Immobilization of thermostable and alkaline protease of thermophilic *Bacillus* sp. APR-4

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Introduction

Proteases are one of the most important groups of industrial enzymes accounting for nearly 60% of the total enzyme sale and find its application in laundry detergents, meat processing, cheese making, silver recovery from photographic film and in peptide synthesis (Rao *et al*., 1998). Although large number of bacterial proteases have been discovered, purified and characterized, yet very few of them have come up for their use in large scale industrial processes due to their high cost, restricted availability, difficulty in recovery from the reaction mixture and above all, the fragile nature of these enzymes (Bhalla *et al.,* 1999). Recent advances in enzyme technology have helped in solving some of these problems by immobilizing the biocatalyst to some extent, with choice of batch or continuous processes, rapid termination of reactions, controlled product formation with an ease in removal of the biocatalyst from the reaction medium without any contamination. The immobilized enzymes are more stable at higher temperatures and are active over a wide pH range. (Kennedy and White, 1985). In the present study an extra-cellular small size thermostable and alkaline protease produced by a thermophilic *Bacillus* sp. APR-4 (Kumar *et al*., 2002a; Kumar and Bhalla, 2004a) was selected for immobilization by adsorption on different matrices and its characterization.

Materials and methods

The 3-amino propyl-triethoxysilane and polyethylenimine were from Sigma Aldrich, USA. The glass wool was from Silver and Micron, Corning Glassworks, New York. Fibre glass was obtained from Fibre Glass India and the silica gel was from Merck Mumbai, India. All other chemicals were of analytical grade.

Microorganism and inoculum preparation

Bacillus sp. APR-4 used in the present study was isolated from cow dung compost (Kumar *et al*., 2002a). Seed culture was prepared by inoculating microorganism in GYP medium containing gL^{-1}(Glucose 0.1, yeast extract 0.05, peptone 0.05, $CaCl_2.2H_2O$ 0.005, $MgSO_4.7H_2O$ 0.0025, sodium chloride 0.05, pH 7.0) and incubated for 24 h at 55 ^oC in an incubator shaker and maintained on GYP casein (0.1 g l^{-1}) agar plates.

Enzyme production and assay

For enzyme production one percent of 48 h seed culture (OD_{660} = 1.0) was added to 50mL of GYP medium in 250 Erlenmeyer flask. This was incubated at 55 ^oC for 48 h in an incubator shaker (155 oscillations min^{-1}), and centrifuged at 5000g for 30 min at 4 ^oC and the supernatant was assayed for the extra cellular protease activity. Protease was assayed by modified method of Manachini *et al*. (1988). The reaction mixture containing 4mL of 0.5% (w/v) casein in 50mM Tris-HCl buffer, pH 9.0 and 0.1 mL of enzyme was incubated at 55 ^oC in shaking water bath for ten minutes. The reaction was terminated by the addition of 5mL of 5% (w/v) trichloroacetic acid and filtered through Whatman No.1 paper. The optical density of filtrate was measured at $OD_{275\ nm}$ (Systronics, UV-VIS Spectrophotometer). All samples were taken in triplicates. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1µg of tyrosine $mL^{-1}min^{-1}$ under assay conditions.

Activation of fibre glass wool and glass fibre discs

The activation of pyrex fibre glass wool and glass fibre discs (1x1cm each) was carried out using Bisswanger (2004) method.

Characterization of alkaline protease after immobilization on different matrices

Immobilization of protease on pyrex fibre glass wool was carried out as described by D'Souza *et al*. (1986). Immobilization of protease on glass fiber discs was carried out by the modified method of Taylor *et al*. (1977). Immobilization on silica gel particles (60-120 mesh size) was done as per the procedure described by Wassermann *et al.* (1980). For Immobilization five ml of enzyme (100 UmL^{-1}) was added to these matrices and the activity of the immobilized enzyme was determined by using 100 mg mL^{-1} of immobilized support under assay condition. The activity yield and other characteristics of immobilized enzyme were compared with that of free enzyme and % yield of immobilized enzyme was calculated using the formula:

% Enzyme yield = Activity in immobilized matrix/ Activity in free enzyme X 100

The Enzyme activity of free and immobilized protease was measured at different pH (7.0-11.0) and temperature (40-80 ^{o}C) under experimental conditions. Thermostability profile of protease in free and after immobilization on all matrices respectively was studied up to 10 h after incubation at various temperatures (40 ^{o}C-70 ^{o}C) by withdrawing and assaying samples at an intervals of 2h each.

Reusability of immobilized enzyme

The thermostable and alkaline protease of *Bacillus* sp. APR-4 immobilized on all three matrices was tested for its reusability using 100mg (100 UmL^{-1}) of immobilized matrix repeatedly up to ten times and percent relative activity determined.

Results and discussion

The thermostable and alkaline protease of thermophilic *Bacillus* sp. APR-4 produced at 55 ^{o}C showed optimal activity at pH 9.0 and 65 ^{o}C respectively (Kumar *et al*., 2002a). This protease was immobilized successfully on to the three supports viz., pyrex fibre glass wool, fibre glass discs and silica particles (Fig.1). All the experiments were carried out in triplicate and the results were analyzed. The immobilization was preceded by silanization to introduce reactive groups onto inert glass surface to increase the surface area for immobilization (Bisswanger, 2004). Immobilization of enzyme with polyethylenimine is one of the very quick and cheap procedures (D'Souza *et al*., 1986) and reported to improve the catalytic and stability characteristics of the biocatalyst.

After immobilization the enzyme activity yield was found to be 58.32, 51.6% and 50.75% for glass wool, glass fibre for silica gel particles respectively. The loss of enzyme activity after immobilization is normal phenomenon (Rosevear, 1988). At least 49% of the activity was retained in controlled pore glass immobilized with *Thermus Rt* 41A protease (Wilson *et al*., 1994). All the enzyme immobilized matrices showed optimum catalytic activity at pH 9.0 and retained 78% and 63.7% activity at pH 8.0 and 10.0 respectively with glass wool and glass fibre (Fig. 2.) The free enzyme also showed the optimal activity at pH 9.0 as reported earlier (Kumar *et al*., 2002).

The extra cellular protease produced by *Bacillus* sp. APR-4 had optimum activity at 60 ^{o}C (Kumar *et al*., 2002a). The maximum activity of protease immobilized on glass wool, glass fibre and silica gel was observed at 65 ^{o}C (100%) and at 70 ^{o}C it was 78%, 48.3% and 48.7% in glass wool, glass fibre and silica gel respectively (Fig. 3). The slight increase in temperature optima was observed with immobilized *Bacillus stearothermophilus* protease (Manolov *et al*., 1995). Taylor *et al*. (1977) recorded high activity with papain immobilized on silanated controlled pore glass, alumina and titania for milk coagulation.

The immobilized protease was stable up to 4 h at 60 ^{o}C and 70 ^{o}C on glass wool and glass fibre and stability up to 2 h was observed at 70^{0}C with silica gel (Fig. 4). The stability of immobilized enzymes at higher temperatures is one of the important improvements that have been achieved with immobilized enzymes on

different supports (Hayashi and Ikada, 1990; Manolov *et al*., 1995). The enhancement in activity and stability of immobilized enzymes is important for their industrial applications (Rosevear, 1988).

The immobilized protease was quite stable and could be reused 6-8 times without any considerable loss in enzyme activity in all supports (Fig. 5). The slight loss in activity after five times was observed in case of glass wool and glass fibre discs. This may be due to abrasion (Rosevear, 1988) of matrices during repeated use.The immobilized protease (100 UmL^{-1}) was evaluated for gelatin degradation from used X-ray films. It took 5 min to completely remove the gelatin with immobilized matrices (Fig. 6) at 60 ^{o}C. Similar results were observed earlier with the free enzyme to remove gelatin from used X-ray film (Kumar *et al*., 2002b).

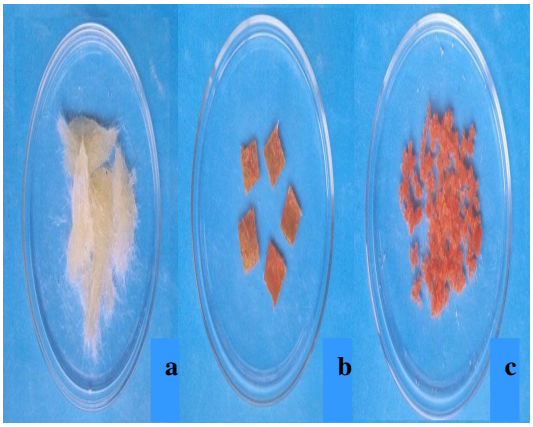

Figure 1. Thermostable and alkaline protease (100 UmL^{-1}) of *Bacillus* sp. APR-4 immobilized onto a) pyrex fibre glass wool, b) glass fibre discs (1x1 cm) and, c) silica gel particles (60-120 mesh size)

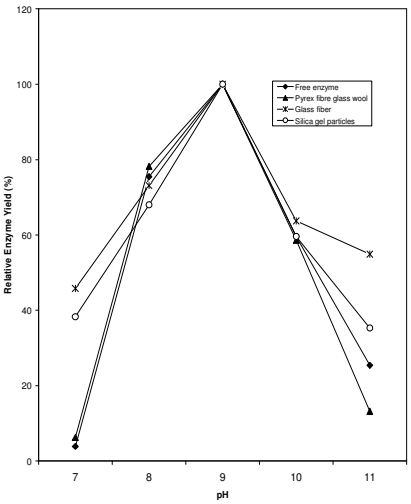

Figure 2. Effect of pH on activity of immobilized protease.Optimum values corresponds to relative yield (100%) of immobilized enzyme on pyrex fibre glass wool (73.33 UmL-1), glass fibre discs (68.26 UmL-1)and silica gel (67.33UmL-1) respectively.

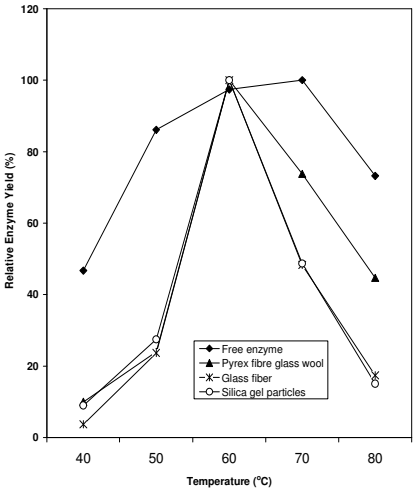

Figure 3. Effect of temperature on immobilized protease. Optimum values corresponds to relative yield (100%) of immobilized enzyme on pyrex fibre glass wool (73.33 UmL-1), glass fibre discs (68.26 UmL-1)and silica gel (67.33UmL-1) respectively.

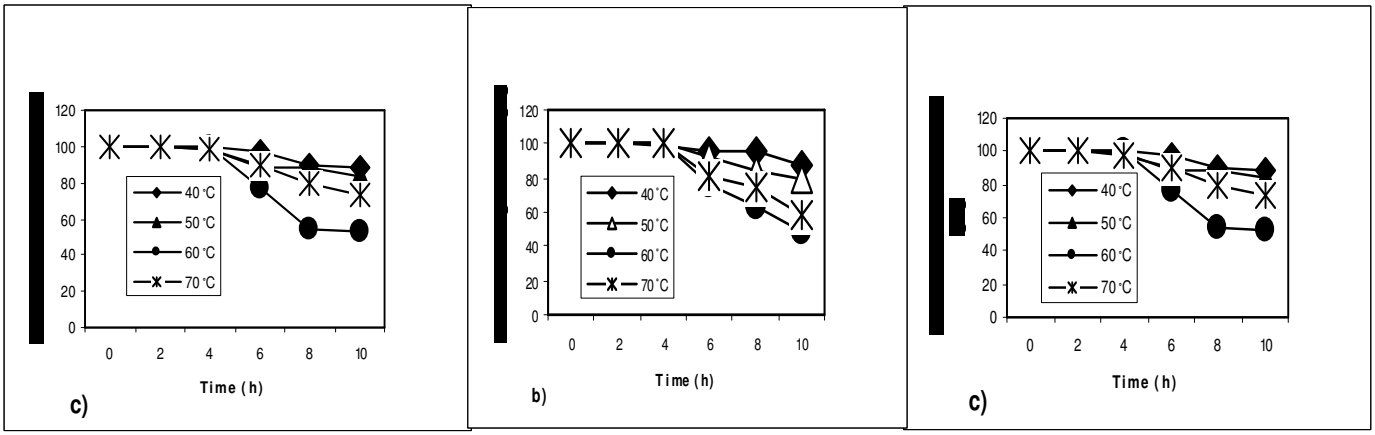

Figure 4. Thermostability profile of immobilized protease. a) pyrex fibre glass wool, b) glass fibre discs and, c) silics gel particles.

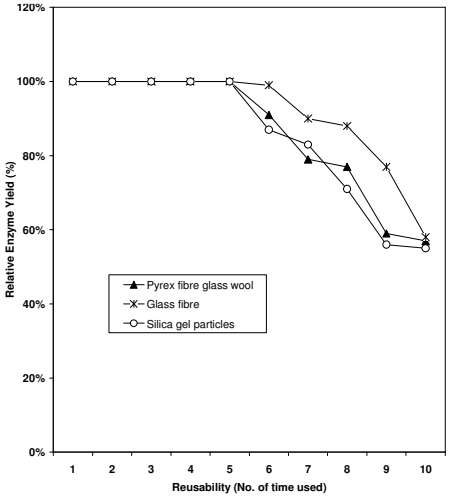

Figure 5. Reusability of immobilized enzyme

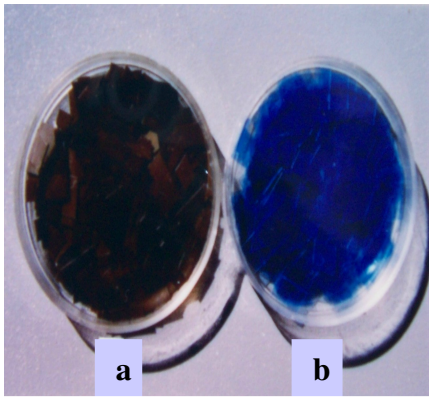

Figure 6. Treatment of used X-ray film pieces with immobilized glass fibre discs (100 UmL^{-1}) for 5 min. a) X-ray film before treatment and, b) after 5 min of incubation under assay (pH 9.0).

Conclusion

Thermostable and alkaline proteases have tremendous commercial importance and, it is worth to employ immobilized thermostable and alkaline proteases for various industrial applications viz. peptide synthesis, protein recovery and stain removal from fabric (Kumar *et al*., 2003a,b; Kumar *et al*., 2004). The use of immobilized alkaline protease in peptide synthesis has been considered as a novel approach (Kumar and Bhalla, 2005) and the potential of this protease in purified and immobilized form could be further explored for the synthesis of biologically active opioid peptides.

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