P-009	**Immobilization of protease produced by new isolate *Bacillus subtilis* MTCC 9226**

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INTRODUCTION

Proteases are one of the important groups of industrial enzymes produced comercially with its major application in laundry detergents, meat processing, cheese making, silver recovery from photographic film and in peptide synthesis (Rao *et al* 1998, Kumar *et al* 2005). A very few bacterial proteases have been exploited in large scale industrial processes pertaining to their high cost, difficulty in recovery and fragile nature (Bhalla *et al* 1999). Immobilized biocatalyst have helped in solving some of these problems with choice of batch or continuous processes, rapid termination of reactions, ease in removal from the reaction medium without contamination and more stability and activity over a wide pH and temperature range (Kennedy and White 1985). In present study an extra-cellular alkaline protease produced by *Bacillus subtilis* MTCC9226 was selected for immobilization by adsorption on two matrices and characterized.

MATERIAL AND METHODS

Enzyme production and assay Glass wool was from Silver and Micron, Corning Glassworks, New York and glass beads were from Biomatrix Technologies, India. *Bacillus subtilis* MTCC9226 was isolated from dung compost and maintained on GYP casein (0.1 g l^{-1}) agar plates. One percent of 48 h old seed culture (OD_{660} = 1.0) was added to 50mL of GYP production 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 9.0) in 250 Erlenmeyer flask and incubated at 40^oC for 48 h in an incubator shaker (155 rpm), and finally centrifuged at 5000g at 4^oC and supernatant assayed for extracellular protease activity (Manachini *et al* 1988). 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 40 ^oC in shaking water bath for ten minutes and the reaction was terminated with 5mL of 5% (w/v) trichloroacetic acid and filtered through Whatman No.1. The optical density of filtrate was measured at $OD_{275\ nm}$. 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.

Characterization of immobilized protease The pyrex fibre glass wool and glass beads were activated using Bisswanger (2004) method. Protease immobilization on pyrex fibre glass wool was carried out as described by Taylor *et al* (1977) and on glass beads as reported by D'Souza *et al* (1986).The activity of immobilized enzyme was determined by using 100 $mgmL^{-1}$ of immobilized support (100 UmL^{-1}) under assay condition and compared with free enzyme at different pH (7.0-11.0) and temperature (40-80 ^oC) under experimental conditions. % yield of immobilized enzyme was calculated using the formula: % Enzyme yield = Activity in immobilized matrix/ Activity in free enzyme X 100
Thermostability of free and immobilized protease was studied at 40 ^oC-70 ^oC up to 10 h by withdrawing and assaying samples at an interval of 2h each. Finally the reusability of immobilized enzyme was studied.

RESULTS AND DISCUSSION

Bacillus subtilis MTCC9226 producing protease at 40^oC showed optimal activity (910 UmL^{-1}) at pH 9.0 and 45^oC respectively. This protease was immobilized successfully on to two supports viz., pyrex fibre glass wool and glass beads (4-5mm) (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 to improve the catalytic and stability characteristics of the biocatalyst (D'Souza *et al* 1986).
After immobilization the enzyme activity yield was found to be 74% and 70% respectively for fibre glass wool and glass beads. 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). Both the enzyme immobilized matrices showed optimum catalytic activity at pH 9.0 and retained 51% and 37% activity at pH 10.0 respectively (Fig. 2a). 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 subtilis* MTCC9226 had optimum activity at 45 ^oC and after immobilization on glass wool and glass beads similar results were observed at 45 ^oC (100%). However, at 50 ^oC activity was 44% and 82% respectively with glass wool and glass beads (Fig. 2b). Taylor *et al* (1977) recorded high activity with papain immobilized on silanated controlled pore glass, alumina and titania used for milk coagulation.
The immobilized protease showed 81-96% stability at 30-60 ^oC up to 2h and after 4 h the stability decreased to 44-68% (Fig. 3). The stability at higher temperatures is one of the improvements with immobilized enzymes (Manolov *et*

al 1995) and is important for their industrial applications (Rosevear 1988). The immobilized protease was quite stable and could be reused 2-5 times without any considerable loss in enzyme activity in both supports. Slight loss in activity after five times in both matrices might be due to abrasion (Rosevear 1988) during repeated use. About 5% of the meat goes as waste after butchering (Kumar et al 2003) and this immobilized protease (100 UmL^{-1}) was evaluated for protein recovery from goat waste bones. It took 120 min to completely remove the protein attached to the goat waste bones at 45 ^{o}C with immobilized matrices (Fig. 4).

CONCLUSION

Thermostable and alkaline proteases have commercial importance viz. in protein recovery from wastes bones and detergents and the potential of this protease in purified and immobilized form could be explored for the synthesis of biologically active opioid peptides.

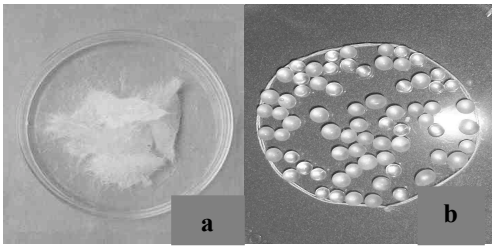

Figure 1: *Bacillus subtilis* MTCC9226 protease (100 UmL^{-1}) immobilized onto a) pyrex fibre glass wool and, b) Glass beads (4-5 mm size)

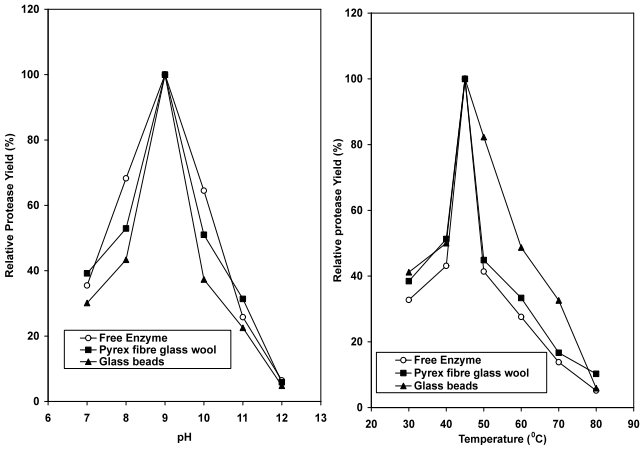

Figure 2: Effect of pH and temperature on immobilized protease. Optimum value corresponds to relative yield (100%) of immobilized enzyme on glass wool (673 UmL^{-1}) and glass beads (637 UmL^{-1})

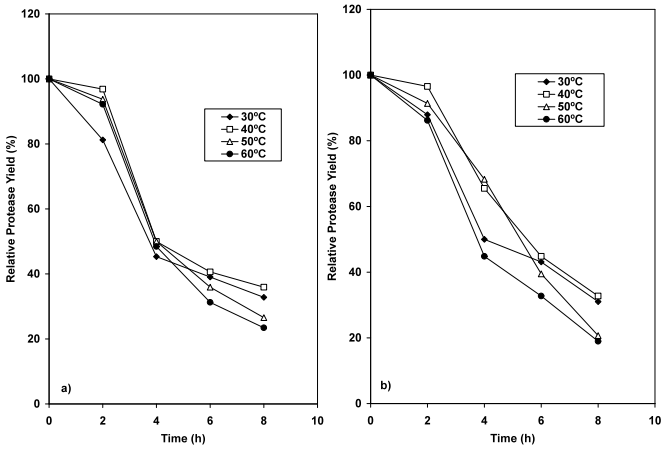

Figure 3: Thermostability of immobilized protease a) Glass wool and, b) Glass beads

Figure 4: Treatment of waste bones pieces with immobilized glass fibre discs (100 UmL^{-1}). a) Waste bones before treatment and, b) after treatment

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