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Production of lipase by immobilized cells of *Aspergillus niger*

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Abstract

Different materials viz. alginate, κ-carrageenan and polyacrylamide gel were examined for immobilization of whole cells of *Aspergillus niger* (ANT 90). The enzyme activity of whole cells immobilised in κ-carrageenan and polyacrylamide gel was low in comparison to these immobilized with alginate. Different concentrations of alginate were tried to study their effect on lipase production. Maximum production was observed with 3% alginate and beads with 60 min curing time (4230 U/l). Olive oil was shown to increase lipase production in both free and immobilized cells.

Keywords: Lipase; Production; *Aspergillus niger*; Immobilization; Alginate; κ-Carrageenan; Polyacrylamide

1. Introduction

Lipases (triacyl glycerol ester hydrolase, EC 3.1.1.3) catalyse the hydrolysis of fats to produce monoglycerides, diglycerides, free fatty acids and glycerol [1,2]. These reactions are reversible so that lipases also catalyse the formation of acylglycerols from glycerol and fatty acids. Lipases also possess characteristic properties like substrate specificity, stereospecificity and the ability to catalyse heterogenous reactions at the interface of water soluble and water insoluble systems. They differ from classic esterases in that their natural substrates are insoluble in water and their activity is maximal only when the enzyme is adsorbed to the oil–water interface [1,3]. Apart from their use in transesterification and ester synthesis, lipases can be used for peptide synthesis, biosurfactant production and resolution of racemic mixtures to produce optically active compounds [4].

Lipases occur in animals, plants and microorganisms. Microbial lipases have a broad spectrum of industrial applications as they are more stable compared with plant and animal lipases and they can be obtained cheaply. Some of the commercially important lipase producing fungi are: *Rhizopus arrhizus*, *Rhizopus japonicus*, *Mucor miehei*, *Aspergillus niger*, *Rhizopus niveus*, *Candida rugosa* [5–7], *Aspergillus terreus* [8], *Penicillium* sp. and *Aspergillus* sp. [9].

Microbial products are usually obtained from free or immobilized cells. Immobilization of cells can be considered as physical confinement or localization of microbial cells. The utilization of immobilized cells as industrial catalysts in continuous processes can be advantageous compared with batch fermentation procedures [10,11]. Immobilized whole cell technology has several advantages over enzyme immobilization [12,13].

The present paper deals with the immobilization of an indigenous *A. niger* mutant ANT 90 for the production of lipase using different entrapment techniques with matrices calcium alginate, κ-carrageenan and polyacrylamide gel. The effect of different incubation periods, optimum concentration of sodium alginate and the reusability of immobilized cells for lipase production were investigated.

2. Materials and methods

2.1. Micro-organism

A lipase producing strain of *A. niger* AU 15 was isolated in this laboratory. The strain was improved for lipase production by mutagenesis. The improved mutant was designated as ANT 90 and used for immobilization studies.

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It was grown on potato dextrose agar slants at 28 °C for 3 days and kept in at 4 °C until further use.

2.2. Preparation of spore suspension

The organism was subcultured on potato dextrose agar medium and incubated for 72 h at 28 °C. The spore crop of each slant was scrapped into 5 ml sterile water and shaken well with sterile glass beads on a rotary shaker for 30 min to break the spore chains and to make a uniform suspension. This suspension was filtered through sterile cotton to remove the hyphal filaments. This spore suspension (5×10^6 spores per ml) was used for preparing the beads.

2.3. Preparation of immobilized cells

2.3.1. Sodium alginate entrapment method

Spores were immobilized in calcium alginate by the traditional external gelation method [14]. About 20 ml of sodium alginate (3% w/v) and 5 ml of spore suspension (5×10^6 spores per ml) were mixed well and this slurry was added drop wise to 0.2 M $CaC1_2 \cdot 2H_2O$ solution at room temperature. The beads (~4 mm) formed were then cured in a refrigerator at 4 °C for l h. The beads were washed two to three times with sterile distilled water and used for the production of lipase.

2.3.2. κ-Carrageenan entrapment method

Eighteen ml of κ-carrageenan (4% w/v) maintained at 40–50 °C was mixed with 2 ml of spore suspension (5×10^6 spores per ml) and poured into sterile 4 in. diameter petriplates and allowed to solidify. It was then cut into equal small blocks (approximately $5 \times 5 \times 4$ mm^3). The κ-carrageenan blocks were cured in 2% KCl for l h in the refrigerator. These blocks were washed thoroughly two to three times with sterile distilled water and used for the production of lipase.

2.3.3. Polyacrylamide gel entrapment method

A chilled cell suspension was prepared by adding 2 ml (5×10^6 spores per ml) spores in 10 ml chilled sterile water. To 10 ml sterile potassium phosphate buffer (pH 7.0, 0.2 M) the following chemicals were added: acrylamide, 2.85 g; bisacrylamide, 0.15 g; ammonium persulphate, 10 mg and 1 ml *N*, *N*, *N′*, *N′*-tetramethyl thylendiamine (TEMED). The chilled cell suspension and chilled potassium phosphate buffer were mixed well and poured into sterile flat bottom 4 in. diameter petriplates. After polymerization, the acrylamide gel was cut into equal size cubes ($5 \times 5 \times 5$ mm^3). The acrylamide cubes were cured in sodium phosphate buffer (pH 7.0, 0.2 M) for l h in the refrigerator. These cubes were washed thoroughly two to three times with sterile water and used for the production of lipase.

All the above three operations were carried out under sterile conditions in a laminar flow unit.

2.4. Fermentation

Immobilized cells prepared by the above three methods, were added to 250 ml Erlenmeyer flasks containing 45 ml growth medium. Medium composition (g/l) was: glucose, 10; peptone, 20; NaCl, 3 and yeast extract, 5; with pH 6.0. Flasks were incubated on a rotary shaker (220 rpm) at 28 °C for 72 h. The growth medium was decanted, the beads washed with sterile distilled water and transferred into 250 ml Erlenmeyer flasks containing 45 ml production medium. The production medium composition (g/l) was: dextrose, 10; peptone, 20; yeast extract, 5; $(NH_4)_2$ SO_4, 5; Mg $(SO_4)_2$ $7H_2O$, 0.2; $Fe(SO_4)_2 \cdot 7H_2O$, 0.01 and NaCl, 5; with pH 6.5. The flasks were incubated on a rotary shaker (220 rpm) at 28 °C for 120 h. Samples were withdrawn at 24 h interval up 5 days and asayed for their lipase activity. Lipase production was also carried out with *A. niger* free cells using the above production and medium and activity produced by immobilized cells compared with that of the free cells.

2.5. Optimization

2.5.1. Repeated batch fermentation

Repeated batch fermentations were conducted with all three types of immobilized cells by running the fermentation for 96 h. At the end of each cycle the production medium was decanted, the immobilized cells were thoroughly washed with sterile water, a fresh production medium was added and the fermentation was continued.

2.5.2. Effect of various concentrations of sodium alginate on lipase production

Three different concentrations of sodium alginate (2–4%) were used for the preparation of beads used for the production of lipase. Fermentations were conducted as described earlier.

2.5.3. Effect of curing time on lipase production

Effect of various curing times (30, 60 and 120 min) on the production of lipase with sodium alginate beads was studied.

2.5.4. Efect of various concentrations of olive oil on the production of lipase

Different concentrations of olive oil were used to study their effects on the production of lipase with free and alginate immobilized cells. The fermentation procedure was carried out as mentioned earlier.

2.6. Lipase activity

Lipase activity in the culture broth was determined by titrimetry (using olive oil as substrate) [15]. One unit of lipase activity is defined as the amount of enzyme required to release 1 μmol of fatty acid at 37 °C under standard assay conditions (pH 7.0, reaction time 30 min).

All fermentations and assays were carried out in triplicate and the mean value are presented.

3. Results and discussion

The experimental results of the lipase production with free and immobilized cells using various entrapment techniques are shown in Table 1. Maximal production of lipase by free and immobilized cells was obtained at 96 h. Petrovic et al. [16] reported that maximum lipase production was obtained at 96 h, incubation by *Penicildum roquefortii*. The present results are consistent with this finding.

Significant process engineering advantages are evident for immobilized cells in repeated batch operations. *A. niger* cells in immobilized form are useful for repeated use for lipase production. Immobilized cells with sodium alginate, κ-carrageenan and polyacrymide matrices were used for the repeated batch fermentations Table 2. The productivity of lipase by cells entrapped in calcium alginate (4230 U/l) was higher than that of cells entrapped in κ-carragreenan (3860 U/l) and polyacrylamide (4070 U/l). The low yields with κ-carrageenan and polyacrylamide blocks may be due to diffusional resistances of nutrients and oxygen into the matrices. The beads prepared from sodium alginate were stable up to three cycles. The immobilization blocks prepared with κ-carrageenan were not able to retain their shape during repeated batch fermentation and disintegrated after three cycles. Blocks prepared with acrylamide were relatively stable but disintegrated after three cycles. This, results are in accordance with studies on *A. niger* [17] and *C. rugosa* [18]. Therefore, alginate was considered to be the best matrix for the production of lipase. As such, cells entrapped by sodium alginate were used for further optimization studies.

Table 1
Lipase production with free and immobilized cells at various incubation periods

Incubation period (h)	Lipase activity (U/l)			
	Free cells	Sodium alginate	κ-Carrageenan	Polyacrylamide
48	–	–	–	–
72	3110 ± 88	3070 ± 40	3160 ± 106	3040 ± 98
96	4090 ± 132	4225 ± 28	3850 ± 58	4075 ± 80
120	2660 ± 92	3220 ± 38	3060 ± 61	3120 ± 38

Table 2
Comparison of repeated batch lipase production with different immobilized cells

Batch number	Lipase activity (U/l)		
	3% Sodium alginate	4% κ-Carrageenan	Polyacrylamide
1	4230 ± 105	3860 ± 83	4070 ± 62
2	3950 ± 43	3800 ± 48	3750 ± 70
3	3830 ± 45	3750 ± 81	3700 ± 77
4	–	–	–

Table 3
Effect of sodium alginate concentration on production of lipase

Sodium alginate concentration (%)	Lipase activity (U/l)			
	Batch I	Batch II	Batch III	Batch IV
2	4095 ± 67	3825 ± 42	–	–
3	4200 ± 79	3905 ± 95	3820 ± 82	–
4	3910 ± 68	3720 ± 40	3320 ± 61	–

Table 4
Effect of curing time on alginate beads for the production of lipase

Curing time (min)	Lipase activity (U/l)			
	Batch I	Batch II	Batch III	Batch IV
30	4000 ± 98	3800 ± 77	–	–
60	4205 ± 91	3830 ± 85	3650 ± 81	–
120	3810 ± 117	3700 ± 70	3050 ± 65	–

Different sodium alginate concentrations were used for the preparation of beads and to study their effects on the production of lipase (Table 3). The production of lipase was maximal with 3% alginate (4200 U/l) in comparison with the other concentrations used. These results are in accordance with the results of Jamuna et al. [19]. Although 4% alginate gave more stable beads, the yield of lipase was lower. The decrease in production of lipase with 4% alginate could be due to the diffusional resistance offered by the beads. Based on this result 3% alginate was selected for subsequent studies.

The effect of curing time on the stability of beads for the production of lipase was studied (Table 4). The beads cured for 60 min were stable and resulted in better lipase production (4205 U/l). These results are also in agreement with the finding of Jamuna et al. [19]. Beads cured for 30 min were gradually disintegrated from second batch onwards, while the beads cured for 120 min were more stable, but yield at less lipase (3810 U/l). As such, 60 min was selected as the curing time for subsequent studies.

The effect of different concentrations of olive oil was also, studied on the production of lipase using free and alginate immobilized cell (Table 5). Maximal lipase production was observed at 1% olive oil in both cases (4290 and 41400 U/l), respectively. This indicated that olive oil is a lipase inducer. These results are in accordance with the studies reported by

Table 5
Effect of different concentrations of olive oil on lipase production

Concentration of olive oil (%)	Lipase activity (U/l)	
	Free cells	Immobilized cells
0.00	3090 ± 132	3225 ± 128
0.25	3193 ± 69	3285 ± 77
0.50	3428 ± 45	3588 ± 83
0.75	3922 ± 73	4078 ± 77
1.00	4290 ± 49	4400 ± 76
1.25	4132 ± 58	4324 ± 66

Yadav et al. [9], Freire et al. [20] and Waller and Comeau [21], who reported an increase in lipase activity when olive oil was incorporated into the production medium.

Based on the above results, it can be concluded that alginate was a good matrix for the cell immobilization for the production of lipase and olive oil is a good lipase inducer.

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