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Research Article

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Enhanced Production of Cellulolytic Enzymes from Immobilized Cells of *Aspergillus niger*

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ABSTRACT

CMCase production by immobilized Aspergillus niger in submerged fermentation was studied.The isolated culture of Aspergillus niger was immobilized in calcium alginate beads. Studies were carried out on different parameters like alginate concentration, incubation time, bead inoculum, $CaCl_2$ concentration and curing time, which affect the productivity and stability of the immobilized system. The maximum CMCase activity of 0.37 IU/ml was achieved with 4% alginate concentration, 48h of incubation time, 40 beads/flask of inoculum, 0.2M $CaCl_2$ concentration and 60 min of curing time.

Keywords: *CMCases, Calcium alginate, Aspergillus niger*

INTRODUCTION

Lignocellulolytic enzymes are of great importance in the present biotechnological era with their all-embracing applications in bioscouring of cotton, degumming of plant fibers, waste water treatment, vegetable oil extraction, tea and coffee fermentation, fruit juice extraction and its clarification, bleaching of paper, poultry feed additives, alcoholic beverages and food industries. Nowadays, there is an increasing demand to replace some traditional chemical processes with biotechnological processes involving microorganisms and enzymes such as pectinases[1,2]. These enzymes not only provide an economically viable alternative, but are also environmental friendly[9]. The reason is that the pH optima of these enzymes are in the range found naturally in materials tobe processed and the enzymes are secreted into the culture media, making the downstream processing easier[3]. Microbial products are usually obtained from free or immobilized cells. The immobilized whole cell technology has several advantages over ordinary suspension culture systems; like elimination of enzyme purificationand extraction step, higher yield of enzyme activity after immobilization, higher operational stability, greaterresistance to environmental perturbations and lower effective enzyme cost[6]. Immobilization of the whole cells is currently a very active area of research because of their wide range of applications.

Use of alginate gel for immobilisation of cells have many advantages like inexpensive,easy to handle,provide mild conditions.

Immobilization of whole cells offer some advantages such as the ability to separate cell mass from the bulk liquid for possible reuse, facilitating continuous operation over a prolonged period[5,10].

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MATERIALS AND METHOD

Materials

The immobilization support (sodium alginate) and all other chemicals used were of analytical grade, supplied by Hi Media Lab, Bombay, India.

Microorganism

A mold strain A*spergillus niger*, isolated from soil and identified. It was observed to produce extracellular lignocellulolytic enzymes i.e CMCase. It was maintained on potato dextrose agar slant, grown at 28^{o}C for 5 days and stored at 4^{o}C with a periodic regeneration. Fungal inoculum was prepared by scrapping 5 days old slant with an inoculation loop into 5ml sterile distillled water. The suspension was filtered through sterile glass wool to form uniform suspension by removing the hyphal filaments. Five ml of spore suspension (1 x 10^6 spores / ml) was used as the inoculum.

Immobilization of microbial cells

Calcium alginate entrapment

The spores were immobilized in calcium alginate by entrapment. Alginate was dissolved in boiling water and autoclaved at 121°C for 15 min. Gel beads were obtained by adding alginate and cell suspension drop by drop into a cold, sterile 0.2 M $CaCl_2$ solution. The beads were now hardened by curing them at 4°C. Finally these beads were washed with distilled water to remove excess calcium ion and un-entrapped cells. Then the beads were transferred to growth medium and cultivated for the required time.

Fermentation

The beads were washed with sterile distilled water and transferred into production medium. The production medium is composed of (g/l): Urea 0.3, $(NH_4)_2SO_4$ 1.4, KH_2PO_4 2, $CaCl_2$ 0.3, $MgSO_4 7H_2O$ 0.3, bactopeptone 0.75, and yeast extract 0.25. Trace elements were also added the pH of the medium was adjusted to 5.5 with 0.1M HCl before sterilizing in an autoclave at 121^{o}C for 15 min. After inoculation the flasks were incubated at 28°C for 96 h. Samples were withdrawn at regular time interval up to 96 hours and assayed for their enzymatic activities.

Optimization of enzyme production

1. Effect of different concentrations of alginate on enzyme production

Four different concentrations of sodium alginate (2%, 3%, 4% and 5%) were used for the preparation of beads and CMCase activities were estimated.

2. Effect of incubation time on enzyme production

The production medium inoculated with beads of immobilized *A. niger* was incubated at 30º C and small aliquot of samples were withdrawn aseptically after intervals of 24, 48, 72 and 96 hours and enzymatic activity was estimated.

3. Effect of curing time on CMCase production: Effect of various curing times (30, 60, 90 and 120 min) on the production of CMCase with sodium alginate beads was studied.

4. Effect of Calcium Chloride concentration

A concentrations range from 0.1 M to 0.3 M of $CaCl_2$ was used for the study of calcium chloride concentration.

5. Effect of bead inoculum on enzyme production

The effect of the bead inoculum was examined by varying the number of beads from 30 to 60 beads/flasks. It was assumed that, increasing initial cell loading in the form of the number of beads could increase CMCase production.

Enzyme assay

Reducing sugars in the culture filtrate were determined by dinitrosalicylic acid (DNS) method (Miller 1959) with glucose as standard. One unit of enzyme activity is defined as the amount of enzyme required to liberate 1µmole of glucose / ml/ min under the standard assay conditions. All the experiments were carried out in triplicate and the mean of the three values was presented.

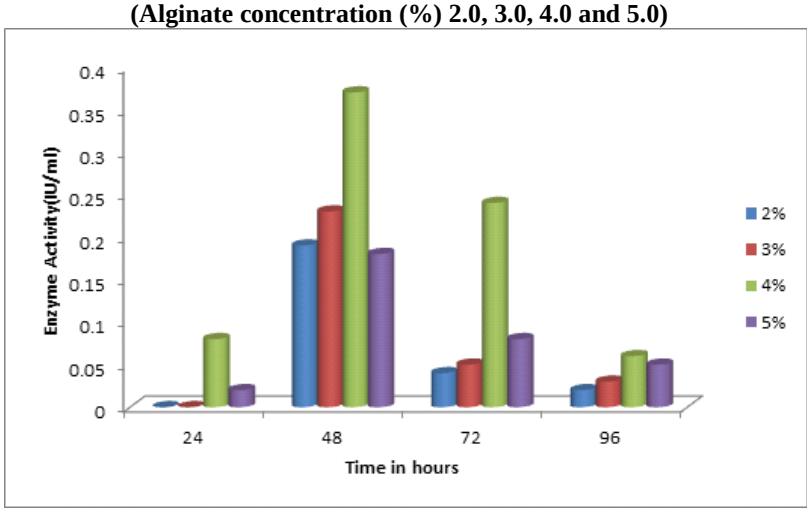
RESULTS AND DISCUSSION

Effect of alginate concentration

Different concentration of alginate i.e. 2%, 3%, 4% and 5% were used to optimize the alginate concentration for immobilization of *Aspergillus niger* cells. The CMCase production increases with

increase in alginate concentration upto 4% and then decreases with 5% beads. The beads of 4% alginate concentration gave maximum CMCase production of 0.37 IU/ml. Beads with 2% and 3% alginate concentration were less stable and shows leakage of cells while at 5% alginate concentration, beads were stable but shows lower CMCase production which may be because of the diffusional resistance offered by the beads[4].

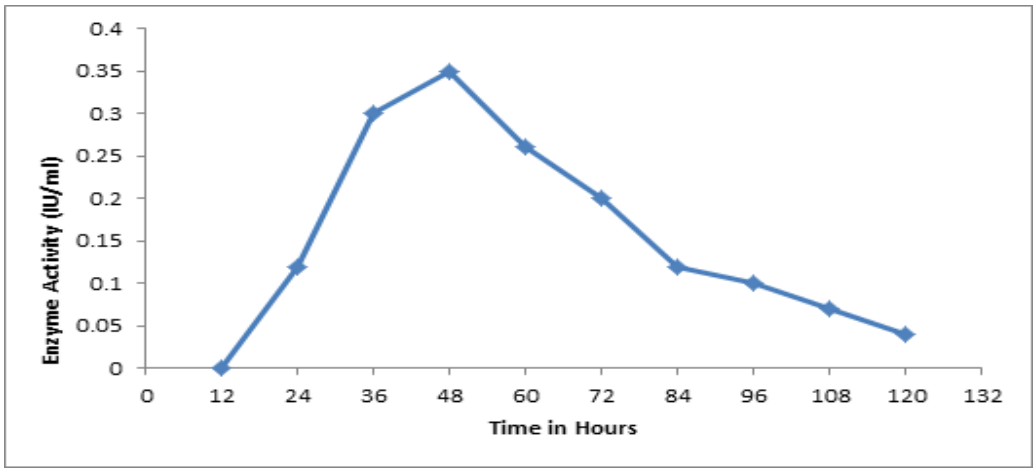
Fig. 1: Effect of CMCase production by *Aspergillus* cells immobilized in sodium alginate beads (Alginate concentration (%) 2.0, 3.0, 4.0 and 5.0)



Effect of incubation time on enzyme production

For optimization the time of maximum enzyme activity, the time of incubation was studied till 120 h. The enzyme activity gradually increased until 48 h, after which, there was a gradual decrease in the enzyme activity, which may be because of the exhaustion of nutrients and/or accumulation of metabolites in the fermentation medium. However, few reports suggest the optimum enzyme production at 96 h using the sodium alginate system[4,6].

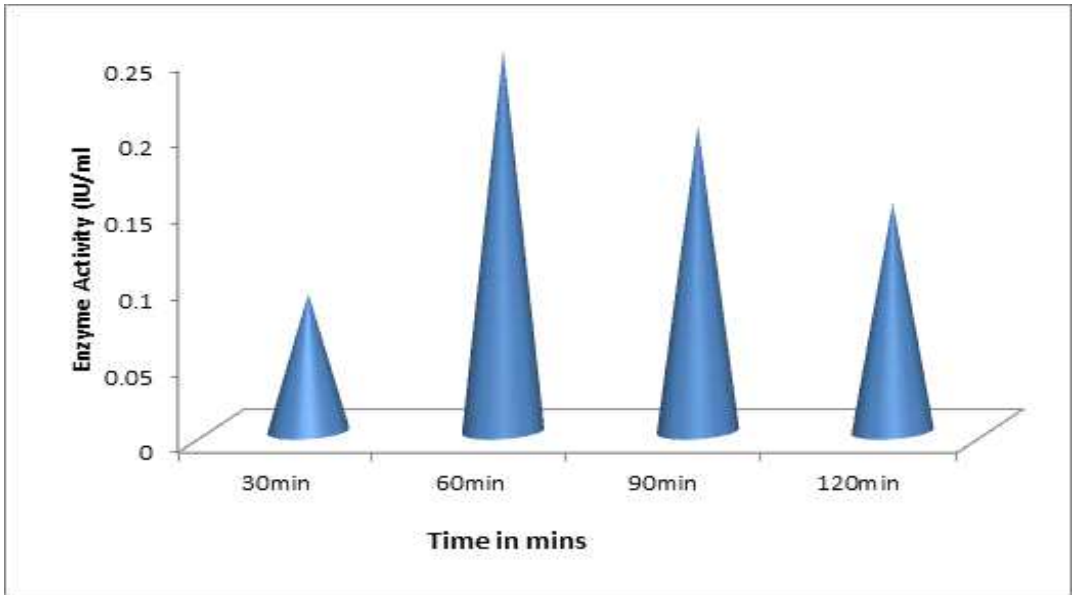
Fig. 2: Effect of incubation time on CMCases production by *Aspergillus niger* cells immobilized in sodium alginate beads



Curing time

To optimize the curing time or immobilization of *Aspergillus niger* cells, various curing time (30,60,90 and 120 mins.) were studied. The results revealed that beads cured for 60 mins were more stable and resulted in better CMCase production. There is no improvement in structural stability of beads because of prolonged curing.

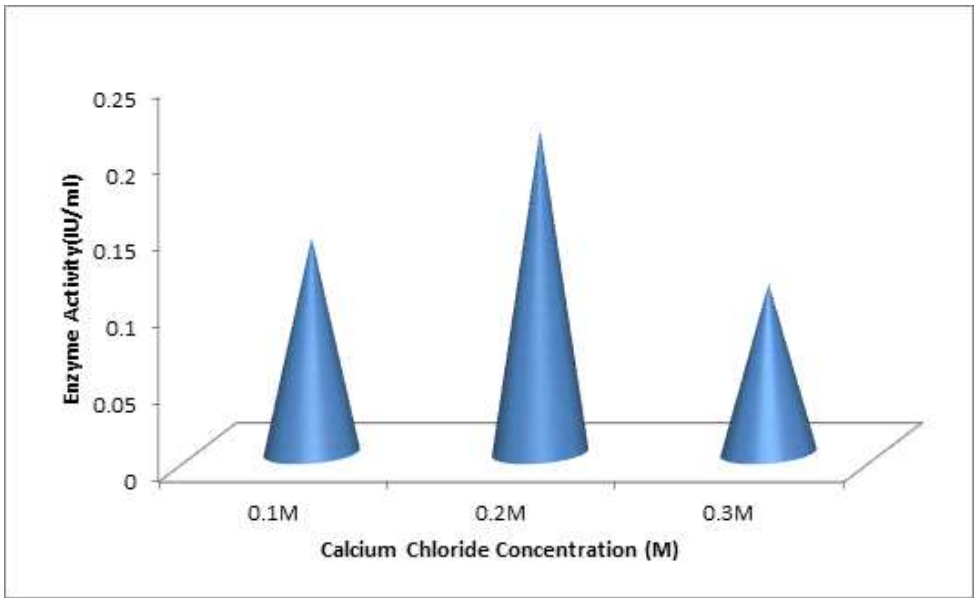
Fig. 3: Effect of curing time of calcium alginate beads



Effect of $CaCl_2$ concentration

Effect of various $CaCl_2$ concentration (0.1M, 0.2M and 0.3M) on stability of alginate beads were evaluated. It was observed that 0.2M $CaCl_2$ gave the highest enzyme activity as well as more stable beads. The reason might be the change in pH which might be one of the factors for affecting the activity of enzyme[8].

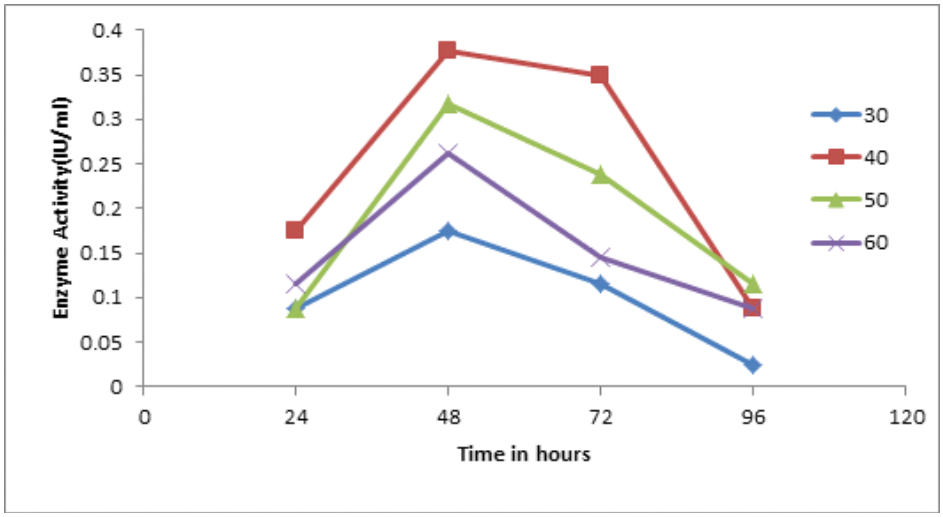
Fig. 4: Effect of $CaCl_2$ concentration on the production of CMCase by immobilized *A. niger* cells



Effect of bead inoculum on enzyme production

Different bead inoculum (30,40,50 and 60 beads per flask) was studied and maximum enzyme production was observed with 40 beads/flask,giving enzyme yield of 0.376 IU/ml. Decrease in enzyme activity beyond 40 beads/flask may be due to decrease in nutrients in the media because of the competition between cells because of which the nutrient concentration available in the flasks may not have been sufficient for optimal growth, leading to low enzyme production. This may be attributed to the fact that, when the number of beads increases, the nutrient/bead ratio decreases, which may become limiting[8].

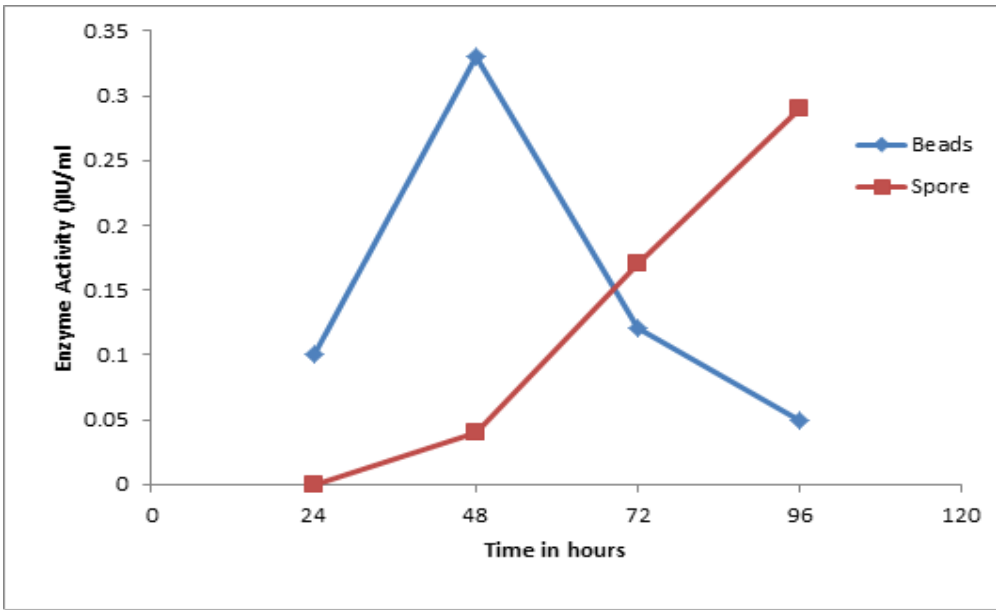
Fig. 5: Effect of bead inoculum on CMCases production by *Aspergillus* cells immobilized in Calcium alginate beads



Comparison of CMCases production by free and immobilized cells

The comparison of CMCase production by immobilized *A. niger* cells with the free cells was evaluated. Alginate entrapped cells have a marginally better product yield than free mycelia. The biosynthesis of CMCase by free mycelium reached maximum at 96hr. While immobilized cells in calcium alginate beads showed a significant increase in the production of CMCase from the beginning and reached maximum at 48 hr.

Fig. 6: Comparison of CMCases production by free and immobilized *Aspergillus* cells under optimized conditions



CONCLUSION

In conclusion, the results show that calcium alginate entrapment is a promising method of *Aspergillus* cells immobilization for CMCase production. CMCase 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 advantage of this technique is long life-term stability, the reusability and possibility of regeneration to be adaptable also to scale-up the obtained data.

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