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IMMOBILIZATION OF *BACILLUS* SP. PROTEASE ON DIFFERENT MATRICES AND ITS ENZYMATIC CHARACTERIZATION

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ABSTRACT

Studies were conducted on the *Bacillus* sp. neutral protease immobilization on different matrices. The immobilized protease was prepared by three methods, such as entrapment on calcium alginate and gelatin; physical adsorption by celite and alumina; ionic binding by amberlite IR-120 and DEAE-cellulose. The maximum immobilization yield was obtained with celite adsorbed protease. The immobilized enzyme was characterized in comparison with free enzyme. The optimal pH of the immobilized protease was shifted to lower values; and exhibited good thermal stability. The reusability and storage stability of the immobilized enzyme on different carriers was analysed. The ability of *Bacillus* sp. protease activity even on immobilization and its characteristics could collectively be useful for commercial production.

KEYWORDS: ***Bacillus*** **sp., protease, matrices, celite and characterization.**

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1. INTRODUCTION

Immobilization of enzymes onto water-insoluble supports is widely used in industries, as they posses several advantages such as enzyme re-utilization, enhanced stability, easy separation of catalyst from the reaction mixture, reducing the delayed during enzyme production and ready application to automated continuous processes [1, 2]. Material used as a carrier should have chemical, physical and biological stability during processing, sufficient mechanical strength, so that they could be used in reactors and industry should be non-toxic for the immobilized cell or bio particle or product. Immobilization techniques involves the coupling of the enzyme with a selected solid support via covalent and (or) noncovalent interactions. Several noncovalent linkages include ionic and metal bindings as well as physical adsorption. Immobilization by adsorption results from weak forces between the protein and the solid surface, such as electrostatic and hydrophobic interactions or hydrogen bonds [3]. The support material decides the efficiency and stability of the enzyme upon immobilization. The water insoluble support material must also have good capacity to bind to enzyme, chemically inert, readily available and mechanically stable. This part of the study deals with the partial purification of neutral protease from *Bacillus* sp. and immobilization by various carriers using entrapment, physical adsorption and ionic binding methods. From these studies an ideal matrix material was determined. The properties of the immobilized enzymes were compared with that of free enzyme to confirm the most apt method of immobilization of protease for commercial uses.

2. MATERIALS AND METHODS

2.1. Materials

Amberlite IR - 120, Bovine Serum Albumin (BSA) and Diethylaminoethyl cellulose (DEAE - cellulose) were procured from Sigma Chemical Co. (St Louis, MO, USA). All other chemicals used were of analytical grade and were purchased from SD Fine Chemicals (Mumbai, India).

2.2. Protease assay

The protease activity was assayed according to Kunitz (1947) with some modifications [4]. The absorbance was recorded spectrophotometrically at 280 nm. Enzyme units were measured using tyrosine (0 - 100 µg) as standard. One unit of protease activity (U) was defined as the amount of enzyme which releases 1 µmole of tyrosine per minute under the assay conditions.

2.3. Immobilization methods

2.3.1. Entrapment of protease by calcium alginate and gelatin

The alginate entrapment of protease was performed according to the method of Johnsen and Flink (1986) [5]. Briefly, 3% sodium alginate solution was prepared and both alginate slurry and protease (3.09 U/g of carrier) were mixed and stirred. The resultant slurry was added drop wise into 0.2 M calcium chloride solution and left for curing at 4 °C with mild stirring at 60 rpm for 1 hour. The beads were then washed for 3 to 4 times with sterile distilled water. The enzyme was added to 20% sterile gelatin, maintained at 45 °C and poured into sterile petriplate. The gel was over-layered with 10 ml of 5% glutaraldehyde for covalent cross linking and hardening at 30 °C. The resulting block was cut into small cubes (4 mm^3) and the cubes were washed thoroughly with sterile distilled water for complete removal of excess glutaraldehyde.

2.3.2. Physical adsorption of protease by celite and alumina

The physical adsorption of protease onto alumina was performed according to the method of Weetall (1979) [6]. 1 g of alumina was incubated with 2.56 U of protease and at 4 °C overnight. 1 g of celite was incubated with 2.56 U of protease at 4 °C. The unbound enzymes were removed by washing with Tris-HCl buffer (50 mM, pH 7.0).

2.3.3. Ionic binding of protease by amberlite IR – 120 and DEAE cellulose

The cation exchanger amberlite IR - 120 was equilibrated with 50 mM acetate buffer of pH 6.0. The anion exchanger DEAE cellulose was equilibrated with 50 mM Tris HCl buffer of pH 7.0. They were incubated with 2.56 U of

protease per gram of carrier in the respective buffer at 4 °C for 12 hours. The unbound enzyme was removed by washing with the same buffer.

2.4. Immobilization yield

The amount of protease immobilized on to different support materials was calculated from the difference of protease activity before and after immobilization in the enzyme solution. The immobilization activity yield is defined as the ratio of the measured activity of immobilized enzyme to that of difference in the enzyme activity of added enzyme activity and unbound enzyme [7]. Immobilization yield is defined as follows

Immobilization yield % = I / A − B x 100

where I is the total activity of the immobilized enzyme, A is the total activity of the added enzyme and B is the total activity of the unbound enzyme.

2.5. Properties of immobilized protease

2.5.1. Effect of pH and temperature on immobilized protease

The effect of pH on the activity of free and immobilized *Bacillus* sp. protease was studied in different buffers (pH 6.0 - 9.0) for 24 hours at 37 °C. The pH was adjusted using the following buffers (50 mM): citrate phosphate (pH 6.0), Tris HCl (7.0 - 9.0). The effect of temperature was studied in different temperatures for 1 hour and the activity was measured.

2.5.3. Thermal stability, reusability and storage stability of immobilized protease

For thermal stability, 250 mg of immobilized protease were incubated in Tris HCl buffer pH 7.0 and 60 °C for 60 minutes. To determine the reusability of the immobilized protease the enzyme was reacted with BSA as substrate. The enzyme recycle study was performed by adding 250 mg of immobilized protease to BSA solution and kept at 45 °C for 1 hour. At the end of every reaction cycle, the product was removed by centrifugation and was assayed. The remaining immobilized protease was washed two times with 2 ml of 50 mM Tris HCl buffer pH 7.0. The same enzyme was used for next 5 reactions in the same conditions and its reusability was calculated. To determine the storage stability immobilized protease was stored at room temperature for 6 months and the enzyme activity was calculated.

3.RESULTS AND DISCUSSION

The protease was immobilized by three method such as entrapment (calcium alginate and gelatin); physical adsorption (celite and alumina) and ionic binding (amberlite IR-120 and DEAE-cellulose). The results of immobilization in terms of its immobilization yield (%) are tabulated in the table 1.

Table 1
Immobilization of protease to different carriers

Type of immobilization	Carrier	Added enzyme (U/g carrier) A	Unbound enzyme (U/g carrier) B	Immobilized enzyme (U/g carrier) I	Immobilization Yield (%) = I / A-B x 100
Entrapment	Calcium alginate	3.09	1.43	0.37	22.26
	Gelatin	3.09	0.90	0.36	16.37
Physical adsorption	Celite	2.56	1.72	0.68	80.39
	Alumina	2.56	1.30	0.52	41.48
Ionic binding	Amberlite IR-120	2.56	1.64	0.57	61.68
	DEAE -Cellulose	2.56	1.37	0.58	48.61

3.1. Entrapment of protease by calcium alginate and gelatin

An immobilization yield by calcium alginate and gelatin of 22.26% and 16.37% was observed respectively. So entrapment of protease was not a suitable method for immobilizing protease from *Bacillus* sp. Several other works have been carried out to immobilize alkaline protease using polyacrylamide with its different concentrations for entrapment [7]. It is possible to use many other polymers as matrix such as

carrageenan, collagen, polyurethane, polyvinylalcohol etc. The decreased yield of protease immobilized to calcium alginate and gelatin may be due to the leak out of the enzyme from the gel at the assay temperature (45 °C). β - amylase isolated from *Bacillus subtilis* DL5 was covalently immobilized in 10% gelatin with activity of 3.33 U/ml and its characteristics was studied [8].

3.2. Physical adsorption of protease by celite and alumina

The results in the table 1 show that celite and alumina gave yield of 80.39% and 41.48% respectively. Celite was found to be the best support material for the immobilization of protease. Its advantages as a physical adsorbant are that it is inexpensive, chemically inert and evenly distributes the catalyst. This support has been used for immobilization of many other enzymes [9]. Alumina was relatively not good carrier for immobilization of protease. Alumina is an inorganic carrier with good mechanical properties, thermal stability and resistance against microbial attack and organic solvents. Chemically activated alumina-phosphate complex formed by the interaction of phosphonate groups in certain selected compounds are responsible for such immobilization of enzymes and other molecules. A number of proteases immobilized on alumina through such phosphate interactions [10].

3.3. Ionic binding of protease by amberlite IR -120 and DEAE cellulose

An immobilization yield of 61.68% was obtained with amberlite IR – 120 and it was the second best carrier amongst the selected materials. This resin was strongly acidic cation exchange resin of the sulfonate polystyrene types. An immobilization yield of 48.61% was obtained with DEAE - cellulose. It is poly cationic in nature and is commonly used for the purification and immobilization of various enzymes as it adsorbs proteins on the basis of ionic interactions. This inhibition of the immobilized enzymes cannot be correlated with enzyme denaturation during immobilization process, because the same protease was partially purified with DEAE cellulose with 0.3 M sodium chloride, showed same activity as that of free enzyme. Therefore this inhibition may be due to the involvement of the fixation process to the active sites of the enzyme. Abdel-Naby et al. 1998 has reported it to be most suitable ion exchanger for enzyme immobilization with highest immobilization yield of 15% [7].

3.2. Properties of immobilized protease

The different properties of immobilized protease in comparison with free enzyme on to four different selected carriers based on their immobilization yield are tabulated in the table 2.

Table 2
Properties of free and immobilized protease to four different carriers

Property	Free enzyme	Carrier of immobilized enzyme			
		Celite	Alumina	Amberlite IR - 120	DEAE -Cellulose
Activity (U/ml)	21	5.92	2.61	6.1	3.63
Optimum pH of the reaction	7	7	6	6	6
Optimum temperature (°C) of activity	50	60	55	60	55
Thermal stability at 60 °C, 60 min (%)	13	58	93	84.4	37.5
Reusability (no of cycles)	Nil	6	4	4	5
Storage stability for 6 months at 4 °C (%)	48	84	39	81	77

3.2.1. Optimum pH and temperature of the reaction

The optimal pH of immobilized protease is attributed to the charge carried by the carrier material. The cationic support materials such as alumina, DEAE - cellulose and amberlite IR - 120 used in the study shifts the pH of the reaction to acidic range (pH 6.0) in comparison to the free enzyme whose optimum pH of the reaction is pH 7.0 (Figure 1). Similar observations are made by Xiao et al. 1990 by immobilized glucoamylase and glucose isomerase [11]. Celite being an inert material, there was no change in the pH of the reaction.

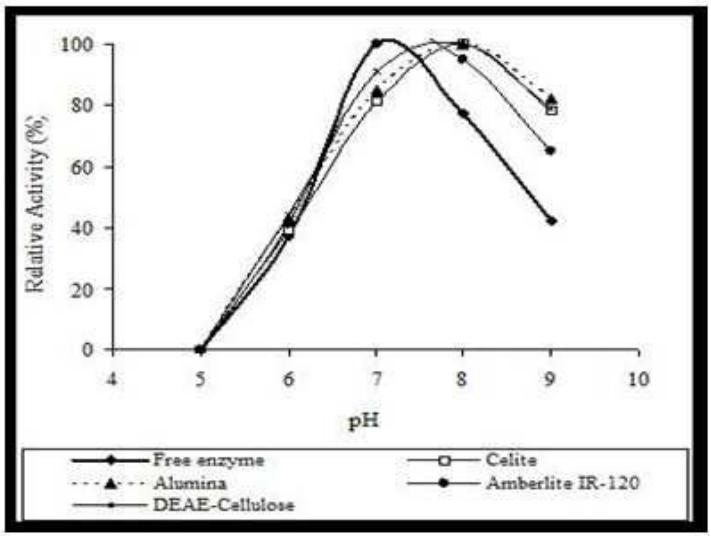

Figure 1
Optimum pH of immobilized protease on different matrices

The optimum reaction temperature was shifted from 50 °C for the free protease to up to 60 °C for celite and amberlite IR - 120 immobilized protease (Figure 2). However, the optimal reaction temperature of the enzymes immobilized on alumina and DEAE - cellulose was changed to 55 °C. It is evident from the results that the enzyme becomes more stable upon immobilization to celite and amberlite IR – 120

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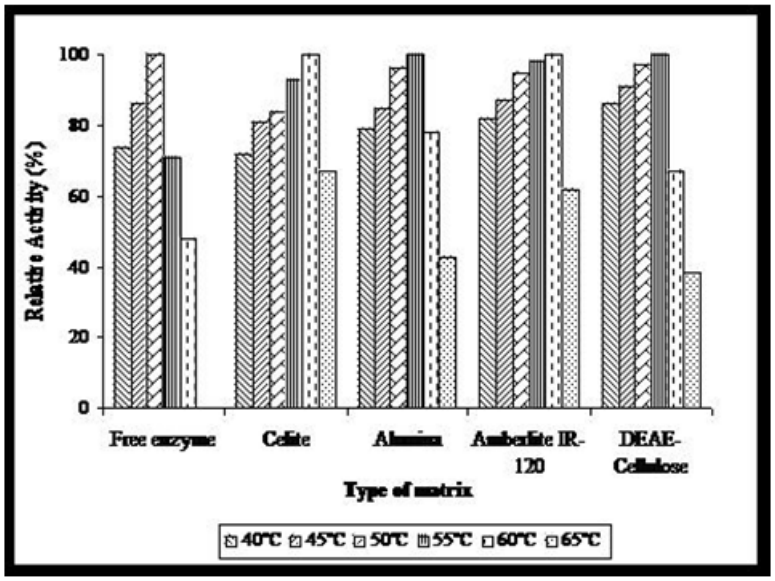

Figure 2
Optimum temperature of immobilized protease on different matrices

3.2.2. Thermal stability

One of the intentions of immobilization process is to improve the thermostability of the immobilized enzyme compared to free enzyme. As shown in the figure 3, the free and immobilized enzyme is subjected to heat treatment at 60 °C for 1 hour. The enzymes adsorbed onto alumina retained 93% of the original activity, while the free enzyme retained 33% of the original activity. The protease immobilized on to amberlite IR -120 retained 84.4% of the original activity where the protease immobilized to celite and DEAE - cellulose retained 58% and 37.5% of their original activities respectively. The above results indicated that Bacillus sp. protease becomes more thermostable when immobilized by alumina and amberlite IR -120.

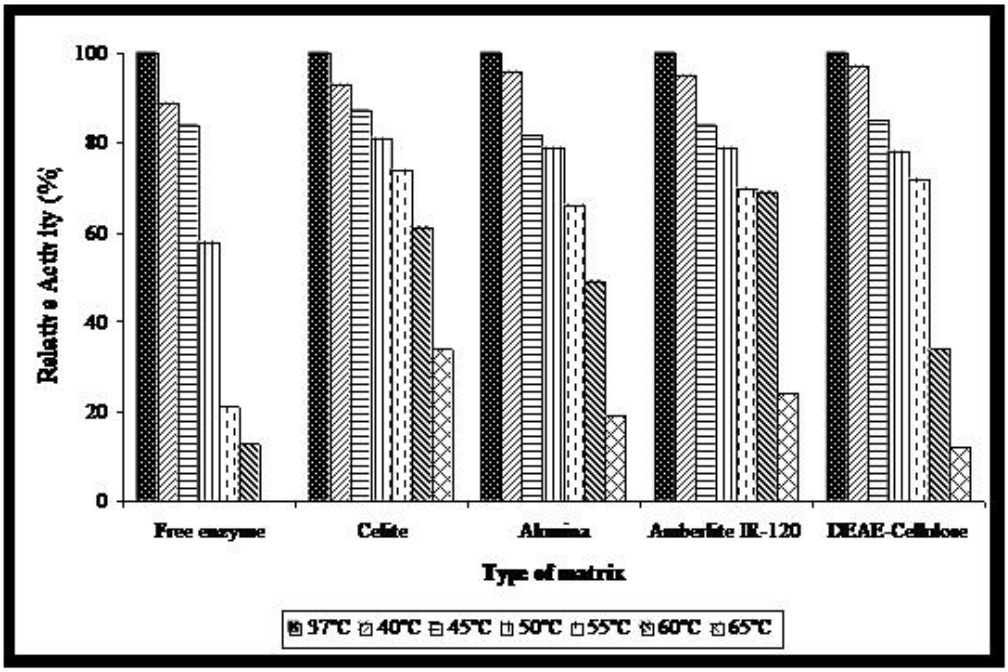

Figure 3
Thermostability of immobilized protease on different matrices

3.2.3. Reusability and storage stability

The results indicated in table 3 that after the second cycle, celite and DEAE-Cellulose matrix retained 70.6% and 84.2% activity respectively. But with Alumina and Amberlite IR-120 as support materials, at every step there was rapid drop in the protease activity. The untightly bound enzyme was be leached out from the carrier which lead to this drop in activity. Among the selected support material, celite adsorbed protease exhibited best operational stability for six cycles. The reuse of the immobilized enzyme is a cost reducing factor on an industrial scale purposes. Results have been reported for immobilized alkaline protease from *Conidiobolus macrosporus* on polyamide retained its activity without any significant loss for twenty-two times performance [11]. It has been reported by Chellpandian (1998) that alkaline protease immobilized on vermiculite retained 78% of its original activity even after five repeated uses [13].

Table 3
Reusability and storage stability of immobilized protease

No	Relative Activity (%)							
	Reusability (no of cycles)				Storage (months)			
	Celite	Alumina	Amberlite IR-120	DEAE-Cellulose	Celite	Alumina	Amberlite IR-120	DEAE-Cellulose
1	100	100	100	100	100	100	100	100
2	70.6	53.4	51.6	84.2	100	79	100	100
3	57.7	14.3	28.4	58.4	93	78	100	98
4	32.4	4.2	3.4	31.6	90	65	95	96
5	23.5	0	0	10.5	86	42	93	85
6	5.9	0	0	0	84	39	81	77

Several industrial implications are possible from such immobilized enzymes. One such study is development useful in textile industries is the novel approach of alternate wool shrink-resist finishing replacing the conventional chlorine treatments is possible from a commercial protease, Esperase, covalently linked to Eudragit S -100 by carbodiimide coupling [14].

4. CONCLUSION

In this study, *Bacillus* sp. protease was immobilized on different types of supports by

different methods: Entrapment by calcium alginate and gelatin, physical adsorption by celite and alumina and ionic binding by amberlite IR – 120 and DEAE - cellulose. Protease immobilized on amberlite IR -120 and celite as support materials was found to be promising. Major accomplishes of these techniques include thermal stability, long-term stability and reusability which are useful for commercial uses and scale up of production. The further studies on immobilized protease can be in the direction of its use for its utilization in different commercial applications.

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