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Research article

ENHANCED PRODUCTION OF PECTINOLYTIC ENZYMES FROM IMMOBILIZED CELLS OF MIXED *ASPERGILLUS SPECIES*

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ABSTRACT: The cells of isolated mixed culture of *Aspergillus fumigatus* and *Aspergillus sydowii* were immobilized in calcium alginate beads. Studies were carried out on different parameters like alginate concentration, incubation time and bead inoculum which affects the productivity and stability of the immobilized system. The best enzymatic activities were obtained with 3% alginate concentration, 48h of incubation time and 200 beads/flask of inoculum. Optimization of these factors causes an increase in enzymatic activities and the possibility of semi-continuous cultivation. Immobilized cells could be reused in five successive reaction cycles with a slight decrease in activities.

Keywords: Pectinases, Calcium alginate, *Aspergillus fumigatus · Aspergillus sydowii*

INTRODUCTION

Pectinolytic enzymes are of significant importance in the current 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. They have a share of 25% in the global sales of food enzyme (Ranveer et al., 2005). Nowadays, there is an increasing demand to replace some traditional chemical processes with biotechnological processes involving microorganisms and enzymes such as pectinases (Bajpai et al., 1999; Bruhlmann et al., 2000). These enzymes not only provide an economically viable alternative, but are also environmental friendly (Viikari et al., 2001). Pectinolytic enzymes used in food processing are predominantly derived from fungi. The reason is that the pH optima of these enzymes are in the range found naturally in materials to be processed and the enzymes are secreted into the culture media, making the downstream processing easier (Dhillon et al., 2004). 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 purification and extraction step, higher yield of enzyme activity after immobilization, higher operational stability, greater resistance to environmental perturbations and lower effective enzyme cost (Hemchander et al., 2001). To improve the economic feasibility of commercially used enzymes in food, pharmaceutical, medical, industrial and technological processes, soluble enzymes are usually immobilized onto a solid support. Immobilization of the enzymes onto solid support is currently a very active area of research because of their wide range of applications. There are several advantages over the use of soluble enzyme preparations including easier separation of reaction products from the incubation mixture, the ability to recover and reuse enzyme, stabilization of tertiary structure of enzyme and increased enzyme stability and operational lifetime. The immobilization procedure on alginate gel is not only inexpensive but also very easy to carry out and provide extremely mild conditions, so that the potential for industrial application is considerable (Busto et al., 2006).

The success of an immobilized enzyme for practical applications depends strongly on the properties of the carriers employed (Karakus and Pekyardimci, 2009). For production of extracellular enzymes, 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 (Zhang et al., 1989; Galazzo and Bailey, 1990).

MATERIALS AND METHODS

Materials

D-Galacturonic acid was procured from Sigma Chemical Co., St. Louis, USA. The immobilization support (sodium alginate) and all other chemicals used were of analytical grade, supplied by Hi Media Lab, Bombay, India.

Microorganism

Pectinase producing microorganisms were isolated from soil and were identified as mixed culture of *A.fumigatus* and *A.sydowii* 7373.09 ITCC from IARI, New Delhi, India. The isolated mixed culture was employed for further experiments and sub-cultured after every 2 week onto the agar medium and maintained at 4°C.

Seed culture

Submerged cultivation was carried out in 250mL Erlenmeyer flask with 100mL of production medium having composition (g/l) : $(NH_4)_2SO_4$, 2.0; K_2HPO_4, 2.0; Yeast extract, 3.0 and carbon source (pineapple residue), 2.0 at pH 5.5. The flasks were incubated for 48h at 37°C at 160 rpm. The culture was centrifuged at 10,000 rpm for 10 min at 4°C and the supernatant obtained was analyzed for pectinase production.

Immobilization of microbial cells

Calcium alginate entrapment

Alginate are available in very wide ranges with varying molecular masses, mannuronate:glucoronate ratios and distribution of units between blocks and alternating sequences. Alginate solution with a concentration range of 2.0-4.0% can be used for cell immobilization. In present study, different sodium alginate concentrations (2, 3 and 4%) were studied. Alginate was dissolved in boiling water and autoclaved at 121°C for 15min. Cells were harvested by centrifugation (10000g, 10min), re-suspended in 2mL of saline and added to 100mL of sterilized alginate solution. Gel beads of approximately 2 mm diameter were obtained by extruding alginate / cell mixture drop by drop into a cold, sterile 0.2 M $CaCl_2$ solution through a sterile 5mL syringe. The beads were now hardened by re-suspending them into a fresh $CaCl_2$ solution for 24h 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 100mL production medium and cultivated for the required time.

Fermentation

The beads were washed with sterile distilled water and transferred into 500 ml of Erlenmeyer flasks containing 100 ml of production medium. The production medium is composed of (g/l): $(NH_4)_2SO_4$, 2.0; K_2HPO_4, 2.0; KH_2PO_4, 2.0; Yeast extract, 3.0 and Pineapple residue, 3.0. The pH of the medium was maintained at 5.5. The flasks were incubated on orbital shaker (150 rpm) at 37°C for 120 h. Samples were withdrawn at regular time interval upto 120 h and assayed for their enzymatic activities. Alginate beads were recovered from the fermentation by filtration and rinsed with sterile 0.05M $CaCl_2$, and afterwards were again incubated in fresh medium, repeating this procedure for five consecutive cycles. Pectinases production was also carried out using free cells, so as to compare the activity produced by free cells with that of immobilized cells.

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Optimization of enzyme production

Effect of different concentrations of alginate on enzyme production

Three different concentrations of sodium alginate (2–4%, w/v) were used for the preparation of beads used for production of pectinolytic enzymes.

Effect of incubation time on enzyme production

The production medium inoculated with beads was incubated at 37°C for 120 h in an orbital shaker (150 rpm) and after intervals of 24, 48, 72, 96, and 120 h, samples were withdrawn aseptically and estimated for enzymatic activity.

Effect of bead inoculum on enzyme production

The effect of the bead inoculum was examined, by varying the number of beads from 50 to 300 beads/flasks. It was assumed that, increasing initial cell loading in the form of the number of beads could increase pectinases production.

Repeated batch cultivation

The advantage of using immobilized biocatalyst is that they can be used repeatedly and continuously. Therefore, the reusability of mixed culture immobilized in alginate gel was examined. The process was carried out in batch mode of operation by decanting the fermented medium at every 48h of cultivation period and replacing it by fresh medium after washing the alginate beads with sterile saline.

Enzyme extraction

The fermented broth of the flask was withdrawn at regular intervals and centrifuged at 8000 rpm for 2 min at 0–4°C. The supernatant obtained was used as the enzyme solution.

Enzyme assay

Polygalacturonase (PG) was assayed by measuring the reducing sugars released from the action of pectinase on citrus pectin using 3,5-dinitrosalisylic acid (DNS) reagent (Miller, 1959) from the reaction mixture containing 0.8 mL of crude enzyme solution and 0.2 mL (1%) citrus pectin in 0.2M acetate buffer of pH 5.0, incubated for 10 min at 40°C. One unit of PG is defined as the amount of enzyme that liberates 1 µmol of galacturonic acid per min under the assay conditions.

Pectin lyase (PL) activity was determined spectrophotometrically by monitoring the increase in absorbance (Albershiem, 1959). The reaction mixture contains the following: 1.25-mL of 0.15M citrate-phosphate buffer (pH 5.5), 0.25 mL of culture filtrate and 1.0 mL of 1% (w/v) citrus pectin. Pre-incubation were carried out at 40°C for 15 min. The reaction was started by adding pectin, control tubes contained the enzyme previously inactivated by incubation for 10 min at 100°C. One unit of PL activity was the amount of enzyme which produced an increase of one unit of A_{235} per minute.

Pectate lyase (PAL) activity was measured by the increase of A_{235} of 4,5 unsaturated reaction product. First, 0.3 mL of 1% (w/v) polygalacturonic acid neutralized by NaOH was mixed with 1.5-mL of $CaCl_2$ solution (0.0005 M in 0.1 M Tris-HCl [pH 9.0]) and 1.1 mL of distilled water. Then, 0.1 mL of enzyme sample was quickly added and the increase in A_{235} was measured (Collmer et al., 1988). One unit of PAL was defined as the amount of enzyme which produces 1 µmol of unsaturated product per min. The molar extinction coefficient for the unsaturated product at 235 nm (ε_{235}) is 4,600 M^{-1} cm^{-1}.

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RESULTS AND DISCUSSION

Effect of alginate concentration

In order to find out the optimum alginate concentration for immobilization of fungal strains, alginate solutions of different concentrations (2, 3 and 4%) were used. The results of experiments are shown in fig.1. The production of pectinases improved with increasing alginate concentration and reached maximum yield of 10850 U/L for PG, 2480 U/L for PL and 4786 U/L for PAL at 3% alginate. At low alginate concentration (2%) the beads were relatively soft and showed rapid leakage of cells compared to high alginate concentration 3% as shown. These results are in accordance with other findings (Angelova et al., 1998; Ellaiah et al., 2004; Shin et al., 2004). Although 4% alginate gave more stable beads, the production of pectinase was lower which could be due to the diffusional resistance offered by the beads (Ellaiah et al., 2004).

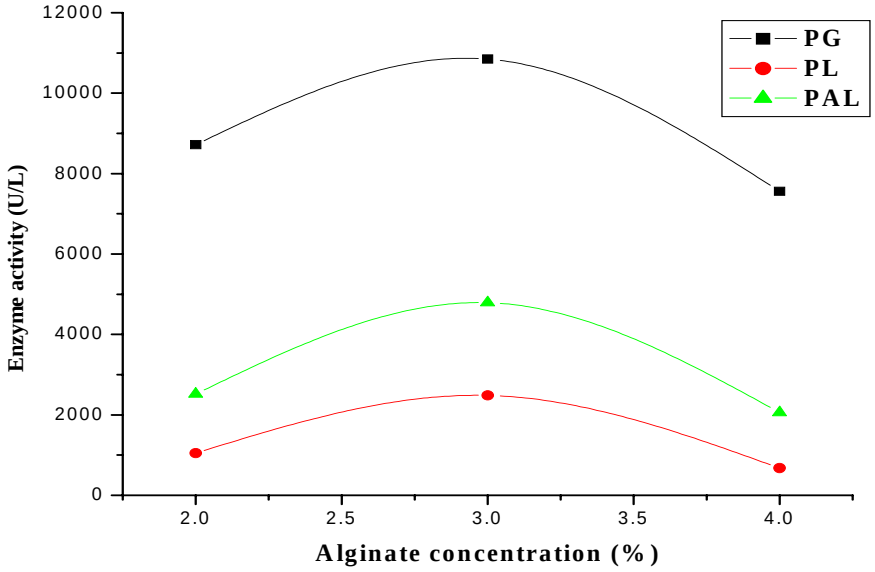

Fig.1 Effect of pectinases production by *Aspergillus* cells immobilized in sodium alginate beads (Alginate concentration (%) 2.0, 3.0 and 4.0).

Effect of bead inoculum on enzyme production

The results revealed that increasing the number of alginate beads/flasks upto 200 was accompanied by increase in the pectinases concentration (fig. 2). This may be because of an increase in the total surface with a greater number of gel beads, which leads to facilitate the mass transfer from Ca-alginate beads.

Results indicated that the most suitable inoculum level giving maximum enzyme yields of 10860 U/L of PG, 2500 U/L of PL and 4560 U/L of PAL were 200 beads per flasks. Increased or decreased inoculum level resulted in reduction of enzyme yield (fig.2). The decreased enzyme activity with increase in bead inoculum may be due to 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.

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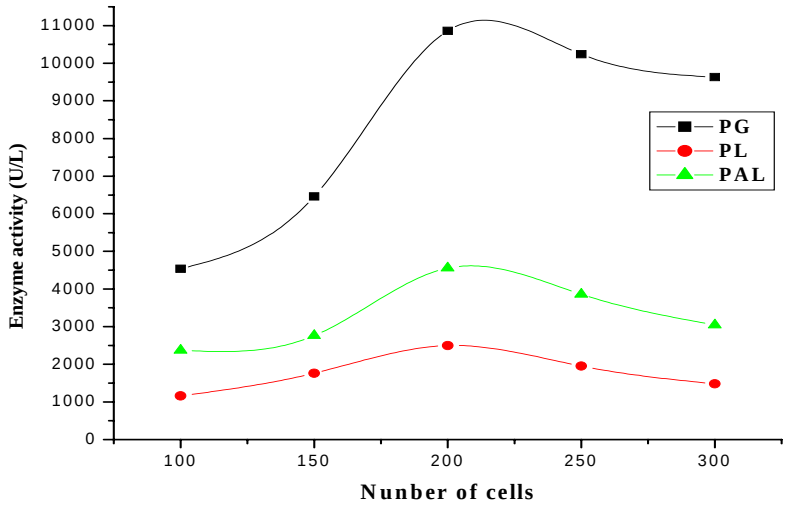

Fig.2 Effect of bead inoculum on pectinases production by *Aspergillus* cells immobilized in sodium alginate beads.

Effect of incubation time on enzyme production

To 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 sharp decrease in the enzyme activity, which may be because of the exhaustion of nutrients and/or accumulation of metabolites in the fermentation medium. However, a few reports suggest the optimum enzyme production at 96 h using the sodium alginate system (Hemchander et al., 2001; Ellaiah et al., 2004). The time of maximum enzyme production is significant because it is considered to be the cycle time for reusability transfer in repeated batch fermentation.

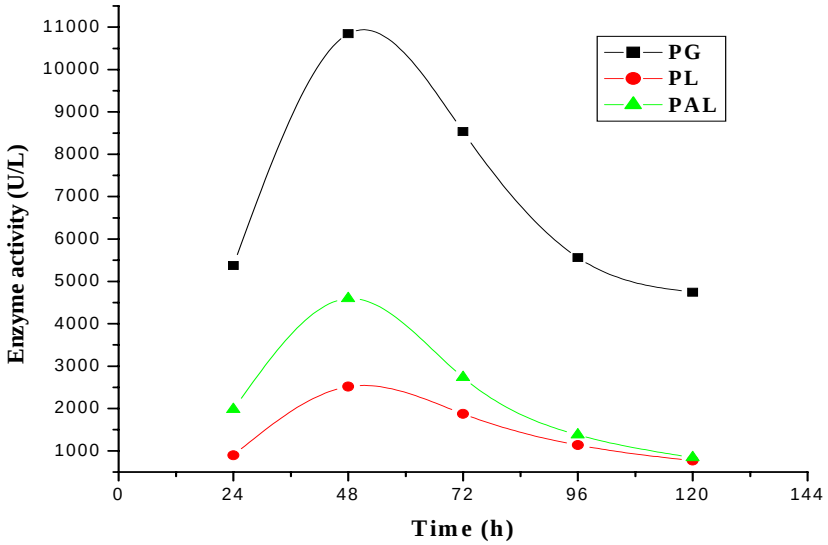

Fig.3 Effect of incubation time on pectinases production by mixed Aspergillus cells immobilized in sodium alginate beads.

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Comparison of pectinases production by free and immobilized cells

The comparison of pectinase production by Ca^{++}alginate immobilized cells with the free cells is given in fig.4. Pectinases production by free cells, increases with time and reached maximum at 48h and kept more or less constant until the end of cultivation. While immobilized cells in Ca^{++}alginate beads showed a significant increase in the production of pectinase enzyme from the beginning of the cultivation process.

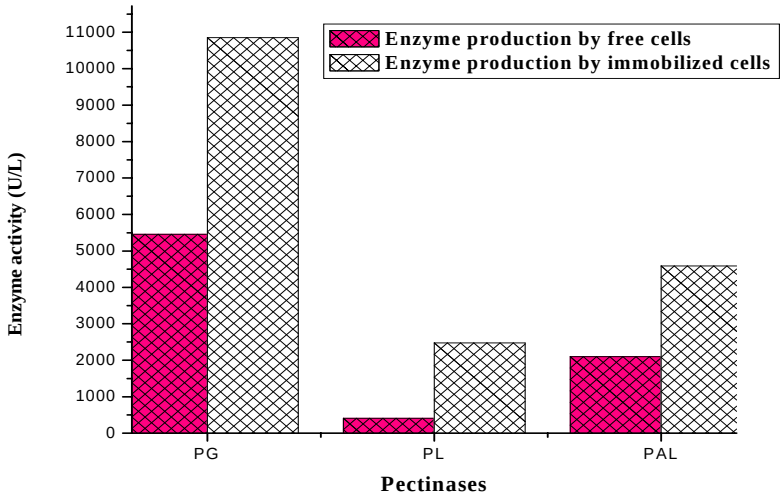

Fig.4 Comparison of pectinases production by free and immobilized *Aspergillus* cells under optimized conditions.

Reuse of the immobilized whole cells for pectinases production in repeated batch shake cultures

The operational stability of the biocatalysts obtained under optimal immobilization conditions (alginate concentration 3%, 200 beads/flask) was followed during 5 cycles (fig.5) to investigate the stability of biocatalyst and its ability to produce pectinases.

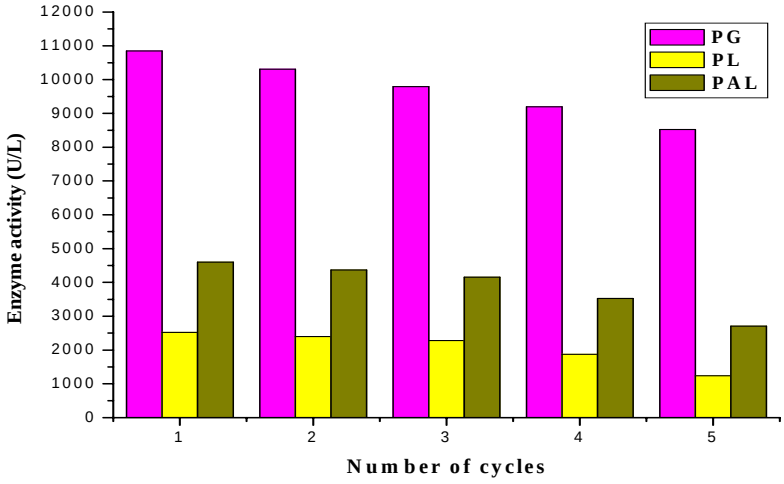

Fig.5 Repeated batch production of pectinases by *Aspergillus* cells immobilized in sodium alginate beads.

Pectinases were produced in repeated batch shake cultures and the time for each batch was 48h. When the estimated maximum activity level had been reached, the culture supernatant was decanted off and 100 mL of fresh medium was added to the Erlenmeyer flasks. The results obtained at the end of the first cycle showed a significant increase (10860 U/L PG, 2525 U/L PL and 4600 U/L PAL) in pectinases activity of entrapped cells compared to those with free cells cultured in parallel. The immobilized cells could be reused effectively for enzyme production three times with a loss of 5% in the enzyme activity. This reusability of this experiment is in accordance with the findings reported by other authors (Hemchander et al., 2001; Angelova et al., 1998; Ellaiah et al., 2004; Slokoska et al., 1999).

CONCLUSION

In conclusion, the results show that calcium alginate entrapment is a promising method of *Aspergillus* cells immobilization for pectinase production. Pectinase 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 rgeneration to be adaptable also to scale-up the obtained data.

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