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Galacto-oligosaccharide synthesis by immobilized *Aspergillus oryzae* β-galactosidase

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Abstract

Aspergillus oryzae β-galactosidase was immobilized by three different techniques, namely adsorption on celite, covalent coupling to chitosan and aggregation by cross-linking. These techniques were compared in terms of the yield of immobilized preparation, enzymatic characteristics, stability and efficiency in oligosaccharide synthesis. Immobilization led to increase in K_m in each case. Immobilization on chitosan gave maximum enzyme yield and oligosaccharide synthesis. At 60 °C, the chitosan-immobilized enzyme was stabilized (by 1.6-fold) due to protection effect of the matrix. However, at 65 °C, the $t_{1/2}$ of cross-linked enzyme aggregates (CLEA) of β-galactosidase was 1.07 h as compared to 0.79 h in the case of free enzyme. Both chitosan-immobilized enzyme and CLEA were used for oligosaccharide synthesis. Using 20% (w/v) lactose, the chitosan-immobilized enzyme gave maximum oligosaccharide yield (17.3% of the total sugar) as compared to free enzyme (10.0%) in 2 h at 40 °C. CLEA were instead found effective in lactose hydrolysis yielding 78% monosaccharide in 12 h.

Keywords: Adsorption; *Aspergillus oryzae*; Celite; Chitosan; Covalent coupling; Cross-linked enzyme aggregates; β-Galactosidase; Immobilization; Oligosaccharide synthesis

1. Introduction

Oligosaccharides and their derivatives are important biomolecules, having a range of important functions in biological systems. They are bifidogenic and also help in: (i) reduction of serum cholesterol level; (ii) synthesis of B-complex vitamins; and (iii) enhance absorption of dietary calcium (Perugino, Trincone, Rossi, & Moracci, 2004; Sako, Matsumoto, & Tanaka, 1999). Due to the possible health benefits associated with the consumption of these compounds, their use as food ingredients has grown rapidly, particularly in Japan and Europe. Their chemical synthesis is tedious, hence enzymatic synthesis using β-galactosidase is advocated (Monsan & Paul, 1995; Sears & Wong, 2001). β-Galactosidase catalyzes lactose hydrolysis as a forward reaction and oligosaccharide synthesis as a reverse reaction (transgalactosylation). Although immobilized galactosidases for hydrolysis have been well worked out, these are not effective for oligosaccharide synthesis (Khare, Jha, & Gandhi, 1994; Szczodrak, 1999). Requirements for β-galactosidase-mediated synthesis are altogether different. The reaction conditions should be those favouring trangalactosylation or reverse reaction, namely high lactose concentration, elevated temperature and low water activity in the reaction medium. β-galactosidase preparations, thermally stable and exhibiting high transgalactosylation activity, need to be developed for efficient oligosaccharide synthesis (Boon, Janssen, & van 't Riet, 2000; Irazoqui, Villarino, & Batista-Viera, 2002). Some of the approaches, such as screening of extremophilic

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enzymes (Akiyama, Takase, Horikoshi, & Okonigi, 2001), protein engineering (Hansson, Kaper, van Der Oost, de Vos, & Adlercreutz, 2001), media optimization (Maugard, Gaunt, Legoy, & Besson, 2003) and immobilization, have been attempted (Albayrak & Yang, 2001; Choi et al., 2003).

Immobilization has been a widely employed technique in industrial applications of enzymes for enhancing stability, imparting reusability and making enzyme-based processes cost-effective and viable (Mosbach, 1987; Tsakiris et al., 2004; van Beilen & Li, 2002). Immobilization of enzymes has been extensively studied on a wide range of matrices; apparently, the suitability of support and method of immobilization vary from enzyme to enzyme and their intended use. It needs to be optimized for each system (Burton, Cowan, & Woodley, 2002; Plessas, Bekatorou, Kanellaki, Psarianos, & Koutinas, 2005). An appropriate immobilized system leading to high temperature stability and transgalactosylation activity is yet to be developed. The present study explores an appropriate immobilization method for β-galactosidase, which can be applicable in oligosaccharide synthesis.

Comparative evaluation of three major immobilization techniques (adsorption, covalent coupling and cross-linked enzyme aggregation (CLEA) of *Aspergillus oryzae* β-galactosidase) is described.

2. Materials and methods

2.1. Material

Aspergillus oryzae β-galactosidase (EC 3.2.1.23) and oligosaccharide standards (maltotriose and maltotetrose) were obtained from Sigma Co., USA. Chitosan was procured from Fluka, Switzerland. Glucose oxidase-peroxidase kit was purchased from Merck, India. All other reagents used were of analytical grade.

2.2. Physical adsorption of enzyme on celite

β-Galactosidase (2 mg) was mixed with celite (1 g, suspended in 10 ml of 0.1 M acetate buffer, pH 4.5) and incubated overnight at 4 °C with constant shaking. The preparation was washed repeatedly with buffer until washings were free of protein and enzyme activity. The celite-bound enzyme was suspended in 5 ml of the same buffer and used as an immobilized preparation for further studies.

2.3. Covalent coupling of enzyme to chitosan

Chitosan was prepared by following the method of Sardar, Roy, and Gupta (2003). Chitosan (0.5 g) was dissolved in 25 ml of 1% acetic acid over 2 h at 30 °C with constant stirring. Afterwards, the pH was adjusted to 8.0 using 3 M NaOH, which caused the precipitation of chitosan. The precipitate was washed with buffer (0.1 M, Tris–HCl, pH 8.0) and suspended in 45 ml buffer (0.1 M, acetate, pH 4.0), followed by addition of 5 ml glutaraldehyde (25%) with magnetic stirring; 40 mg of enzyme were added to this suspension and the mixture was incubated at 30 °C for 1 h with continuous shaking. The preparation was centrifuged at 8000*g* for 10 min at 25 °C. The pellet containing the bound enzyme was washed with acetate buffer until washings were free of enzyme activity. It was finally suspended in 5 ml of acetate buffer.

2.4. Preparation of β-galactosidase cross-linked aggregates

β-Galactosidase (20 mg) was dissolved in 1 ml phosphate buffer (0.1 M, pH 7.0) and maintained at 4 °C. $(NH_4)_2SO_4$ (0.55 g) was added to it, followed by the addition of another lot of ammonium sulfate in solution form (1 ml of 55% $(NH_4)_2SO_4$, w/v in distilled water). To this solution, 80 μl of glutaraldehyde (25%) was added and kept for stirring at 4 °C for 16 h. The aggregates formed were centrifuged and washed thoroughly with phosphate buffer until no activity was observed in the washings. These were finally suspended in 3 ml buffer (0.1 M, glycine–HCl, pH 3.0) and used for further studies.

2.5. Protein estimation

Protein was determined by the dye binding method (Bradford, 1976) using bovine serum albumin (BSA) as standard protein.

2.6. β-Galactosidase assay

Activity of free and immobilized β-galactosidase enzyme was estimated by following the method of Batra, Singh, Banerjee, Patnaik, and Sobti (2002), using *o*-nitrophenyl-β-D-galactopyranoside (ONGP) as substrate. 1 ml of a suitable dilution of free and immobilized enzyme was taken in appropriate buffers (free and celite immobilized enzyme: 0.1 M acetate buffer, pH 4.5; chitosan-immobilized enzyme: 0.1 M, acetate buffer, pH 4.0; CLEA: 0.1 M glycine–HCl buffer, pH 3.0) with 2 ml of substrate (5 mM in respective buffers). This mixture was incubated at 55 °C for 10 min and the reaction was stopped by addition of 2 ml of 1 M sodium carbonate. Absorbance was recorded at 405 nm.

Enzyme activity is defined as the amount of enzyme that liberates 1 μmol of *o*-nitrophenol (ONP) per minute under standard assay conditions.

2.7. Thermal stability of native, immobilized and CLEA preparations

The free, celite and chitosan-immobilized and CLEA preparations of β-galactosidase were incubated at different temperatures (50, 55 and 60 °C). Samples were withdrawn at various time intervals and the residual enzyme activity was determined in each case under assay conditions.

2.8. Oligosaccharide synthesis

Enzyme preparations (free, immobilized and CLEA, 4 U each) were incubated with 20% lactose (w/v) solution in appropriate buffers (free and celite-immobilized enzyme: 0.1 M acetate buffer, pH 4.5; chitosan immobilized enzyme: 0.1 M, acetate buffer, pH 4.0; CLEA: 0.1 M glycine–HCl buffer, pH 3.0) at 40 °C in sealed vials with continuous shaking. Samples were withdrawn periodically from the reaction mixtures and kept in a boiling water bath for 10 min. Oligosaccharides formed were analyzed by a normal phase HPLC (4.6×25 cm, Supelcosil LC-NH_2 column, Supelco Co., USA) using refractive index detector (Shimadzu, Japan). Acetonitrile and water (80:20, v/v), at a flow rate of 1.5 ml/min, were used as mobile phase for elution. Percentage of each carbohydrate was calculated from its HPLC peak area divided by the total peak area due to all sugars formed at that time.

2.9. Reusability of chitosan immobilized enzyme

The reusability of immobilized preparation was assessed at 37 °C by carrying out the hydrolysis of lactose (2%, w/v) in 0.1 M acetate buffer, pH 4.0, containing 1 U immobilized enzyme activity and monitoring the amount of glucose liberated after each cycle using a glucose oxidase-peroxidase kit. After each cycle of hydrolysis, the pellet containing the immobilized enzyme was recovered by centrifugation at 8000*g* for 5 min at 25 °C and reused for further cycles similarly.

3. Results and discussion

β-Galactosidase catalyses both hydrolysis and transgalactosylation reactions. Tansgalactosylation is favoured at high substrate concentration, low water content and high temperature (Huber, Gupta, & Khare, 1994). Hence, efforts to get an immobilized preparation stable, at high temperature, low water content and giving high transgalactosylation activity, was attempted. Adsorption, entrapment, covalent coupling and aggregation are four major techniques for immobilization of enzymes, each having its own merits and demerits. Adsorption is simple but causes problems of leaching; covalent coupling overcomes this, but harsh conditions lead to undesirable loss of activity, at times (Khare & Nakajima, 2000). Entrapment can be applied to only low molecular weight substrates and products due to diffusion limitations (Khare & Gupta, 1988a). Aggregation often generates a very stable preparation but low yield and poor packing properties are associated drawbacks in this case (Broun, 1976; Khare & Gupta, 1990). In the present work, all the above techniques were tried for immobilization of *Aspergillus oryzae* β-galactosidase, to obtain a suitable preparation for effective oligosaccharide synthesis. Entrapment was not attempted because it may not be useful here, due to the formation of high molecular weight products (oligosaccharides).

A. oryzae β-galactosidase was adsorbed on celite, covalently coupled to chitosan and aggregated by gluteraldehyde. Immobilization conditions were optimized for each of these techniques. Table 1 compares the results of each of these techniques in terms of yield of immobilized enzyme and kinetic parameters. Chitosan-immobilized enzyme preparation showed the maximum

Table 1
Characterization of immobilized and cross-linked preparation of *Aspergillus oryzae* β-galactosidase

Parameters	Free enzyme	Chitosan-immobilized enzyme	Celite-adsorbed enzyme	CLEA
Activity yield (%)	100	18.4	2.0	13.5
pH optima	4.5	4.0	4.5	3.0
Temperature optima (°C)	55	55	55	55
$t_{1/2}$ (h)				
55 °C	14.60	18.00 (1.23)	14.17 (0.97)	17.37 (1.19)
60 °C	2.26	3.63 (1.60)	4.09 (1.81)	3.12 (1.38)
65 °C	0.52	0.79 (1.51)	–	1.07 (2.06)
k_i (h^{-1})				
55 °C	0.06	0.04	–	0.03
60 °C	0.19	0.30	–	0.138
65 °C	0.88	1.32	–	0.546
K_m (mM)	2.63	6.38	–	4.13
V_{max} (μmol ONP/min)	0.36	0.74	–	0.24

Numbers in brackets show the stabilization factor for the enzyme afforded by immobilization.
k_i, inactivation kinetic constant.
$t_{1/2}$, half-life.

enzyme yield. There were not much changes in pH and temperature optima upon immobilization. Increase in K_m, as a result of immobilization, was observed in all the methods. Similar behavior, subsequent to immobilization, has been reported in the case of other enzymes. *E. coli* β-galactosidase attached to con A by covalent coupling has been reported to give 10% yield, without any change in pH and temperature optima, but increase in K_m by 1.94-fold (Khare & Gupta, 1988b).

Since, chitosan immobilization and cross-linked aggregation of β-galactosidase showed higher enzyme recovery, these preparations were selected for further characterization and oligosaccharide synthesis.

The chitosan-immobilized β-galactosidase and CLEA preparation were thermally more stable than the free enzyme. The plot of log% residual activity versus time for free and immobilized enzymes at 60 °C, showed first-order inactivation kinetics (Fig. 1). Immobilization led to enhancement in the half-lives of enzyme in both chitosan and aggregate preparations at all the temperatures employed (Table 1). The inactivation constant (k_i) of immobilized chitosan at 60 and 65 °C was higher than that of free enzyme, indicating higher thermostability due to immobilization. Enhancement in thermal stability due to immobilization is well documented (Calsavara, Moraes, & Zanin, 2000; Tyagi & Gupta, 1995).

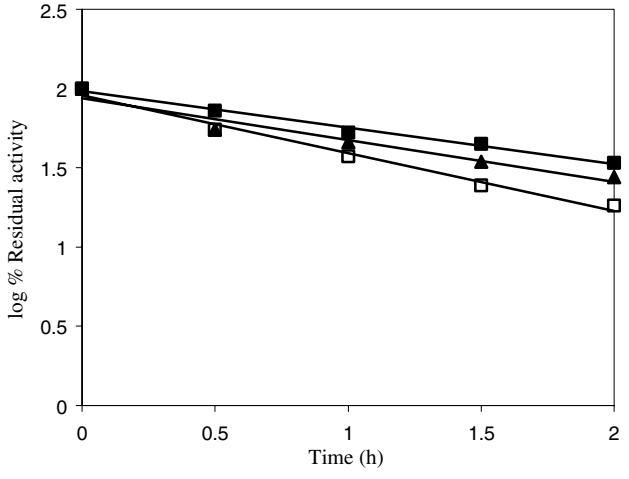

Fig. 1. Thermal stability of *A. oryzae* β-galactosidase. Free, chitosan-immobilized enzyme and CLEA were incubated at 60 °C. Residual enzyme activity was determined in the periodically withdrawn samples using ONGP as substrate. Free β-galactosidase (□); chitosan-immobilized β-galactosidase (■) and CLEA (▲).

To assess the suitability for oligosaccharide synthesis, the chitosan-immobilized and CLEA preparations were incubated with 20% lactose at 40 C (optimized conditions for synthesis). In case of chitosan-immobilized enzyme, 17.3% trisaccharide yield was obtained within 2 h as compared to 10.0% obtained with free enzyme (Table 2).

However, the trisaccharide yields by free enzyme and immobilized preparation became almost the same (22.6 versus 25.5%) when reaction was carried out for 12 h. In fact, tetrasaccharide formation from trisaccharide starts in a longer reaction time, as is evident from very small peaks corresponding to higher oligosaccharides.

However, immobilized enzyme still has advantage over free enzyme because of its reusability. Secondly, if the synthesis is performed at 60 °C or conditions for synthesis are optimized for immobilized enzyme, the difference in oligosaccharide yield may be more pronounced. Mozaffar, Nakanishi, and Matsuno (1989) have reported increase in trisaccharide yield from 6% to 12% upon modification of β-galactosidase with glutaraldehyde. The CLEA of β-galactosidase resulted in low oligosaccharide synthesis. These were found to be more effective in lactose hydrolysis, yielding 78% monosaccharides in 12 h as compared to 3.9% observed with free enzyme.

One of the limitations associated with the industrial application of enzymes is their high cost and instability under operational conditions. The overall process becomes cost-effective if the preparation shows higher efficiency and reusability. Chitosan-immobilized enzyme, in the present study, could be reused for four cycles of

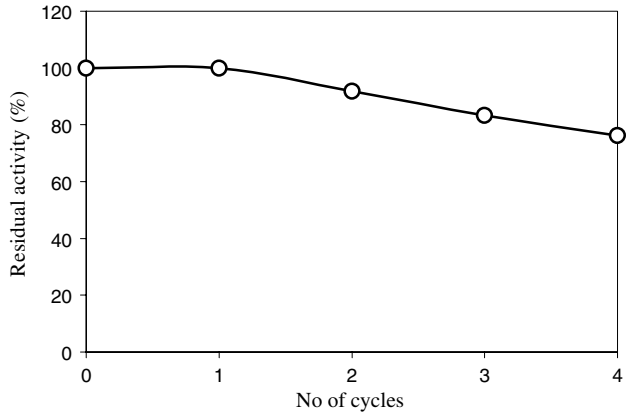

Fig. 2. Reusability of chitosan-immobilized enzyme. The immobilized preparation was used consecutively for four cycles, as described in Materials and methods section.

Table 2
Oligosaccharide synthesis by free (a), immobilized (b) and cross-linked (c) *A. oryzae* β-galactosidase

Time (h)	Monosaccharides (%)			Disaccharides (%)			Trisaccharides (%)			Higher oligosaccharides (%)		
	(a)	(b)	(c)	(a)	(b)	(c)	(a)	(b)	(c)	(a)	(b)	(c)
0	3.0	2.9	2.8	96.9	97.0	97.1	0.0	0.0	0.0	0.0	0.0	0.0
2	4.9	7.7	50.3	85.0	75.0	45.0	10.0	17.3	4.6	0.0	0.0	0.0
12	6.9	15.9	78.0	68.7	58.5	20.3	22.6	25.5	1.4	1.5	0.0	0.0

lactose hydrolysis without significant loss in activity (Fig. 2). Considering the ease of the colorimetric method, the reusability of enzyme was studied for lactose. It is believed that reusability of immobilized enzyme, for both the reactions, could normally be the same (Khare, Snape, & Nakajima, 2001).

It is therefore concluded that covalent coupling to chitosan seems to be an appropriate method for β-galactosidase immobilization for oligosaccharide synthesis application, as compared to adsorption or aggregation. Enzyme aggregates appear to be more suitable for lactose hydrolysis applications.

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