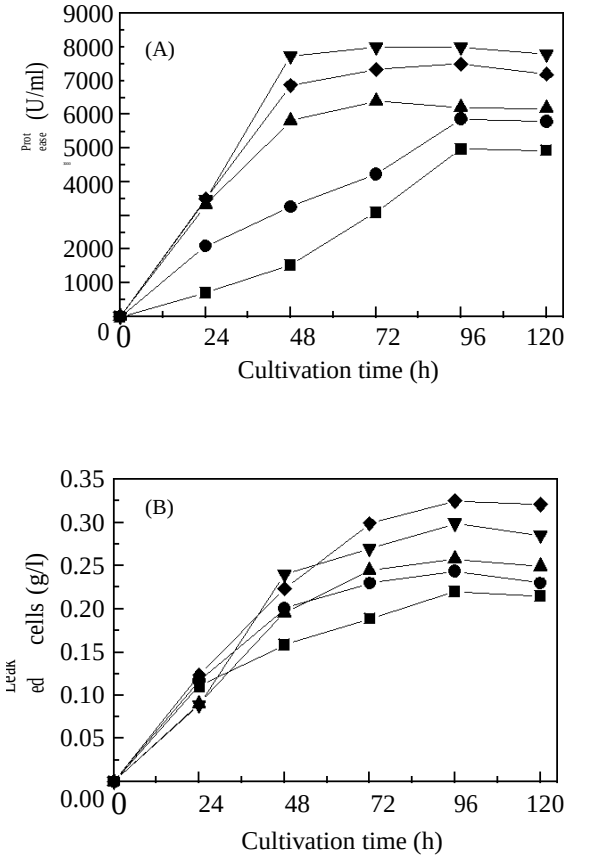

Figure 2. Effect of cell loading on both protease production (A) and cell leakage (B) by *T. turnirae* cells immobilized in Ca-alginate beads (No. of beads/flask: 100 ( ), 200 ( ), 300 (▼), 400 (▲), 600 ( )).

an increase in the total surface with a greater number of gel beads, which leads to facilitate the mass transfer from/to the Ca-alginate beads. The results also indicate that using 400 beads/flask was the most suitable inoculum level giving a maximum enzyme yield of 8000 U/ml. Higher or lower inoculum levels resulted in reduced enzyme yield (Figure 2).

Comparisons of protease production by *T. turnirae* as free and immobilized cells

Figure 3 shows the protease production by Ca- alginate immobilized cells as compared to free *T. turnirae* cells. In case of free cells, protease production started to increase after about 6 h and reached a maximum activity of 2890 U/ml at the end of logarithmic phase at 48 and kept more or less constant until the end of cultivation. While, immobilized *T. turnirae* cells in Ca-alginate beads showed a significant increase in the production of protease enzyme from the beginning of the cultivation process.

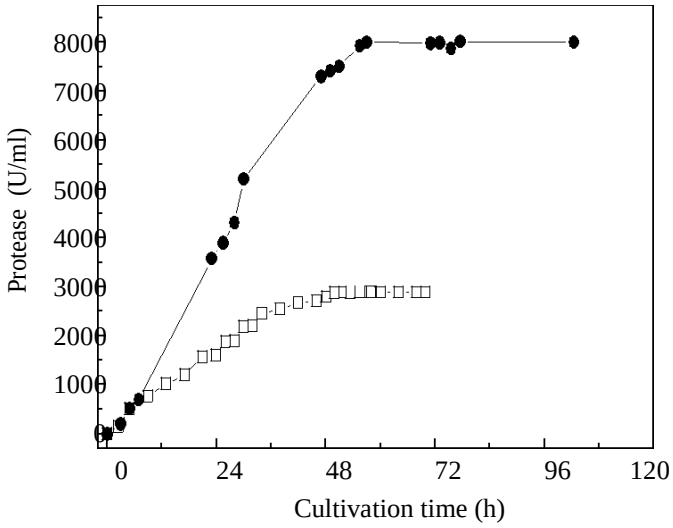

Figure 3. Alkaline protease production by free ( ) and immobilized *T. turnirae* cells ( ) in Ca-alginate beads.

Figure 3 also shows that protease production by immobilized cells reached 3000 U/ml after about 20 h, which could be obtained after 48 h with free cells. This activity continued to increase and reached a maximum value of 8000 U/ml at 48 h and kept more or less constant. Thus, the alkaline protease concentration from alginate-immobilized cells was 176.8% higher than that obtained by free cells as control.

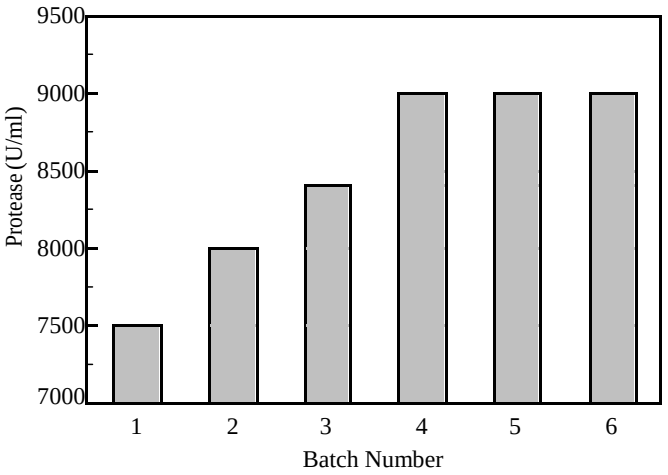

Figure 4. Repeated batch production of alkaline protease by *T. turnirae* cells immobilized in Ca-alginate beads.

Reuse of the immobilized biocatalyst for alkaline protease production in repeated batch shake cultures

The semi-continuous fermentation was terminated in order to investigate the stability of the biocatalyst and its ability to produce alkaline protease under repeated batch cultivation conditions. Therefore, the operational stability of the biocatalysts obtained under optimal immobilization conditions (alginate concentration 25 g/l, 400 beads/flask) was followed during 5 cycles (Figure 4). Alkaline protease was produced in repeated batch shake cultures and the time for each batch was 48 h. When the estimated maximum activity level had been reached, the culture supernatant was decanted off and 50 ml of fresh medium was added to the Erlenmeyer flasks. The results obtained at the end of the first cycle showed a significant increase (176.8%) in alkaline protease activity of entrapped cells compared to those with free cells cultured in parallel. This high alkaline protease production continued after transfer into fresh medium at the end of the third cycle of cultivation (192 h), the alkaline protease yield of the biocatalysts was 311.4% as compared with the control (free cells) . The highest activity of 9000 U/ml obtained during the repeated batch shake flask experiment was reached in the third batch after about 6 days from the beginning of the repeated batch series. Further replacements of the medium had no positive effect to improve the enzyme productivity more than 9000 U/ml to the fifth cycle (10 days).

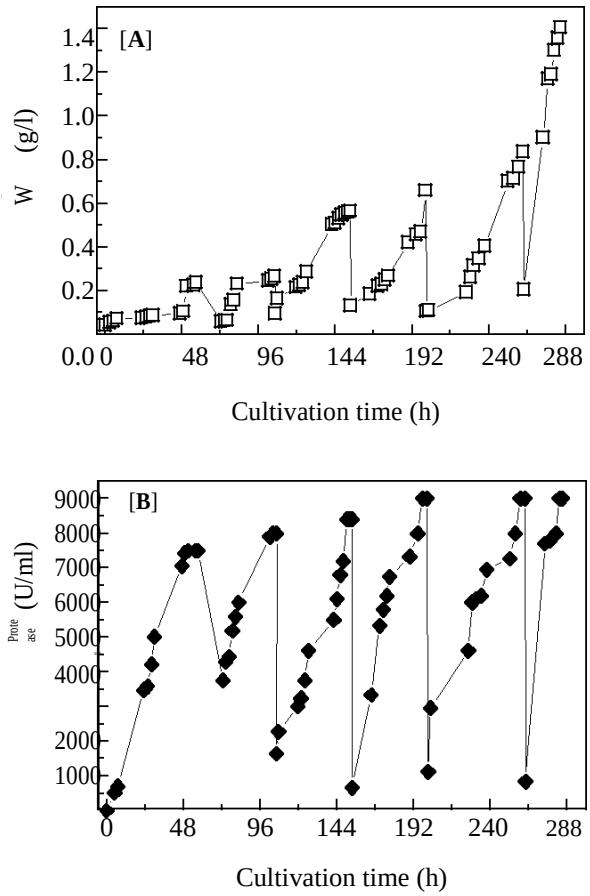

Figure 5. Time course of semi-continuous cultures with immobilized *T. turnirae* cells in Ca-alginate beads.

Analysis of the semi-continuous cultures with immobilized *T. turnirae* cells

Figure 5 shows the changes of cell concentration in medium and protease activity in 5 repeated replacement cultures of the immobilized *T. turnirae* cells. The slightly increase of cell concentration in the medium was caused by the release of cells from the gel beads and their growth. The change of cell mass in gel beads $\frac{dx}{dt}_{Gl}$ and in the medium $\frac{dx}{dt}_{M}$ could be expressed as follows:

$$\frac{dx}{dt}_{Gl} \quad \frac{dx}{dt}_{Gr} \quad \frac{dx}{dt}_{Re} \qquad (1)$$

$$\frac{dx}{dt}_{M} \quad \frac{dx}{dt}_{Gr} \quad \frac{dx}{dt}_{Re} \qquad (2)$$

$\frac{dx}{dt}_{Gr}$ in equations (1) and (2) represents the changes of cell mass by growth in the gel beads and in the medium, respectively.

$\frac{dx}{dt}_{Gr}$ in the medium can obtained experimentally from Figure 5. While, $\frac{dx}{dt}_{Gr}$ in the gel beads and $\frac{dx}{dt}_{\mathrm{Re}}$ as well as specific growth rates ( ) of cells in gel beads and free cells could be calculated.

Similar mass balance for the production of alkaline protease in Ca-alginate beads and in the medium can be expressed as follows:

$$\frac{dE}{dt}_{Gl} \qquad \frac{dE}{dt}_{pg} \qquad \frac{dE}{dt}_{\mathrm{Re}} \qquad (3)$$

$$\frac{dE}{dt}_{M} \qquad \frac{dE}{dt}_{Pm} \qquad \frac{dE}{dt}_{\mathrm{Re}} \qquad (4)$$

Scanning electron microscopy studies

The internal structure of Ca-alginate beads in the presence of immobilized *T. turnirae* cells was studied under a scanning electron microscope. It was observed from the SEM photographic plates that the cells were randomly distributed in alginate beads (Figure 6) . The region in the periphery of the beads appeared to be more densely packed compared to the central region due to higher growth rate aiming to better supply of nutrients.

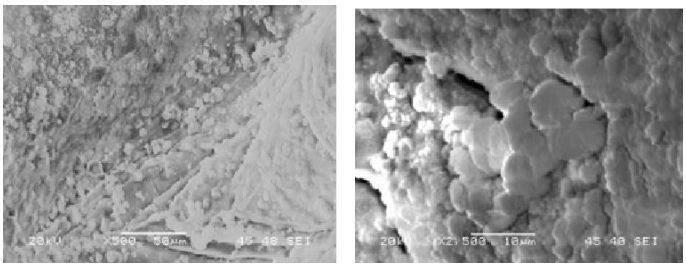

Plate A Plate B

Figure 6. Scanning electron microscopic observations of *T. turnirae* cells entrapped in Ca-alginate beads (Near the surface of the bead (A), in the center of the bead (B)).

DISCUSSION

Cell immobilization is one of the common techniques for increasing the over-all cell concentration and productivity. The separation of products from immobilized cells is easier compared with suspended cell systems. Immobilization of cells may allow continuous operation of cultivation processes at high dilution rates. Last but not least, immobilization is a strategy for protecting cells from shear forces. Many different techniques for immobilizing cells have been proposed (Brodelius and Vandamme, 1987; Beshay et al., 2002; Abd-El-Haleem et al., 2003). Using polysaccharides for gel entrapment or encapsulation has developed into a challenging method and the use of alginate gel beads stands out as the most promising and versatile method yet. Alginic acid and its derivatives are commercially available in a wide range of types, having different viscosities and gelling properties. Calcium alginate gels form rapidly in very mild conditions and provide suitable media for the immobilization by entrapment of whole microbial cells. Only a single type of sodium alginate (Fluka) was used in this work, it was flexible enough in its properties to be suitable for successful immobilization of *T. turnirae* cells.

In this study, the author have chosen to vary the concentrations of alginate solutions to prepare the biocatalyst beads in the aim to determine the impact of different kind of constraints on the physiology of *T. turnirae* cells. Actually, the gel network is very different depending on the conditions in which gel beads are obtained (Nava et al., 1996). The variation of the supramacromolecular structure of the matrix affected the morphology and physiological behaviour of immobilized *T. turnirae* cells. Figure 1 indicates that, further increase in alginate concentration beyond 25 g/l was accompanied by decreasing the protease yield. This may be attributed to the fact that the bead durability was improved at higher alginate concentrations but that diffusion limitation due to strong gel consistency leads to lower enzyme yields. These results are in agreement with other investigations (Martinsen et al., 1992; Dobreva et al., 1996). Another explanation for decreasing protease production with further increasing of alginate concentration is that the rate of substrate mass transfer and the lower porosity of the gel beads may have led to a decrease in enzyme production as judged by previous reports (Shinmyo et al., 1982; Fumi et al., 1992).

In addition, the dependence of enzyme yield on initial cell loading (ICL) in the form of the number of alginate beads/flask was studied (Figure 2). The positive effect of increasing cell loading, which led to improve enzyme yield was the same results obtained for the production of gluconic acid by *Aspergillus niger* immobilized in Ca-alginate beads (Rao and Panda, 1994). Enzyme yield decreased at high ICL (up to 400 beads/flask). This could be attributed to the fact that, when the number of beads increases, the nutrient/bead ratio decreases, which may become limiting.

Figure 4 shows the possibility for re-use the Ca-alginate biocatalysts to produce alkaline protease in semi-continuous mode. The results revealed that protease production increased gradually with repeated batch

cycles. Increasing of protease productivity of the immobilized cells during the early use cycles may be caused by the growth of cells in the gel. The cells gradually grew on the surface of the gel with increasing cycles. The surface of the beads was suitable for the growth of cells, because the supply with oxygen and nutrients was better. These findings for the production of alkaline protease by repeated batch of *T. turnirae* cells immobilized in Ca-alginate beads were in accordance with those obtained previously for the production of protease by immobilized *Serratia marcescens* and *Myxococcus xanthus* in calcium alginate beads (Vuillemard et al., 1988) . It was found that protease production by immobilized *S. marcescens* increased with repeated growth cycles, and reached a maximum after 5 cycles. In addition, the mechanical stability of the biocatalysts was high enough, since no fragments of alginate beads were found in culture broth. In contrast, free cells showed lower enzyme productivity than the immobilized ones and their activity decreased markedly with repeated batch cycles (data not shown). While the immobilized biocatalyst had been in use for about 2 weeks, after which it still possessed significant alkaline protease production. It has been shown that immobilized cells were able to produce alkaline protease consistently and that they might be used for continuous alkaline protease production.

In conclusion, the results show that calcium alginate entrapment is a promising method of *T. turnirae* immobilization for alkaline protease production. Alkaline protease production by immobilized cells is superior to that of free cells because it leads to higher volumetric activities within the same time of fermentation. Specific advantages of this technique such as long life-term stability, the reusability and possibility of regeneration to be adaptable also to scale-up the obtained data. In addition, experiments with repeated batch of bacterial growth by introducing fresh nutrients every 48 h leads to an enzyme activity which is 3.2 times higher than that obtained with free cells.

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