Research Article

Indian Journal of Agricultural Sciences **85** (1): 14–9, January 2015/Article

Immobilization kinetics of CMCase and β-glucosidase from *Aspergillus niger* in calcium alginate beads

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Received: 20 August 2013; Revised accepted: 3 July 2014

ABSTRACT

Carboxymethyl cellulase (CMCase) and β-glucosidase were produced by *Aspergillus niger* using mango peel residue as substrate and immobilized on calcium alginate beads through entrapment technique. The catalytic properties of the immobilized CMCase and β-glucosidase were compared to free enzyme. The enzyme efficiency of the immobilized CMCase and β-glucosidase were found to be 89.52 and 86.61%, respectively. Maximum immobilization efficiency was achieved on beads formed by 5% (m/v) of sodium alginate and bead size of 2 mm and 1.5 mm, for CMCase and β-glucosidase, respectively. The immobilized enzyme showed higher stability over a wider pH and temperature range. Metal ions, viz. Cu^{2+}, Mo^{2+}, Mn^{2+}, Ca^{2+}, B^{3+}, Zn^{2+}, Mg^{2+} and Fe^{2+} showed positive effect for immobilized β-glucosidase activity, while Mg^{2+} on immobilized CMCase activity. The immobilized enzymes could express 20-25% relative enzyme activity even after 4th repeated use. Immobilization improved the thermal stability over the free enzyme and affected the enzyme kinetic constants, viz. K_M, V_{max} and activation energy.

Key words: *Aspergillus niger*, β-glucosidase, CMCase, Immobilization, Mango peel

Cellulases are used widely in textile, food, animal feed, detergent, paper and pulp industry for hydrolyzing β-1, 4 glucan linkages of lignocellulosic materials. Commercially cellulases are produced by either solid or submerged fermentation using media containing cellulose of different purity which result in high cost. The cost of enzyme production may be reduced substantially if lower value lignocellulosic substrate including horticultural waste is used (Xin and Geng 2010).

Mango, being a major fruit in India is processed into a variety of products right from the immature to ripe stage. Mango processing industries generates huge amount of solid waste in the form of peel and stones amounting to 40-45% of total fruit processed. Mango peel contributes to 35-40% of the solid waste (Tandon and Garg 1999). However, it is either utilized for cattle feed (Garg *et al*. 1994, 1998, 2000) or deposited as landfills causing environmental pollution. It is a good source of pectin and fibre but its biochemical composition may vary according to the mango cultivar (Larrauri *et al*. 1996). Mango peel has been reported as substrate for pectinase (Garg and Asfaque 2010) and cellulase (Kumar *et al*. 2012) production. Earlier, *Aspergillus niger* was used for cellulase production using mango peel as substrate (Garg *et al*. 2008). However, the recovery and long term storage of purified enzyme hinders its commercial implementation. Immobilization of the enzymes on solid supports for increased stability and operational life time makes downstream applications easier (Drevon 2002, Doaa *et al*. 2009, Kumar *et al*. 2012). Use of entrapment technique on porous gel support such as calcium alginate beads leads to immobilized enzyme with high retention of specific activity and stability (Anwar *et al*. 2009, Kumar *et al*. 2012). The present study reports the immobilization of cellulase obtained from solid state fermentation of mango peel residue by *Aspergillus niger* on calcium alginate beads. The conditions for immobilization, *viz.* concentration of calcium alginate and bead size were optimized for highest enzyme activity and expressed in terms of immobilization efficiency. The process parameters, such as, pH, temperature and thermal stability affecting the performance of the immobilized enzyme were optimized and kinetic parameters of the immobilized enzyme were compared with that of free enzyme.

MATERIALS AND METHODS

Mango peel was obtained from mango processing industries of Chittoor industrial area. Pectin was removed from peel by boiling at 90°C for two hour with 3% (m/v) Sodium hexametaphosphate (SHMP) as per method of Garg and Ashfaque (2010) and filtered by muslin cloth. The pectin containing filtrate was discarded and the residue left was dried at 60±2.0^{0}C till 5-7% w/w moisture, powdered to 100 mesh size and used as substrate for carboxymethyl

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cellulase (CMCase) and β-glucosidase production.

The cellulolytic microorganisms were isolated from cellulose rich organic substrates. Best cellulolytic microbial culture screened as per method discussed by Kumar 2012, was identified as *Aspergillus niger* NAIMCC-F-02958.

All the chemicals used were of analytical grade. Sodium alginate, calcium chloride, carboxymethyl cellulose and ammonium sulphate were purchased from E-Merck, while Dinitrosalicylic acid (DNS) from Sigma.

For production of CMCase and β-glucosidase using mango peel as a substrate by *A. niger*, the process standardized by Kumar *et al.* (2012, 2013) was followed. 100g of sterilized substrate, i e pectin extracted mango-peel residue (PEMPR) with 70% moisture in 250ml Erlenmeyer flasks was inoculated with 10^8 CFU of *A. niger* and allowed to ferment for 4 days at pH 5, temperature 40°C for β-glucosidase and after 5 days at pH 6, temperature 40°C for CMCase. Protein in culture filtrate (25 ml) obtained after fermentation was partially purified by ammonium sulphate precipitation (70% saturation) followed by centrifugation at 12000 rpm and dialysis (using Himedia LA 390 dialysis membrane) twice in acetate buffer (50mM, pH 5.0).

The partially purified CMCase and β-glucosidase with enzyme activity 0.28 and 0.19 U/ml, respectively were mixed independently with sodium alginate solution in 1:1 ratio by varying the final concentration of the alginate between 1-9% (m/v). The enzyme-alginate mixture was added drop-wise into calcium chloride 2% (m/v) solution with continuous shaking at 4°C to obtain beads size 1-3 mm which were kept for 4 hr. The beads thus formed were washed 3-4 times with de-ionized water and finally with 50mM acetate buffer of pH 5.0, dried and weighed before using for further studies.

The assay reactions for the immobilized CMCase and β-glucosidase were set up according to the protocols described by Plahuta and Raspor (1996) with minor modifications. The reaction mixture in acetate buffers (50mM, pH 5) containing 200 mg of enzyme immobilized calcium alginate beads; 400 µl of 1% (m/v) carboxymethyl cellulose solution and cellobiose for CMCase and β-glucosidase respectively, was incubated at 30ºC for 60 min and the products formed were assayed using DNS reagent. The reaction was stopped by incubating in 100ºC water bath for 10 min. Enzyme activity was expressed in units, where, one unit is defined as the amount of CMCase or β-glucosidase that produced 1 µmole of reducing sugar under assay condition per gram of bead.

Protein concentration was estimated by Lowry's method using with bovine serum as a standard (Lowry *et al.* 1951).

Immobilization efficiency was determined from the difference in enzyme activity in the solution before and after the immobilization (Jabasingh and Nachiyar 2011).

Immobilization Efficiency (%) = (IE/AE-UE) × 100

where, AE = added enzyme (U/g of bead); UE = unbound enzyme (U/g of bead); IE = immobilized enzyme (U/g of bead).

The activity of free and immobilized CMCase and β-glucosidase was assayed at various temperature, ie. 20–70ºC (10ºC intervals) and pH 3.5-7.0 (0.5 intervals) by adopting the strategy of Figueira *et al.* (2011) and by following assay conditions as per Dey *et al.* (2001) with minor modifications.

Free and immobilized enzymes were incubated at 60ºC in 50 mM acetate buffer for 3 h. Sample were taken at different intervals and activity were measured by method as described earlier. The change in activity as a function of time was measured as the temperature stability (Zeng *et al.* 2009).

Kinetics of the immobilized and free CMCase was determined at pH 4.5 and temperature 50ºC while, for β-glucosidase pH 5 and 50ºC, respectively. Michaelis-Menten constant (K_M) of immobilized CMCase and β-glucosidase were determined by hydrolysis of carboxymethyl cellulose sodium salt and cellobiose, respectively at varying concentrations (0.25-20 mg/ml and 0.65-25 mg/ml) from Lineweaver-Burke plot. The activation energy (-Ea) of the immobilized and free enzymes were estimated from Arrhenius plot by plotting the log of the reaction rate against 1/T.

RESULTS AND DISCUSSION

CMCase and β-glucosidase, enzymes are produced extracellularly by fungi. In the present study, pectin was removed from the peel in the first step and then the pectin extracted mango peel residue was used as substrate for production of CMCase and β-glucosidase by *Aspergillus niger*. Ammonium sulphate precipitation and dialysis of culture filtrate resulted in 6.037 and 3.054 fold purifications, respectively. The specific activities calculated for CMCase and β-glucosidase after purification were 1.77 and 3.82 U/mg, respectively. This partially purified enzyme was immobilized in calcium alginate beads via entrapment technique and the enzymatic properties were characterized.

Immobilization efficiency as affected by sodium alginate concentrate

Immobilization efficiency depends on the sodium alginate concentration and shows a linear relationship which in turn influences the specific activity of enzyme. This is because the rate of hydrolysis is affected by the size of the final lattice formed by the bead. A concentration of 0.14 and 0.098 U of CMCase and β-glucosidase activity, respectively, was used per ml of sodium alginate-enzyme mixture in formation of Ca-alginate beads. Beads formed by 5% (m/v) of sodium alginate in 2% (m/v) calcium chloride resulted in maximum immobilization efficiency for CMCase (87.31%) and β-glucosidase (85.25%) (Fig 1A). This theory of calcium alginate-enzyme immobilization has been explained by Anwar *et al.* (2009) and similar results have been achieved in case of amylase immobilization on calcium alginate beads by Kumar *et al.* (2012). Calcium alginate immobilization of cellulase from various bacteria and fungi has been reported earlier (Attitalla and Salleh 2010, Andrini *et al.* 2012).

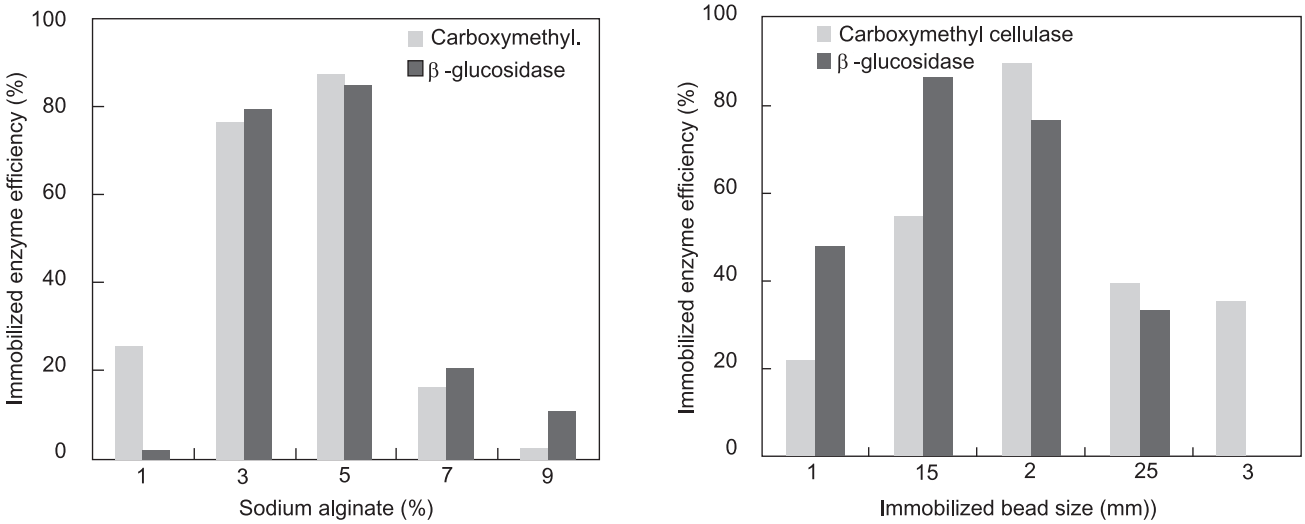

Fig 1 Optimization of sodium alginate concentration (**A**) and beads size (B)* on immobilized CMCase and β-glucosidase activity.

Immobilization efficiency on beads size

Bead size is another major factor that influences the immobilization efficiency. The substrate has to diffuse into the beads for the enzymatic reaction to take place in the immobilized enzyme system Maximum CMCase and β-glucosidase immobilization efficiency was achieved in 2mm and 1.5mm bead size, about 89.52% and 86.61% respectively (Fig 1B). Immobilized CMCase of *Trichoderma reesei* is reported to have retained 80% of its original activity in calcium alginate beads (Busto *et al*. 1998).

Comparative study of immobilized vs. free enzyme at different pH and temperature

The enzyme activity of the immobilized CMCase and β-glucosidase were compared to free enzyme at a range of pH (3.5-7.0) and temperature (20-70ºC). The results indicated that immobilized CMCase were more stable compared to free enzyme. The free enzyme showed optimum activity at pH 4.5 while immobilized CMCase in a broader pH range between 4.0-5.0. Wongkhalaung *et al*. (1985) reported more stable activity of immobilized cellulase at acidic pH than the free enzyme. The β-glucosidase enzyme showed optimum activity at pH 5.0 in immobilized as well as free enzyme though, better stability was observed in immobilized enzyme (Fig 2). The optimum relative activity reduced sharply to 61 and 57.8% in immobilized enzyme, 60.7 and 47.3% in free enzyme at pH 4.5 and 5.5, respectively (Fig 2A). The immobilized CMCase was active at the temperature range of 50-70°C with optimum activity at 50ºC (Fig 2B). In case of free enzyme 40ºC was the optimum temperature and the activity decreased sharply (up to zero) by increasing temperature at 60°C. These results indicate that thermo stability could be increased by immobilization.

Immobilized β-glucosidase was stable in the temperature range of 35-50ºC and for free enzyme at 40ºC and its activity reduced to 78 and 56% with variation of temperature from 20 to 50°C (Fig 2B). Iqbal *et al*. (2012) reported that thermo-stability of immobilized cellulase enhanced up to 75% at 80°C as compared to the free enzyme. Earlier, Chakrabarti and Storey (1988) and Yuan *et al*. (1998) have also reported that immobilized cellulase had better stability with respect to pH and temperature as compared to free.

Operational thermal stability

Thermal stability of the immobilized CMCase and β-glucosidase after heat inactivation at 70ºC was found to be more than that of free enzyme over a longer time interval compared to that of free enzyme. The immobilized CMCase enzyme retained up to 60.9% relative activity compared to free enzyme (which retained only 13.33% of the optimum) even after 180 min. Similarly, immobilized β-glucosidase retained up to 54.23% activity of the optimum compared to free enzyme where it was 15.68% after 180 min (Fig 2C).

The immobilization material has a protecting effect due to the changes in the conformational flexibility of the enzyme. This step increases the enzyme thermal stability (Abdel-Naby 1993). Immobilized CMCase and β-glucosidase were used repeatedly up to four times and 20-25% relative activity was observed during 4th time use. Similar results were observed for immobilized amylase from *Fusarium solani* (Kumar *et al*. 2012) and immobilized cellulase from *Aspergillus niger* (Kumar *et al*. 2012). Liang and Cao (2012) reported that the immobilized cellulase retained its initial activity after repeated five cycles of hydrolysis reaction.

Effect of metal ions on immobilization

Metal ions act as cofactor in enzymatic reactions. The results indicated that Mg^{2+} expressed positive while, Cu^{2+}, Mo^{2+} , Mn^{2+}, Ca^{2+}, B^{3+}, Zn^{2+} and Fe^{2+} negative effect on free CMCase activity. The effect of Cu^{2+}, Mo^{2+}, Mn^{2+}, Ca^{2+}, B^{3+}, Zn^{2+}, Mg^{2+} and Fe^{2+} ions was recorded as positive on immobilized CMCase and β-glucosidase activity (Fig 3). Similar results have been reported by Narasimha *et al*.

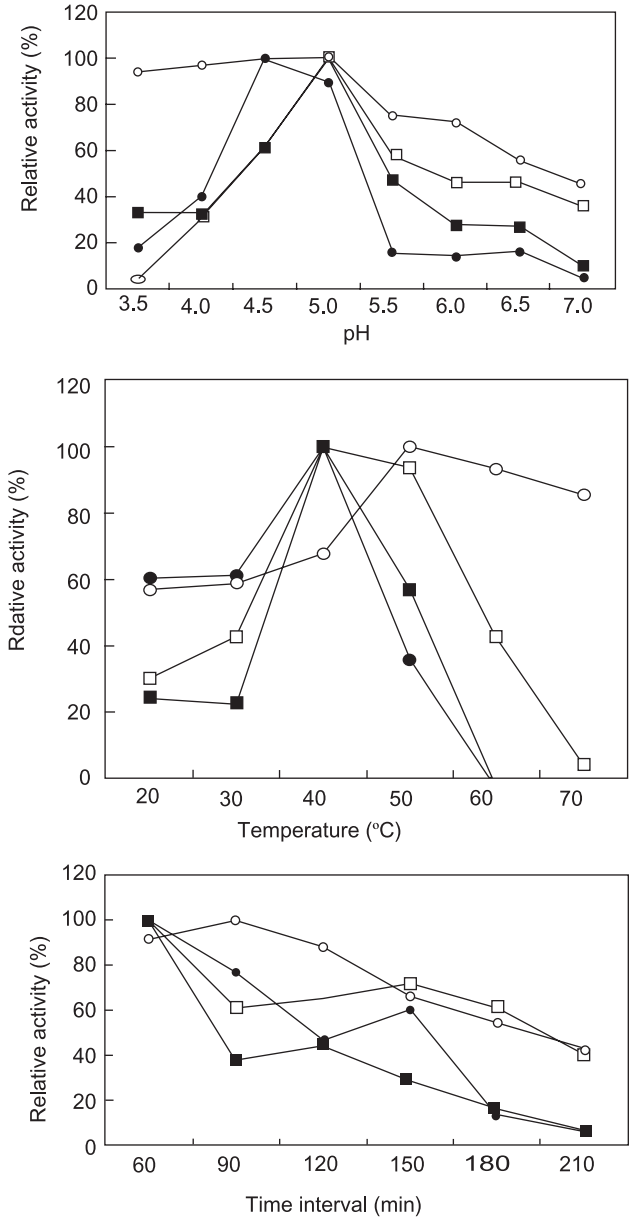

Fig 2 Optimization of pH, temperature and time interval for maximum enzyme relative activity. (-●-) Free CMCase; (-○-) immobilized CMCase; (-■-) free β-glucosidase and (-□-) immobilized b-glucosidase

(2005). Chen *et al*. (2004) reported inhibitory effect of Ag ion on the activity of carboxymethyl cellulase from *Sinorhizobium fredii*.

Kinetic properties of immobilized CMCase and β-glucosidase

The kinetic properties of immobilized and free CMCase and β-glucosidase are reflected in Table 1. The K_M values were 10.1 and 10.0 mg/ml while the Vmax values were 0.056 and 3.2 mole/min/ml for immobilized and free CMCase, respectively. Similarly, K_M values were 1.26, and 1.2 (mg/ml) while the Vmax were 0.084 and 0.29 mole/min/ml for immobilized and free β-glucosidase, respectively. The increase in K_M (0.83 mmol) and lower V_{max} (38.2

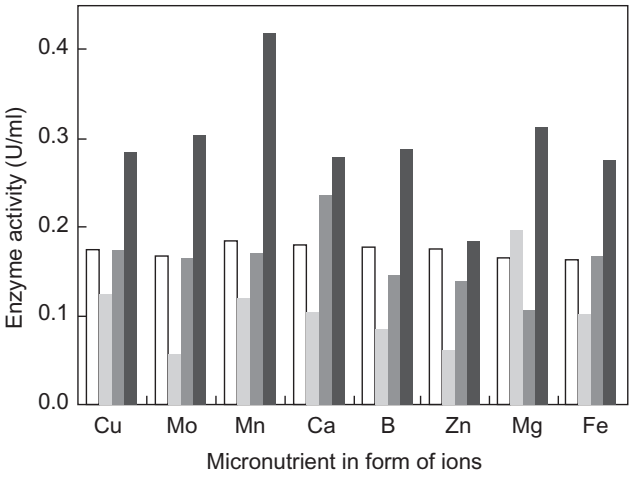

Fig 3 Effect of metal ion on immobilized and free enzyme. Immobilized CMCase (□); free CMCase(▒) ; immobilized β-glucosidase (▒) and free β-glucosidase (■)

Table 1 Kinetics profile of carboxymethyl cellulase (CMCase) and b-glucosidase

Kinetic parameter	CMCase		β-glucosidase	
	Free	Immobilized	Free	Immobilized
K_m (mg/ml)	10.0	10.2	1.20	1.26
V_{max} (mole/min/ml)	0.32	0.56	0.29	0.084
Activation Energy (K cal/mole)	9.32	5.41	53.24	3.1

μmol/min) as a result of immobilization has been reported by Jabasingh *et al*. (2011). Zhang *et al*. (2006) also reported lower K_M and higher V_{max} for free cellulase compared to immobilized enzyme.

The Arrhenius plot showing the activation energy (Ea) of immobilized and free CMCase 5.41 and 9.32 while, 3.1 and 53.24 Kcal/mol for immobilized and free β-glucosidase, respectively as given in Fig 4. However, the decline in activation energy might be due to reduced conformational flexibility of the immobilized enzyme. The calcium alginate immobilization of CMCase and β-glucosidase from *A. niger* enhanced the enzyme stability over wider pH at temperature for longer shelf-life. Similar results of longer shelf-life of immobilized cellulase by superparamagnetic nanoparticles were reported by Xu *et al*. (2011).

CONCLUSION

The study indicated that immobilization of extracellular CMCase and β-glucosidase in 2 and 1.5 mm sized calcium alginate beads, respectively resulted in better pH and thermo stability over a longer period of time, though the relative enzyme activity was reduced slightly. The low catalytic efficiency and instability of enzymes are generally considered as drawbacks for the use in industrial processes. However, taking into consideration the reduction in cost due to reusability and better pH and thermal stability, immobilized enzyme may find wider application in industries where these are used on a large scale.

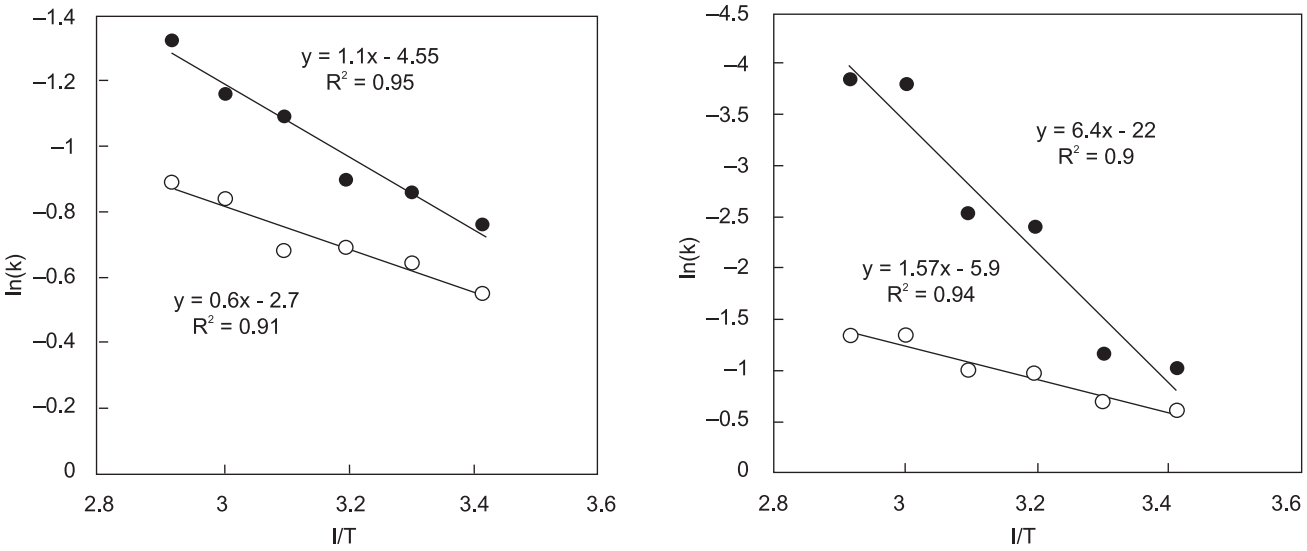

Fig 4 Arrhenius plot of energy of thermal deactivation for immobilization vs. free enzyme: (**A**) CMCase and (B) β glucosidase. Immobilized enzyme (-○-) and free enzyme (-●-)

ACKNOWLEDGMENT

This research was supported by funding from Application of Microorganisms for Agriculture and Allied Sector (AMAAS) networking project of Indian Council of Agricultural Research.

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