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A study on the immobilization of acid xylanase on chitosan from chitin of shrimp shells

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ABSTRACT

This study aims to immobilize Xylanase, an enzyme which degrades Xylan, onto Chitosan polysaccharide found abundantly in shrimps. Immobilization gives several advantages such as improved stability, reuse and increased activity of the enzymes. Chitosan was extracted from Chitin after the deacetylation process and used as a support material in the form of beads prepared by a novel method without the aid of any cross linking agent. The effect of parameters like pH, temperature and contact time was carried out to optimize the process. The optimum pH was found to be 5, temperature 50^0C and contact time 90 min. Finally, at the optimized conditions assay of immobilized and the free Xylanase enzyme was carried out and a fivefold increase in the activity of immobilized enzyme was observed when compared to the free enzyme, thus proving Chitosan to be one of the best supports for the immobilization of xylanase.

KEY WORDS: Xylanase, Xylan, Chitosan, Immobilization, Optimization.

1. INTRODUCTION

Xylanase is a class of enzyme that catalyzes the hydrolysis of xylans the most abundant natural, noncellulosic polysaccharide found around the world thus breaking down hemicellulose, one of the major components of plant cell wall along with other enzymes that hydrolyze polysaccharides. These enzymes are produced mainly by microorganisms affluent on plant sources for the degradation of plant matter into usable nutrients (Polizeli, 2005). Xylanases has a lot of biotechnological applications in various industries and in processes such as forage crops and lignocellulosics biomass pretreatment, in poultry industry for increased consumption of cereal based nutritive diets, improvement of flour in bakery and food products, enzymatic conversion of wastes from farm land, industries and municipality, reducing the use of chlorine containing chemicals in the bleaching of cellulose pulps and also in the treatment of textile cellulosic waste, thus avoiding the use of sulphuric acid (Bhatt, 2000; Beg, 2001; Ninawe and Kuhad, 2006; Pawan and Duni, 2012). The purpose of this study is to increase the activity of the xylanase enzyme by immobilization technique. In addition, an attempt was made to optimize the physiological conditions like pH, temperature and reaction time affecting the activity of the enzyme. A matrix or a support material increases the operating performance of the immobilized system greatly and the immobilized enzyme has the properties of both the matrix and the enzyme. Some of the important characteristics of the support material for an enzyme immobilization process are its mechanical stability, strong affinity, accessibility of the active site of the enzyme for chemical alterations with ease, hydrophilicity, rigidity and simple preparation process in different geometrical configurations such as beads, gel matrix etc (Carrea, 2000; Guilbault, 1983). In case of use in the pharmaceutical, food and poultry industry the support material should be biocompatible and non-toxic; besides the support should also be biodegradable and cheap. Chitosan the deacetylated form of Chitin is an ideal support material, as it offers most of the above mentioned characteristics (Krajewsk, 2004). This study also features a novel method of Chitosan bead preparation as a support matrix without the aid of any cross-linking agent.

2. MATERIALS AND METHOD

2.1. Enzyme Preparation: Xylanase enzyme was procured from Tex Biosciences Private Limited Chennai in the form of dry powder. 1 % enzyme solution was prepared using Sodium Acetate buffer.

2.2. Extraction of Chitosan from Chitin (Felicity, 2007): 1g of Chitin (Himedia) was dissolved in 50% sodium hydroxide (1:15 ratio) and autoclaved for 30 minutes. The resulting solution was cooled to room temperature and then filtered. The process was repeated a few times to retain the solids. The sample was oven dried at 120°C for 24 hours. The raffinate obtained was Chitosan, a creamy white hydrogel formed from Chitin via shrimp shells that can dissolve easily in acetic acid.

2.3. Preparation of Chitosan beads: 1g of Chitosan was dissolved in 30ml of 5% acetic acid (v/v) in a conical flask and concentrated by boiling at 75^0C. This was allowed to cool at room temperature for 2 hours. The Chitosan hydro gel thus formed was introduced as droplets into 100 ml of 30% sodium hydroxide solution using a sterilized syringe. The Chitosan beads prepared were thoroughly rinsed with distilled water until neutral pH was reached and stored in distilled water under room temperature. The beads were reasonably spherical in shape, and their diameters were measured. The circumference for 10 beads was taken and the average size of the beads was calculated by using the formula, Circumference $=2\pi r$, where r is the radius of the bead, r=2D, D is the diameter of the beads.

2.4. Immobilization of Acid Xylanase: 6g of Chitosan beads were packed tightly to a height of 22 cm in a 42cm column (37cm working volume + 5cm head space; Bed height/Column height=0.6). 5 ml of 1% enzyme solution was allowed to pass through the column and retained for 30 minutes for adsorption at room temperature and eluted. The activity of free and the immobilized enzyme were studied by xylanase assay.

2.5. Acid Xylanase assay: The total protein content of xylanase was calculated by Lowry's method (Lowry, 1951) with Bovine Serum Albumin as standard. Xylanase assay was carried out according to the standard protocol by Bailey (1992), in which xylanase acts on the substrate xylan (Sigma) from Beech Wood to release xylose that reacts with DNS to form a color (Miller, 1959). 1% xylose standard with concentrations ranging from 0.5 to 2.5 μmoles was prepared using 0.05 M sodium acetate buffer with pH 5.0. 5ml of the working standard from each concentration was pipette out in a test tube and 0.5ml of sodium acetate was added to it. 1 ml of DNS was added to stop the reaction. The solution was boiled for 10 min and cooled to room temperature. Finally 3 ml of RO water was added and the absorbance was read at 540nm.A standard graph was plotted with concentration and time. For the test sample, 1% Birchwood Xylan substrate was prepared using sodium acetate and 0.5 ml of it was taken for the assay. It was pre-incubated at 50°C for 5 min. 1:100 dilution of free enzyme and immobilized enzyme was prepared using 100 ml sodium acetate separately. 0.5 ml of it was added to the substrate and the solution was incubated at 50°C for 5 min. 1 ml of DNS was added to each test tube and boiled for 10 min and allowed to cool down to room temperature. Finally 3 ml of RO water was added and the absorbance was read at 540nm. The assay was done in triplicates to reduce the errors and the concentration of xylose was calculated from the standard graph. The enzyme activity and the specific activity for both the free enzyme and immobilized enzyme were calculated. Enzyme activity was expressed as micromoles of xylose equivalent liberated per minute per ml of enzyme solution (U/ml) and specific activity was expressed as U/mg protein.

2.6. Optimization process: In order to study the effect of Chitosan concentration, 0.5 % to 3.5 % of xylan substrate (in 0.5 M sodium acetate buffer) was added to the reaction medium and the assay was done under standard conditions. The optimum pH for immobilized enzyme activity was analyzed by varying the pH of 0.5 M sodium acetate buffer in the range of 3 to 7. After incubation at room temperature for 30 min, xylanase activity was measured and calculated as described above. To optimize the temperature, the assay was carried out at different temperatures between 30°C to 70°C. The effect of contact time on enzyme activity was determined by incubating the reaction media at standard conditions and every 10 minutes once the enzyme was eluted and the assay was done. This was repeated up to 120 minutes. Under the optimized conditions the immobilization process was carried out and the specific activity of the immobilized xylanase and free xylanase was measured.

3. RESULTS AND DISCUSSION

3.1. Yield of Chitosan: After the deacetylation process with NaOH (50%), 39% of Chitosan was obtained which was higher than the yield (15.4%) reported by Hossain and Iqbal, 2014 and Monarul Islam et al., 2011 who also obtained 15.21% of Chitosan using 70% NaOH at room temperature with the reaction time of 72hrs. Similarly a yield of 34% was reported by Yateendra (2012) and this increase in yield may be due to the prolonged reaction time.

3.2. Chitosan beads: For the immobilization process Chitosan was prepared as beads by a novel methodology without the addition of any other cross linking or gel forming agents which is not reported yet (Fig.1). Maximum xylanase activity was observed at 3% Chitosan concentration and did not change significantly when Chitosan concentration was further increased as shown in Fig.2. Low concentration of Chitosan makes the bead, soft and fragile and thereby cell seepage is high, whereas high concentration improves pellet firmness (Anisha and Prema, 2008). Gilson and Thomas (1995) also suggested that beads with less than 1.5% are soft and easily breakable and suffers no damage when its concentration increases.

3.3. Effect of pH: Most of the enzymes exhibit a maximum activity at a specific P^H. The activity reduces above and below this pH. The enzyme's 3D structure responsible for its catalytic activity is stabilized by hydrogen bonds, hydrophobic interactions and disulfate bonds. Changing the hydrogen concentration modifies the equilibrium of these forces, irreversibly deactivating the enzyme. P^H change also affects factors like charge of the matrix substrate, the immobilization method and enzyme's acid and alkaline behavior (Busto, 1997). From Fig 3 it is inferred that the optimal pH was 5.0 for xylanase, which was also reported by Sheila (2014).

3.4. Effect of temperature: In case of the effect of temperature, similar to all other chemical reactions, the enzyme activity increases exponentially as the temperature increases over a determined range in which enzyme is stable. Since enzymes are delicate protein structures generally active at normal physiological temperatures, their activity is lost above 40^0C due to denaturing of various enzyme molecules present, but immobilization methods enhanced heat stability (Vipan, 2010). From Fig.4 it is seen that the enzyme activity continuously increased with increase in temperature, with a maximum activity at 50°C.

3.5. Effect of contact time: The optimal time for the enzyme to bind to Chitosan during the immobilization process was found to be 90 minutes (fig.5) in this study, which is in compliance with the observations of Sheila (2014). Debabrata and Vineet (2013) reported 48 hours of contact time, which are towards the higher side than reported in

this study. However, Yin (2010) observed 30 minutes optimum contact time for xylanase activity from *Bacillus sp*. YJ6 whereas a maximum activity was reported to be 50 minutes by Seyis (2005). Sanghi (2010) reported 5 min contact time for purified xylanase of *Bacillus subtilis* which is very much contradictory to the current study. Contact time has an intense effect on enzyme activity since particular duration of reaction time is necessary for the substrate to bind to the active sites of the enzyme and the amount of products also increases up to a certain point. After a specific reaction time no active sites are available for the substrate to bind and there is no increase in the activity of the enzyme (Pawan and Duni, 2012).

3.6. Comparison of xylanase activity by free and immobilized cells under optimized conditions: A comparison of the specific activity between free enzyme and immobilized enzyme was done and the results are tabulated in table 1. Higher specific activity of 44.6 U/mg of protein was found under immobilized conditions. This might be due to the fact that the cells did not encounter the adverse environment and unnecessary extracellular proteins are much less in immobilized systems when compared to free cells. Though there was a change in the pH during the process, the degree of alkalinity in the immobilized system was less compared to the free cell system (Debabrata and Vineet, 2013).

Table.1. Comparative study of the specific activity of free and immobilized enzyme

Xylanase	Enzyme Activity (U/ml)	Specific Activity (U/mg of protein)
Free enzyme	17.5	9.1
Immobilized enzyme	87.8	44.6

Fig.1. Chitosan beads prepared by a new method without a cross linking agent

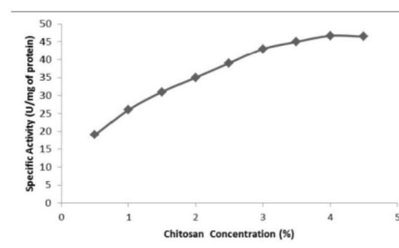

Fig.2. Effect of Chitosan concentration on the activity of immobilized xylanase enzyme

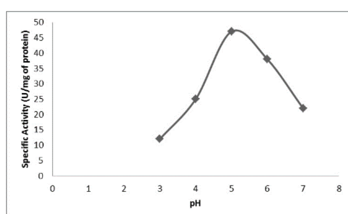

Fig.3. Effect of pH on the activity of xylanase enzyme immobilized on Chitosan beads

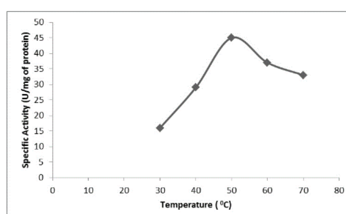

Fig.4. Effect of temperature on the activity of xylanase enzyme immobilized on Chitosan beads

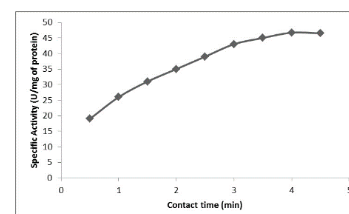

Fig.5. Effect of contact time on the activity of xylanase enzyme immobilized on Chitosan beads

4. CONCLUSION

In this study a novel method was adopted for the preparation of Chitosan beads for immobilization of Xylanase. The specific activity of the immobilized Xylanase enzyme was 44.6 U/mg of protein, which was fivefold higher than the activity of the free enzyme (9.1U/mg of protein). Thus Chitosan can be used as a support matrix in immobilized systems and applied in various industries like enzyme production, poultry industries, textile industries etc. because of its biocompatibility and sustainability.

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