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CHEMICAL COUPLING OF GLUCOAMYLASE PRODUCED BY *Arthrobotrys conoides* ONTO COTTON CLOTH AND *Ocimum basilicum* SEEDS AND CHARACTERIZATION OF THE IMMOBILIZED ENZYME

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ABSTRACT

Glucoamylase produced by *Arthrobotrys conoides* was immobilized onto cotton cloth pieces (CC) and seeds of *Ocimum basilicum* (OB) employing five different approaches. Mechanical stability of CC and large surface area provided by the microfibrillar structure in the mucilage of OB seeds offers the advantage for their use as immobilization matrices. Periodate treated enzyme coupled to polyethylenimine treated support gave the best results with immobilization percentage of 67.5 and 53 for CC and OB seeds respectively. Immobilized enzymes exhibited broader pH and temperature activity profiles as compared to those of native and PI oxidized forms. Immobilization conferred stability to the enzyme in acidic region and also improved its thermo-stability. Km values for starch were found to be 0.08 and 0.105 $mg.ml^{-1}$ for native and PI treated forms and, 2.3 and 2.6 $mg.ml^{-1}$ for enzymes bound to CC and OB respectively. Although the enzyme preparations were optimally active at 50°C, recycling studies indicated optimum temperature of 40°C for saccharification of starch. CC and OB bound preparations could be recycled 13 and 11 times respectively every 2h at 40°C, with retention of 50% activity. Immobilized preparations were able to convert starch to an extent of 61-64%. Conversion percentage improved to 73% when CC preparation was incubated with starch at increased speed of agitation indicating diffusional limitations as one of the factors influencing the apparent decrease in the affinity of the immobilized enzyme for its substrate. Cloth bound preparation was found to be superior in its performance in comparison to enzyme coupled to OB.

Keywords: Glucoamylase, Immobilization, Cotton, *Ocimum basilicum*, periodate treatment, polyethylenimine treatment

INTRODUCTION

Glucoamylases ((EC 3.2.1.3.) are exo acting α-1,4-glucan glucohydrolases which act on glucan polysaccharides yielding glucose as the major product. The enzyme is used by industries involved in saccharification of starch for glucose and alcohol production. *Arthrobotrys* species are a group of nematophagous fungi, which kill and consume microscopic animals. These fungi are known to produce pectinases, amylases, cellulases and various other hydrolytic enzymes. We have reported production of glucoamylase by *Arthrobotrys* species **(Jaffar *et al.*, 1993; Shetty, 2016).** Immobilization of glucoamylase has been widely studied as a means of reducing enzyme cost in the manufacture of glucose and high fructose corn syrups. Various supports and techniques have been assessed for immobilization. Activated alginate beads, ion exchangers, chitosan, earth materials and equivalents, magnetic micro-particles etc. have served as matrices for immobilization of amylolytic enzymes **(Pieters *et al.,* 1992; Goncalves *et al.*, 1997; Iyer *et al.*, 2003; Shkutina *et al.,* 2005; Eldin, *et al.*, 2011; Wang *et al.*, 2013).** Choice of a suitable matrix and technique of binding are of critical importance in immobilization. Attrition and/ flotation are often the problems associated in the long run in agitated reactors using shear sensitive support materials **(Regan *et al.*, 1974).** Particulate supports have the limitations of high pressure drop and hindered flow characteristics in packed bed reactors on continuous use **(Svec & Gemeiner, 1995; Suen, 2015).** Methods of immobilization such as adsorption and entrapment, though simple and efficient, do not create strong bonds between the enzyme and the support and often, enzyme leaks into the solution. Diffusional restrictions encountered further limit the benefit of entrapment systems which act on polymeric substrates. Covalent binding is a promising technique which takes care of such issues associated with adsorption and entrapment. One of the key issues associated with covalent linkage is the need for activation the support and often modification of the enzyme as well, which may result in loss of activity to a significant extent. Glucoamylases are glycoproteins by nature **(Shenoy *et al.*, 1985)** Binding of the enzyme through its glycosyl residues can be a method of choice for immobilization.
The present investigation deals with immobilization of glucoamylase produced by *A conoides* onto cotton cloth pieces and the seeds of *Ocimum basilicum* (locally known as Sabja). Various techniques were evaluated for immobilization. The enzyme bound preparations were characterized and assessed for their recycling and saccharification efficiency.

MATERIALS AND METHODS

Microorganism

The organism *A conoides* (ATCC 44454) was maintained on corn meal agar slants as recommended by ATCC catalogue, 1983.

Enzyme production

Enzyme was produced in a medium containing ($g.l^{-1}$), pH 7.2: Corn starch, 10.0; peptone, 2.0; $NaNO_3$, 3.7; KH_2PO_4, 3.4; KCL and $MgSO_4.7H_2O$, 0.5g each; $FeSO_4.7H_2O$, 10 mg; $ZnSO_4.7H_2O$, 0.5 mg, thiamine HCl, 0.1 mg and biotin, 5μg. The medium (25 ml) was dispensed into 250 ml Erlenmeyer flasks and inoculated with around 10^6 spores. Incubation was carried out at 25±1°C for 12 days under stationary conditions. The broth was filtered and the culture filtrate was used for further studies.

Assay methods

Amylolytic activity of the free enzyme was measured in 2 ml of the reaction mixture containing appropriately diluted enzyme and 0.25% soluble starch in 25 mM acetate buffer, pH 5.6. It was then incubated at 40°C for 20min and the resultant reducing sugars were measured by DNSA. Glucose produced was estimated using glucose oxidase-peroxidase reagent (GOP method). The assays were carried out by DNSA method unless mentioned otherwise.
Immobilized activity was measured by addition of 5 ml of soluble starch (1.5% in 25mM acetate buffer, pH 5.6), to the immobilized support. After an incubation period of 1h at 40°C in a shaker water bath at 40 rpm, reducing sugars were estimated in the supernatant by DNSA method.

Unit of activity is defined as the μmole of reducing equivalent (glucose) released per minute under the assay conditions.
Protein was estimated by Lowry's method.

Detection of reaction products by paper chromatography:

One unit of the enzyme was incubated in 2ml of 0.25% starch at 50°C. The aliquots containing 20µg of reducing sugars (as determined by DNSA) was loaded onto Whatman No.1 filter paper. Descending paper chromatography was carried out in n-butanol:pyridine: water (6:4:3) solvent system. Sugars were detected by dipping in 0.1% silver nitrate in acetone, followed by development of color with 1.4% NaOH and destaining in 5% $Na_2S_2O_3$ solution **(Touchstone and Dobbins, 1978).**

Concentration

The culture filtrate was dialyzed against PEG 20000 and further concentration was carried out by salting out at 80% saturation with ammonium sulfate at 4°C. This preparation was used for immobilization.

Treatment of the enzyme

Periodate (PI) treatment: To 95 ml of the sample containing 100units of the enzyme (specific activity of 3.92) in 0.05M acetate buffer, pH 5.6, 5 ml of 100mM sodium meta periodate was added and kept in dark for 3h. Ethylene glycol, 1 ml was then added and kept for 30min. The solution was dialyzed overnight in refrigerator.
Polyethylenimine (PEI) treatment: To 95ml of the sample containing 100units of the enzyme, 5ml of 4% PEI was added and the pH was adjusted to pH 7. After 3h, the reaction mixture was dialyzed.

Treatment of the supports

White Cotton cloth was washed, rinsed in D/W and air-dried. Cloth pieces were cut (4x4cm, 105-115mg) and used for immobilization. OB seeds, 0.2g each were soaked in D/W for 2h and water was filtered off using a strainer. The strainer was placed on a pad of filter paper to absorb excess water.
The supports were subjected to following treatments.
PEI treatment: To one cloth piece/o.2g of swollen OB, 5ml of 0.2% PEI, pH 7.0 (adjusted with HCl) as added. After incubating for 2h at ambient temperature (30±1°C), the supernatant was discarded and the treated support was rinsed with D/W.
PI treatment: The support was immersed in 5 ml of 100mM sodium meta periodate for 3h in dark and rinsed with D/W.

Immobilization of the enzyme

Different methods employed for investigation were as follows, Method I- The enzyme was added to PEI treated support; Method II- PI treated enzyme was added to PEI treated support; Method III- PI treated enzyme was added to PEI treated support. After 3h, the support was rinsed with D/W and treated with 5 ml of 0.5% glutaradehyde for 1h; Method IV- Untreated enzyme was applied onto PI treated support; Method V- PEI treated enzyme was applied onto PI treated support.
Enzyme 2U was added per piece of cloth/0.2 g of OB seeds. Contact time for immobilization in all the methods was 3h at 30±1°C. The supernatant was assayed for unbound activity. Enzyme bound support was assayed for immobilized activity. The supports were stored under moist conditions (in 0.2 ml D/W) in refrigerator when not in use.
Desorption studies: The enzyme bound support was incubated in 5 ml of 0.2M NaCl for 10min at 40rpm. Residual activity in the support was assayed.
Optimization of enzyme dosage: Different units of the enzyme ranging from 1.35 to 5.4 were subjected to Method II of immobilization.

Characterization of the enzymes

Effect of temperature and pH on the activity of the free enzymes/ immobilized preparation was studied.
Enzyme was incubated at 30°C for 2h each in 5 ml buffer at various pHs ranging from pH 4 to 12.5 (Acetate, phosphate and glycine-NaOH buffers) for pH stability studies. Residual activity was then assayed at pH 5.6. Thermal stability was tested by incubating the enzymes at temperatures ranging from 35 - 55°C for 60 min followed by assay.
The enzyme was incubated with varying concentration of starch and the Km was determined from M-M and L-B plots.

Saccharifiaction studies

Recycling efficiency of the immobilized preparation: Immobilized preparation was incubated in 5 ml of the 1.5% starch solution for 2h at 35-50°C for 2h. The reducing sugars in the supernatant was estimated. The enzyme bound support was washed and resuspended in fresh batches of substrate under similar conditions.
Continuous use of immobilized preparation for starch saccharification: The immobilized preparations (10 cloth pieces/2g of OB seeds) were added to 30 ml of 8% starch solution contained in a 250 ml beaker and placed in the water bath maintained at 40°C at 40/100rpm. Aliquots of 50-100µl were taken periodically and analyzed for reducing sugar content. Percent conversion was calculated based on the consideration that 0.9g of starch produces 1g of glucose on saccharification.
Storage stability: Immobilized preparations were stored at 10°C in moist state for various time periods from 0-40days and then assayed.

RESULTS AND DISCUSSION

Concentration of the enzyme

Amylolytic activity elaborated by the fungus *A conoides* was assayed by DNSA and GOP method for estimation of total reducing sugars and glucose respectively. More than 95% of the reducing sugars comprised of glucose proving that the enzyme is glucoamylase. Paper chromatography results also proved that the major product of enzyme action was glucose (figure 1).

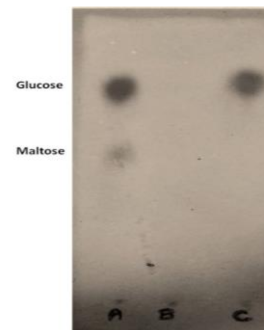

Figure 1 Paper chromatography for detection of reaction products. Lane A: Standards (glucose and maltose-20µg each); Lane B: 0 min; Lane C: 15min incubation

The yield of the enzyme was around 1.2U/ ml. The enzyme was concentrated by dialysing against PEG 20000, followed by salting out at 80% ammonium sulfate saturation. Concentrated enzyme was purified 3.26 folds with a specific activity of 3.92 and recovery of around 85%.

Immobilization of the enzyme

Five different approaches were employed for immobilization of the enzyme onto both the supports. **Melo *et al* (1986)** have suggested OB as a suitable pellicular support for immobilization of cells and enzymes. **Melo and D'Souza (1992)** have reported immobilization of invertase onto OB seeds. Sucrose, the substrate of invertase, is a small molecule with a molecular mass of 342 daltons. However, the substrate for amylase are starches which are large molecules. The average degree of polymerization of starch varies with origin and type, ranging from about 250-4000 anhydrous glucose units approximating to molecular masses of 40-650 kDa (Rutenberg, 1980). The performance of an immobilized enzyme acting on its high molecular weight substrate is affected to a great extent on the mode of attachment and rigidity conferred to the enzyme consequent to its binding. Immobilization of such enzymes to surface of the matrices may be advantageous to minimize diffusional limitations. Immobilization of glucoamylases, pectinases and cellulases onto activated surfaces of gels have been reported **(Tomar & Prabhu, 1985; Li *et al.*, 2008; Zhang et al., 2016).** Variety of supports with amino pendant groups have been used as enzyme carriers **(Yamazaki *et al*., 1984; D'Souza & Godbole, 2002; Alahakoon *et al.*, 2012)**. Various supports have been converted into anion exchangers by treatment with PEI **(Bahulekar *et al*., 1991; Wasserman *et al*., 1982).** Proteins can bind to the PEI treated support via their negatively charged functional groups. Method I involved coupling of the untreated enzyme to PEI treated support. Glucoamylases are reportedly glycoproteins in nature **(Shenoy *et al*., 1985)**. Glycoenzymes offer an opportunity to perform immobilization through their carbohydrate chains. Periodate (PI) ion is known to oxidize hydroxyl groups of adjacent carbon atoms in the glycosyl residues of the enzyme resulting in fission of the intervening C-C bond with formation of aldehyde groups **(Kiernan, 1990; Wong and Wong, 1992)**. The enzyme molecules can then be linked via the newly formed aldehyde groups through schiff's base to the PEI treated matrix. In Method II, PI treated enzyme was immobilized onto PEI coated support. An attempt was made in method III to further strengthen the binding using the bifunctional crosslinking reagent glutaraldehyde. The capsular mucilaginous layer of the OB is comprised of polysaccharides. As both the supports consist of carbohydrates, PI oxidation was employed as a strategy for activation of the supports in method IV. An attempt was made to couple the enzyme to PI treated support, wherein the aldehyde groups generated in the support were coupled to the amino groups of the enzyme. In method V, the amino pendant groups introduced into the enzyme by PEI treatment were coupled to PI treated carrier. The results are summarized in figure 2.

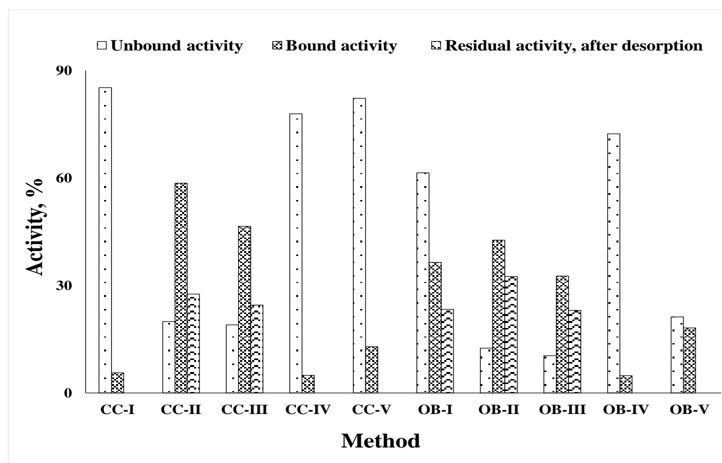

Figure 2 Binding of glucoamylase onto Cloth pieces and seeds of *Ocimum basilicum* by various techniques

Of the various techniques employed, methods II and III proved efficient for both supports. It was interesting to note that in methods III and V, the unbound fraction of OB exhibited only around 10.4 and 21% of activity respectively, implying that around 80-90% of activity was bound to the swollen seeds. However, only around 32.6% and 18.1% of activity was expressed as immobilized units. The pellicular nature of the support may have resulted in unproductive cross-linking between the enzyme and the support. In both the supports, maximum amount of immobilized units were obtained by methods II and III. In case of OB, method I also gave good yield of immobilized activity. These immobilized systems were subjected to desorption using 0.2M NaCl. As shown in fig.2, enzyme immobilized by method II could retain the enzyme to a greater extent in both the supports. For further studies, immobilization was carried out by binding PI modified enzyme to the PEI treated supports (method II). The result for optimization of enzyme dosage for immobilization is presented in fig.3.

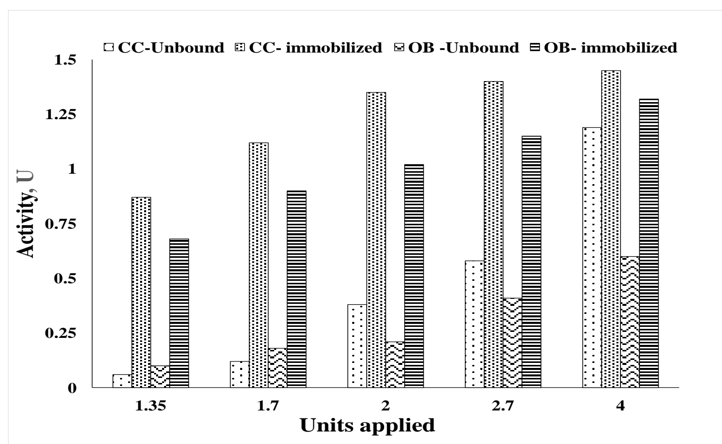

Figure 3 Optimization of enzyme dosage for immobilization of glucoamylase by method II

Based on these results, 2 units of PI treated enzyme was found to be the optimum load for cloth piece as maximal retention to an extent 67.5% occurred. Dosage of 1.68 units/ 0.2g of OB was optimal with a retention of 53% activity. It is well known that significant loss of activity occurs on covalent binding. The coupling efficiency and recovery of the enzyme by method II gave promising results. Enzyme immobilized by method II was characterized.

Characterization of the immobilized enzymes

The physico-chemical properties of the immobilized system were investigated and compared to those of free and PI treated forms. All the enzymes were optimally active at around the pH of 5.6-5.8. Immobilized enzymes however, exhibited broader pH-activity profiles as compared to those of native and PI oxidized forms (figure 4).

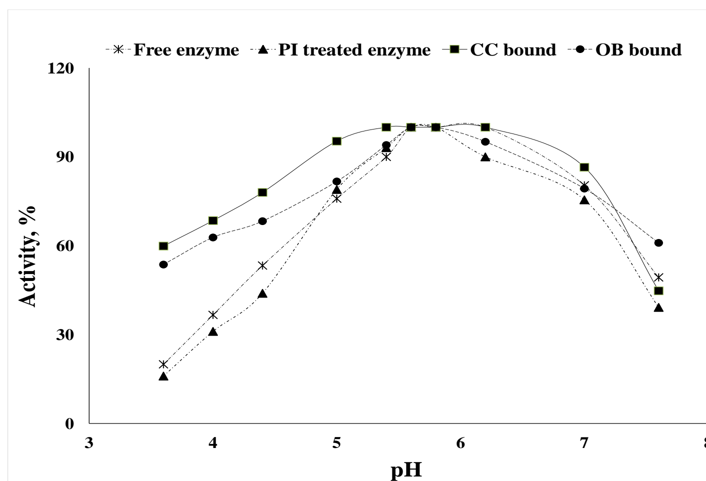

Figure 4 Effect of pH on the activity of glucoamylase preparations

The shift of pH and temperature profiles as a consequence of immobilization has been documented. Shift in pH/temperature-activity/stability profile or narrowing or broadening of the profiles as a consequence of immobilization have been reported **(Klibanov, 1983; Bachler *et al*., 2004; Guzik *et al*., 2014).** Both the immobilized forms were much more active at the acidic side of the pH optima. An enzyme attached to a poly electrolyte carrier may encounter micro-environmental effects in its immediate vicinity. Hydroxyl ions may accumulate at the poly-cationic carrier surface and hence the pH in the microenvironment of the enzyme is likely to be higher than the bulk solution. This may result in a shift in the pH versus activity profile to acidic side. Stability studies showed a significant degree of inactivation of free enzymes below pH 5 (fig 5A).

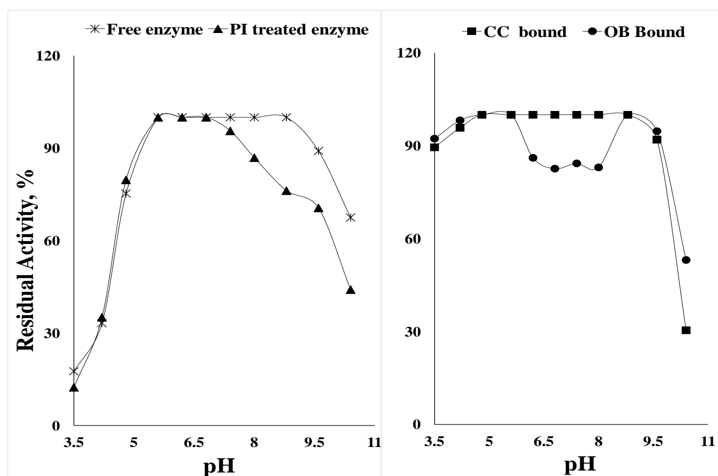

Figure 5 Effect of pH on the stability of the immobilized glucoamylase

The native enzyme which was stable in the range of pH 5.5- 8.8, lost its stability above pH 7 on oxidation with PI. The PI treated enzyme regained the stability in the alkaline side of pH and also attained stability in the acidic side of pH on immobilization to CC (fig 5b). Thus pH stability curve broadened on immobilization especially in case of enzyme coupled to CC. Enzyme immobilized on OB showed significant stabilization in acidic region. Interestingly, the residual activity was around 83-84% in phosphate buffer at pH 6.2 to 8.0. The stability increased to 100% at pH 8.8 and remained relatively stable till pH 9.6, decreasing thereafter. It appears that loss of activity at pH 6.2-8.0 may not be due to direct impact of pH on the stability of the enzyme. It was observed that the OB seeds swell further in size when incubated in phosphate buffers. This expansion might have had an impact on the structure of enzyme or the bond between the enzyme and support resulting in decreased activity. Effect of temperature on the activity of immobilized enzyme is exhibited in fig 6.

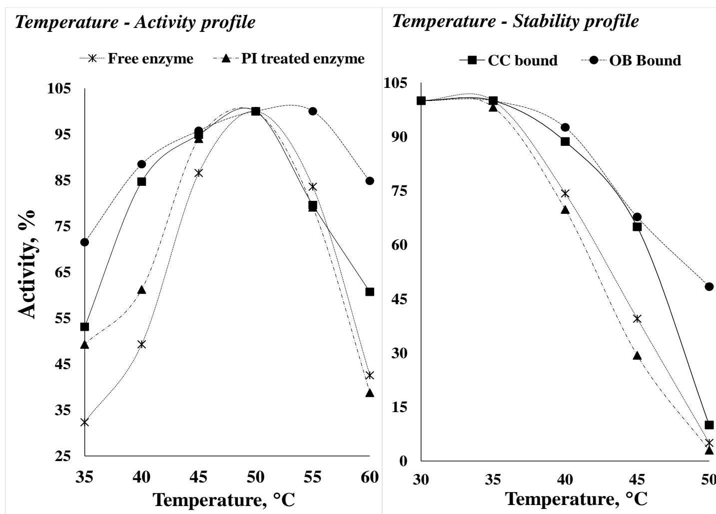

Figure 6 Effect of immobilization on the temperature-activity and thermo-stability profiles of the glucoamylase

The native enzyme, PI treated enzyme and both the immobilized forms were optimally active at 50°C. Enzyme immobilized on OB was optimally active at 50-55°C. Overall, both the immobilized forms exhibited relatively broader temperature zone of maximal activity and the free forms showed comparatively a narrow bell shaped curve. If unfolding is recognized as an indispensable step during thermal denaturation of an enzyme, then the more firm the protein moiety of the enzyme is fixed onto the support, the more difficult it is to unfold and inactivate the enzyme. Immobilization conferred protection to the enzymes against thermal denaturation to a significant extent as shown in figure 6.

The Km for native, PI treated enzyme, cloth bound and OB bound forms were 0.08, 0.105, 0.23 and 0.26 mg.ml^{-1} respectively. The apparent increase in the Km values by 22-25 fold could be due to the limited diffusion of the substrates towards the enzyme active site and the movement of the formed products from the microenvironment of the enzyme to the bulk solution or, due to possible changes in the enzyme structure on being immobilized. Diffusional limitation may be more pronounced in case of high molecular mass molecules such as starch.

Starch saccharification studies

It was observed that the rate of starch hydrolysis was relatively linear up to 120min for both the bound forms when fed with 5 ml of 15 mg.ml^{-1} starch. The immobilized systems were recycled 14 times (120min cycle each) over a period of 28h (figure.7).

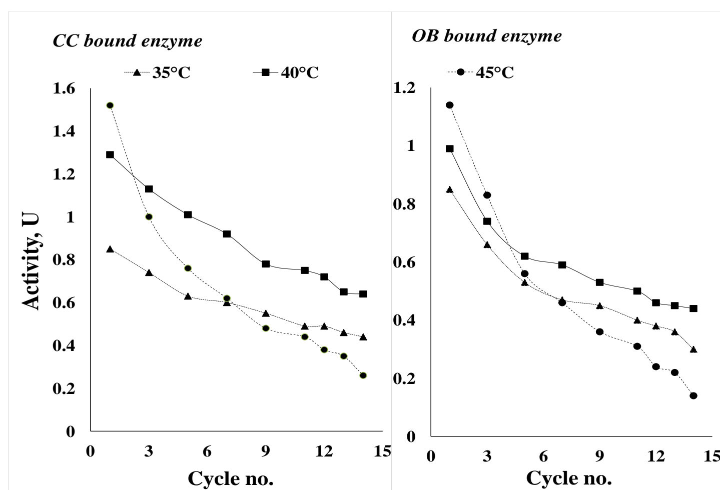

Figure 7 Recycling efficiency of the immobilized glucoamylase preparations

Enzyme coupled to CC could retain 50% of its activity at 40°C at the end of 13th cycle. Enzyme bound to OB seeds could retain 50% of its activity at 40°C at the end of 11th cycle. Both the systems lost 50% of their activity by the end of 5th cycle at 45°C. Total amount of glucose produced by immobilized cloth preparation in 14 cycles at 35°C, 40°C and 45°C were 182, 275 and 207mg respectively and 153, 187 and 155mg respectively by the OB bound enzyme. Product formation was maximum at 40°C for both the immobilized forms. Saccharification process was therefore carried out at the suboptimal temperature of 40°C (figure 8).

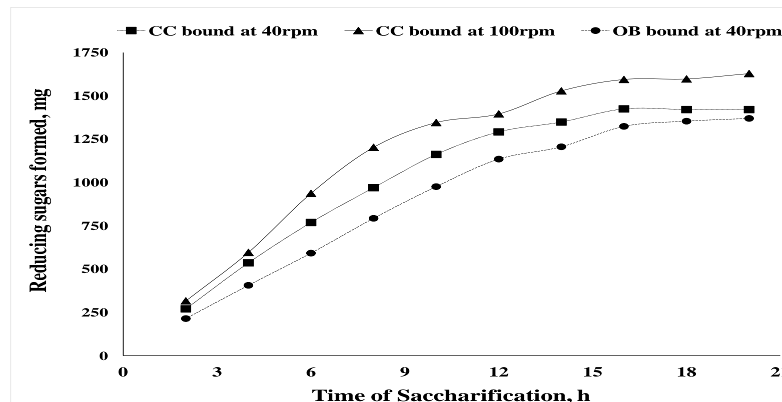

Figure 8 Continuous saccharification of starch by the immobilized preparations of glucoamylase

The enzymes in free state offered around 91.5-92% conversion. However, CC and OB bound preparations were able to convert starch only to an extent of 63.9 and 61.6% respectively at 40rpm. Decrease in % conversion of starch has been one of the major drawbacks suffered by most of the immobilized glucoamylases. As discussed earlier, affinity of the enzyme for the substrate decreased on immobilization. To assess the diffusional limitation as a factor influencing the apparent Km values, the immobilized enzyme was incubated with the substrate in a shaker water bath at 100 rpm to determine the Km. The Km values of 2.3 and 2.6 mg.ml^{-1} (at 40rpm) decreased to 1.98 and 1.86 mg.ml^{-1} for the enzyme coupled to cloth and OB seeds respectively. Diffusional limitation of the solutes, therefore appears to be one of the factors responsible for the apparent decrease in the affinity of the immobilized enzyme for starch. Functioning of CC bound preparation was relatively better than the OB preparation. The pellicular, mucilaginous coat of the OB seeds may undergo attrition at higher speeds of agitation when used continuously over long periods of time. Cloth does not encounter such issues. Saccharification of starch at 100rpm was therefore performed using cloth bound preparation. Saccharification to an extent of 73% could be achieved.

Both the enzyme preparations stored at 10°C (refrigeration) were stable upto 15 days. After 40 days of storage, the cloth bound enzyme lost 19.7% of its initial activity, whereas the OB bound seeds lost around 44% of activity.

CONCLUSION

Immobilization of the enzyme through its carbohydrate moieties appears to be a promising technique for immobilization of the glucoamylase produced by *A conoides* onto both the supports activated by treatment with PEI. Immobilzation conferred stability to the enzyme in the acidic region, hinting at micro-environmental effect due to cationic nature of the PEI treated supports. Immobilised enzymes were relatively more thermostable than in its free state. Although the enzyme preparations were optimally active at 50°C, optimum temperature for saccharification (continuous use) of the enzyme was found to be 40°C. Km of the enzyme increased on immobilization. Starch being a high molecular weight substrate, the immobilized preparation appears to encounter diffusional limitation. Conversion of starch by CC bound preparation improved when saccharification was carried out at higher speed of agitation CC preparation may hence, be well suited for use in fluidized bed reactors. . The support chosen for immobilization must be low cost, easy to use, and easily available and must bind to the enzyme via a simple and inexpensive activation method. In addition, for use in food industries it has to be non-toxic, chemically inert (under conditions of use) and non-biodegradable (**Contesini *et al*., 2013**) Cloth fulfils all these criteria and overall, cloth bound enzyme preparation was superior in its performance to the OB bound glucoamylase.

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