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RESEARCH ARTICLE

IMMOBILISATION OF *BACILLUS* SP.JB-99 FOR THE PRODUCTION OF ALKALINE PROTEASE

[1]*Shivasharana C.T., Naik, G.R[2] and Kaliwal, B.B[1]

[1]Post Graduate Department of Biotechnology and Microbiology, Karnatak University, Dharwad- 580003, Karnataka-India.
[2]Department of Biotechnology, Gulbarga University, Gulbarga-585106, Karnataka-India.

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ABSTRACT

The thermoalkalophilic *Bacillus* sp.JB-99 was employed in this study to immobilize it in sodium alginate and produce alkaline protease using chemically defined medium. The cell concentration/ml/gm of beads was $6x10^6$ cfu. The parameters optimized for the production of alkaline protease are; 25g/l sodium alginate, 300 beads/flask, 8.5-9.0 medium pH, 72h incubation period yielded a maximum of 3700 U/ml and ends up with 120h yielded 3800U/ml. This was about 3 fold lower than that obtained by free cells (12000 U/ml). The cell leakage was almost proportionate with initial cell loading and time of incubation. The repeated batch cultures of cells have shown viability till 6 cycles. At every 48h intervals upto 120h shown gradual increase in enzyme production till 6^{th} cycle with a max of 4000U/ml.

INTRODUCTION

Proteases are ubiquitous, being found in a wide diversity of sources such as plants, animals, and microorganisms. They are degradative enzymes which catalyze the hydrolysis of proteins. Proteases not only play an important role in the cellular metabolic processes (Tremacoldi *et al.*, 2007), but also have many applications in detergents, leather processing, silver recovery, medical purposes, food processing, feeds, the chemical industry, as well as waste treatment (Ma et al., 2007). Proteases constitute 60-65% of the global industrial market most of which are alkaline proteases (Banerjee et al., 1999; Ellaiah et al., 2003). Among the various proteases, Bacterial proteases are more significant compared with animal and fungal proteases (Joo et al., 2003). And among Bacteria bacillus species are the specific producers of extracellular proteases.

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. Immobilization of whole cells for the production of extra cellular 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). The method of immobilization seems to be promising in the development of biotechnology, includes continuous technology, retention of plasmids bearing cells, prevention of interfacial inactivation, stimulation of production, excretion of secondary metabolites and production against turbulent environment (Kulkarni and Kaliwal, 2009). However, proper selection of immobilization techniques and supporting material is needed to minimize the disadvantages of immobilization. One of the most suitable methods for cell immobilization is entrapment in sodium 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 bimolecular and microorganisms (Orive *et al.*, 2003). Beads of sodium alginate are prepared under mild condition and have been used extensively for microencapsulating and entrapping cells.

In this investigation, we have undertaken to study optimal culture conditions for the production of protease by immobilizing *Bacillus* sp. JB-99 in sodium alginate. Moreover Binocular microscope was set to elucidate the morphological features of *Bacillus* sp. JB-99 cells entrapped in the alginate matrix. We have evaluated the efficiency of immobilized biocatalysts in repeated batch fermentation for production of alkaline protease.

MATERIALS AND METHODS

Microorganism and maintenance

Bacillus sp. JB-99 was previously isolated in our laboratory (Johnvesly and Naik, 2001) and was regularly maintained on chemically defined medium.

Sodium alginate entrapment of cells

The alginate entrapment of cells was performed according to the previously described method (Johnsen and Flink, 1986). Alginate was dissolved in boiling water and autoclaved at 121°C for 15min. Cells were harvested during the mid logarithmic growth phase by centrifugation (5000g for 10min), resuspended in 2 ml of saline and added to 100 ml of sterilized alginate solution. This alginate/cell mixture (with

* Corresponding author: +91 (O) 836-2779533
E-mail address: b_kaliwal@yahoo.com

stirring on magnetic stirrer) was aseptically extruded drop by drop into a cold, sterile 0.2M $CaCl_2$ solution through a sterile 5ml burette. Gel beads of approximately 2mm diameter were obtained. The beads were hardened by resuspending into a fresh $CaCl_2$ solution for 24h 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 50ml production medium and cultivated for the required time.

Measurement of cell concentration in sodium alginate beads

The contents of the cells entrapped in the alginate beads were measured by dissolving the gel beads in 10 ml of sodium pyrophosphate (1% w/v) followed by serial dilution and plating on nutrient agar plates. The number of bacteria/ml/gm sample were calculated as follows.

$$\text{Organisms/ml/gm sample} = \frac{\text{Number of colonies (average of 3 replicates)}}{\text{Amount plated x dilution}}$$

Optimization of enzyme production by immobilized cells

Enzyme production was optimized in immobilized cells by using various concentrations of sodium alginate (20, 25 and 30g/l) for cell entrapment and by varying the bead numbers (100 to 500 beads/flask), the influence of the initial cell loading (ICL) was tested. The pH of the chemically defined medium varied from 7.0-9.0, by addition of sterilized sodium carbonate solution. Both the cell growth in freely suspended cultures and cell leakage from the gel beads were determined.

Fermentation conditions for both free and immobilized cells

The reusability of beads of *Bacillus* sp. JB-99 cells immobilized in alginate gel (3%) was examined by batch fermentation and inoculating them into a 250ml Erlenmeyer flasks containing 50ml of chemically defined medium (pH 8.0), incubated at 37°C at 120rpm for 24-120 hours. This process was carried out by decanting the spent medium every 48h and replacing it by fresh medium after washing the alginate beads using saline. The alkaline protease enzyme was produced using free cells on the same medium, but with pH 10.5 and incubation at 45°C.

Extraction of enzyme and assay

The enzyme from culture filtrate was extracted by centrifuging at 10000 rpm for 10min at 4°C. The supernatant was used as source of alkaline protease. Assay of protease was carried out as per the method of Kembhavi *et al.*, (1993). One unit of protease activity is defined as the amount of enzyme required to liberate 1 µg of tyrosine per ml per min under experimental conditions.

Microscopic examinations

The sections of immobilized cell beads were fixed, stained and observed under a Binocular Compound Microscope (HFX-DX 218411 Nikon Japan) to observe the entrapped cells.

RESULTS

Measurement of cell concentration in sodium alginate beads

Upon serial dilution about 60 colonies were observed on a $1:10^5$ dilution and their number recorded was 6×10^6 cfu/ml/gm.

Effect of alginate concentration

Effect of alginate concentration of cell entrapment was shown in Fig-1. The production of alkaline protease was improved using increasing the alginate concentration from 20 to 25g/L and reached a maximum of 3500 U/ml at 25 g/l alginate at 72 h of incubation. This was about 3 fold lower than that obtained by free cells (12000 U/ml). Moreover, at low alginate concentration (20 g/l) the beads showed rapid leakage of cells compared to high alginate concentration 30 g/l (Fig-2). However, further increase in alginate concentration beyond 25 g/l resulted in lower enzyme yields.

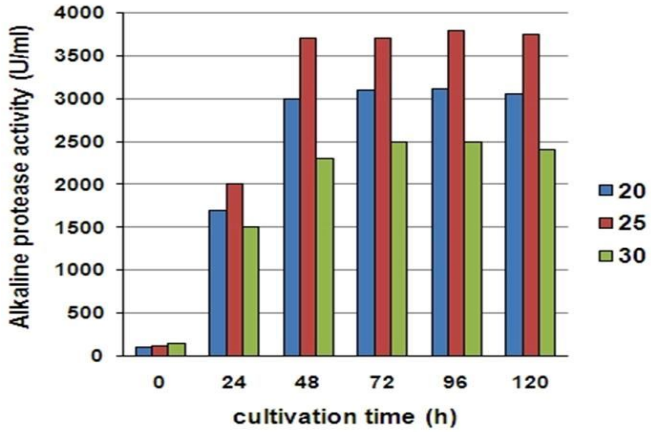

Fig.1 Effect of sodium alginate concentration (g/l) on enzyme production (U/ml

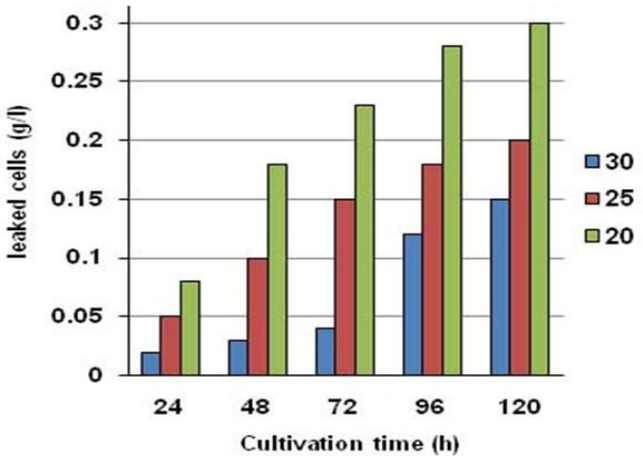

Fig.2 Effect of alginate concentration on cell leakage (g/l)

Effect of Ph

The pH optimized for alkaline protease production was 8.5-9.0; further alkalinity resulted in dissolution of beads. Around 4000 U/ml of enzyme activity was observed with the optimized pH. The decrease in the enzyme titers could be seen in immobilized cells when compared to free cells might be due to the less pH of the medium (pH 8.5).

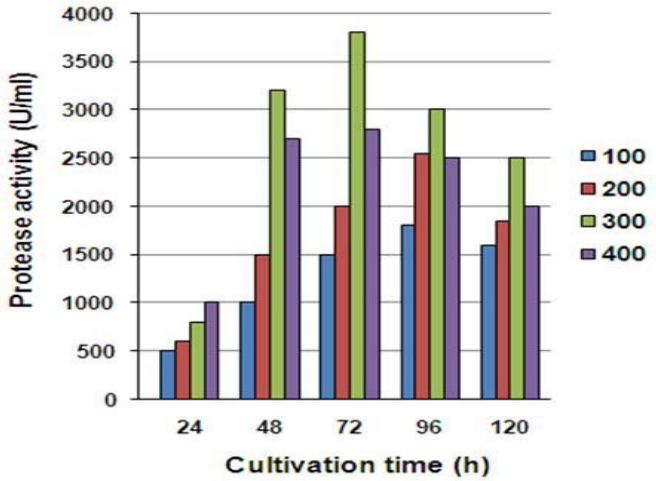

Fig. 3 Effect of initial cell loading in numbers on Alkaline protease production (U/ml)

Effect of initial cell loading (ICL)

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 were accompanied by increase in the protease concentration (Fig-3)**.** This might be due to increase in the total surface with a greater number of gel beads, which leads to facilitates the mass transfer from/to the sodium alginate beads. The results also indicate that using 300 beads/flask was the most suitable inoculam level yielded 3200 U/ml at 48h and proceeds up to 120h and yielding a maximum of 3800 U/ml at 72 h of incubation. Higher or lower inoculam levels resulted in reduced enzyme yield. The cell leakage was almost proportionate in all the initial cell loads, started from 24h and terminates till 120h of incubation (Fig-4).

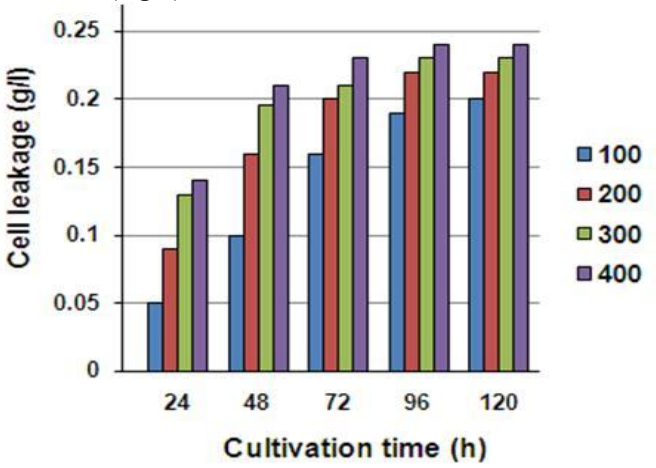

Fig. 4 Effect of initial cell loading on cell leakage (g/l)

Comparison of protease production by Bacillus sp. JB-99 using free and immobilized cells

The protease production by sodium alginate immobilized cells as compared to free *Bacillus* sp. JB-99 cells were shown in Fig-5. In case of free cells, protease production started to increase after about 6h and reached a maximum activity of 11500 U/ml at the end of logarithmic phase at 24 h and gradually decreases until the end of the cultivation. While, immobilized *Bacillus* sp. JB-99 cells in sodium alginate beads showed a significant increase in the production of protease enzyme from the beginning of the cultivation process. The enzyme activity begins from initial stages and and reached a maximum of 4000 U/ml at 48h. The activity remained constant up to 144h of incubation thereafter slowly declined.

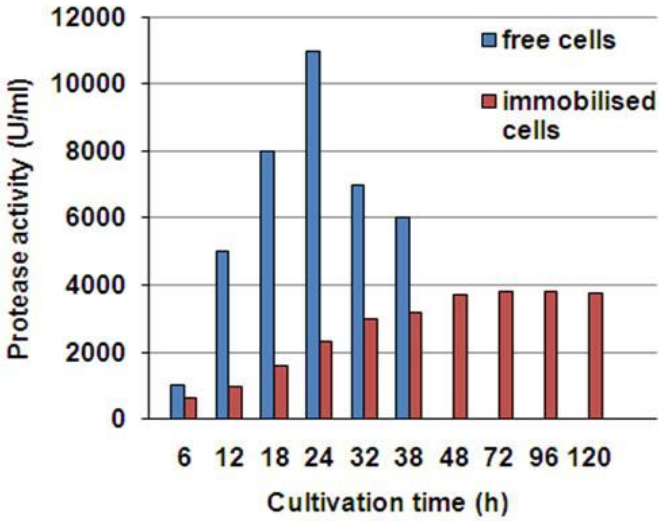

Fig. 5 Alkaline protease production by free and immobilized cells

Reuse of immobilized cells for alkaline protease production in repeated batch 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 6 cycles (Fig-6).

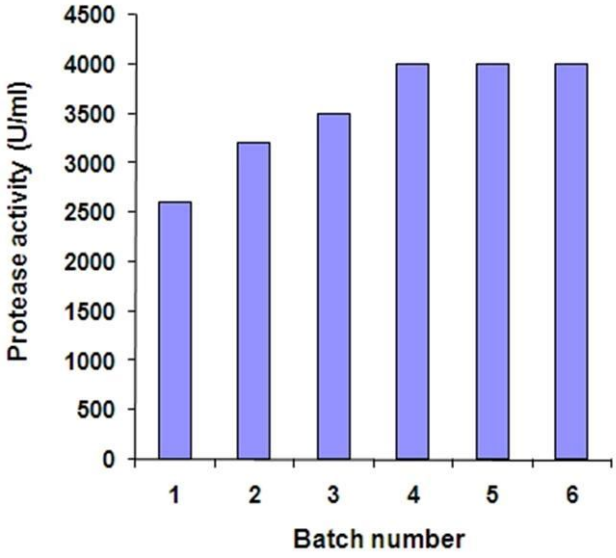

Fig. 6 Repeated batch production of alkaline protease by immobilized cells in sodium alginate beads

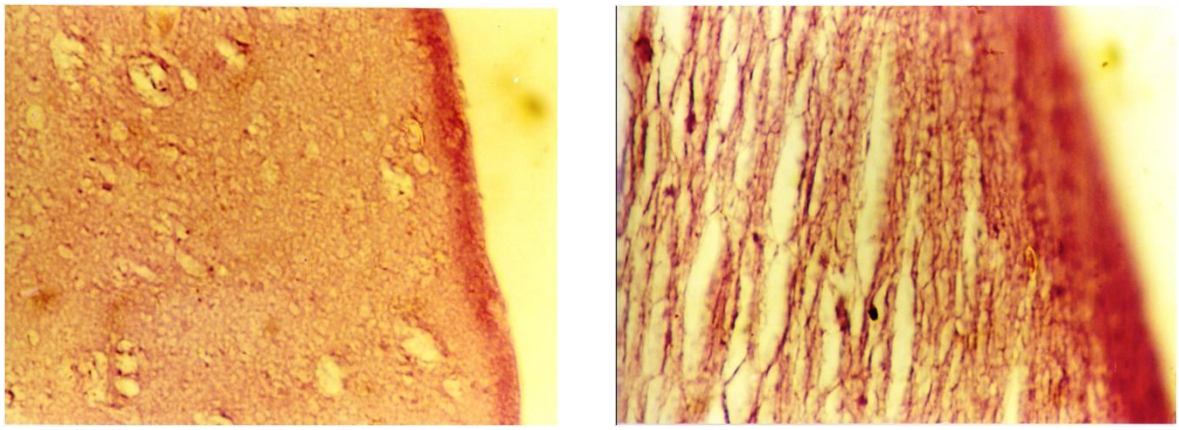

Fig 7 Sodium alginate beads with unentrapped (left) and entrapped cells (Right)

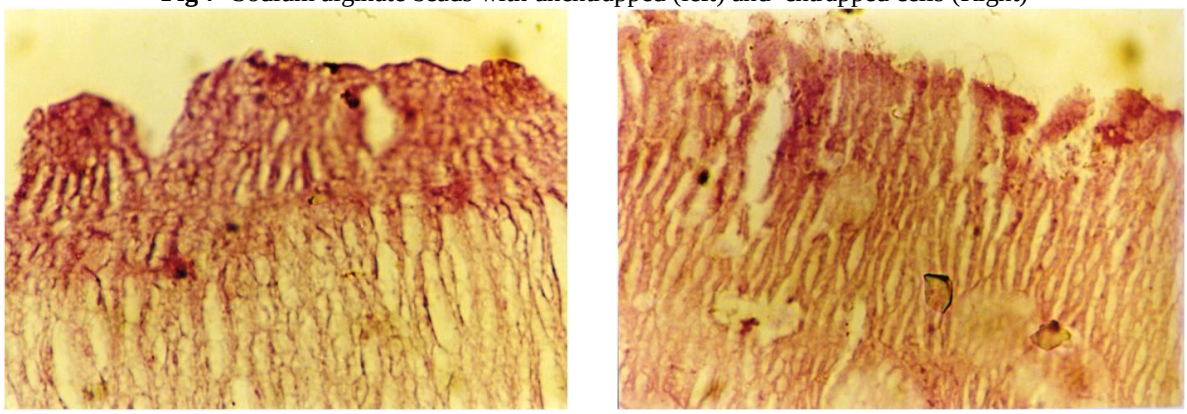

Fig.8 Entrapped cells in sodium alginate beads accumulated at the edge (left) of the beads and total entrapment (Right)

Alkaline protease was produced in repeated batch shake cultures and the time for each batch was 48h. When the maximum activity was reached, the culture supernatant was decanted and added with 50 ml of fresh medium. Alkaline protease activity of entrapped cells compared to those with free cells cultured in parallel, the results obtained at the end of the first cycle showed about 2600 U/ml of activity, a significant increase could be observed in the second cycle, i.e., 3200 U/ml. This high alkaline protease production continued after transfer into fresh medium at the end of the 3rd cycle of cultivation (108h), the alkaline protease yield of the biocatalysts was 3500 U/ml as compared with the first cycle. The highest activity of 4000 U/ml obtained during the repeated batch shake flask experiment was reached in the 4th 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 4000 U/ml up to the sixth cycle (12 days).

Binocular Compound Microscopy (BCM) studies

The internal structures of sodium-alginate beads in the presence and absence of *Bacillus* sp. JB-99 cells were studied under a Binocular Compound Microscope (Fig-7 & 8). The beads without immobilized cells in the left and randomly distributed cells could be seen in Fig-7. 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 in the medium (Fig-8).

DISCUSSION

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 (Beshay, 2003). 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. Immobilization is a strategy for protecting cells from shear forces. Many different techniques for immobilizing cells have been proposed (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. Sodium 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 was used in this work, it was flexible enough in its properties to be suitable for successful immobilization of *Bacillus* sp. JB-99 cells.

In this study, we varied the concentrations of alginate solutions to prepare the biocatalyst beads to determine the impact of different kind of constraints on the physiology of *Bacillus* sp. JB-99 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 behavior of immobilized *Bacillus* sp. JB-99 cells. Fig-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 and 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 and Beshay, 2003). At low alginate concentration (20g/l) the beads shown rapid leakage due to their relative softness, compared to the harder beads (30g/l), where cell leakage is restricted (fig-2).

Relativity of enzyme yield on initial cell loading (ICL) in the form of the number of alginate beads/flask was studied (Fig-3 and 4). The positive effect of increasing cell loading which led to improve enzyme yield and also cell leakage. Enzyme yield decreased after optimum ICL levels. This could be attributed to the fact that when the number of beads increases the nutrient/bead ratio decreases, which may become limiting. Similar results obtained for the production of gluconic acid by *Aspergillus niger* immobilized in Ca-alginate beads (Rao and Panda, 1994).

The possibility for re-use the sodium-alginate biocatalysts to produce alkaline protease in semi-continuous mode revealed that protease production increased gradually with repeated batch cycles. Increasing of protease productivity of the immobilized cells during the early 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 *Bacillus* sp. JB-99 cells immobilized in sodium-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) and it was found that protease production by immobilized *Saccharomyces marcescens* increased with repeated growth cycles, and reached a maximum after 5 cycles. Elibol and Moreira, (2003) reported approximately 3.5 fold increase in volumetric productivity of protease after the fourth cycle.

In conclusion, the results show that sodium alginate entrapment is a promising method of *Bacillus* sp. JB-99 immobilization for alkaline protease production. Alkaline protease production by immobilized cells is not superior to that of free cells because it leads to lower 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 48h leads considerable enzyme activity which is higher than that obtained with previous experiments.

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