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Immobilization of Pectinex Ultra SP-L to produce galactooligosaccharides

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Abstract

Pectinex Ultra SP-L, a commercial enzyme preparation obtained from *Aspergillus aculeatus*, containing β-galactosidase activity, was immobilized onto Eupergit C and was used for the production of galactooligosaccharides (GOS). Immobilization resulted in 100% binding yield and higher GOS yield (24%, w/v) with respect to soluble enzyme. Optimum conditions were not affected by immobilization, and optimum pH and temperature for free and immobilized enzyme were 4.0–5.0 and 55–60 °C, respectively. Immobilized enzyme was more stable at high pH and temperatures. The amount of GOS produced from 30% (w/v) lactose solution using the free and immobilized enzyme was determined to be 12.8 and 15.8% (w/v) of the total sugar in the reaction mixture, respectively. The kinetic parameters for the free and immobilized enzyme were also determined. The immobilized Pectinex Ultra SP-L could be used for the production of galactooligosaccharides, since the immobilization yield is high (124%) and immobilized enzyme retains its activity for 20 days without any decrease.
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Keywords: Eupergit C; Immobilization; β-Galactosidase; Galactooligosaccharides; *Aspergillus aculeatus*; Pectinex Ultra SP-L

1. Introduction

GOS are non-digestible carbohydrates comprised of 3–10 molecules of galactose and one glucose [1]. The ability of GOS to promote the growth of *Bifidobacterium* sp. in the large intestine is beneficial to human health. They are added to nutritional foods such as infant formula and yogurt as functional ingredients to improve the intestinal flora [2].

GOS are usually produced by transgalactosylation during the enzymatic hydrolysis of lactose [3]. In addition to normal hydrolysis of the β-galactoside linkage in lactose, some β-galactosidases may catalyse the formation of GOS through transfer of one or more D-galactosyl units onto the D-galactose moiety of lactose [4]. The linkage between the galactose units and the components in the final product depend on the enzymes and conditions used in the reaction [5].

Synthesis of GOS from lactose using β-galactosidases has been studied by several groups [3–10]. However, due to low yield of GOS, researchers are working on the improvement of GOS yield. When thermostable or immobilized β-galactosidases were used in the GOS production, yield was increased [11,12]. Pectinex Ultra SP-L has also been used for the production of GOS from lactose [1,2]. The product of these reactions was 6′-galactosyl lactose as demonstrated by NMR analysis [1,2]. The aim of this study is to immobilize Pectinex Ultra SP-L covalently onto Eupergit and to investigate the effect of immobilization on the yield of 6′-galactosyl lactose. Eupergit C having active epoxy groups, is an excellent matrix for immobilization of enzymes [13,14]. Many enzymes have been immobilized onto Eupergit C. Epoxy groups on matrix react with amino, sulphydryl and hydroxyl groups of biomolecules depending on pH of buffer used. Immobilization procedure is quite simple and involves the reaction of Eupergit C beads with aqueous enzyme solution at room temperature, or 4 °C for 12–120 h [13]. Immobilization of enzymes onto Eupergit C is affected by amount of Eupergit C, duration of immobilization, pH and concentration of buffer used [15].

2. Materials and methods

2.1. Materials

Pectinex Ultra SP-L, a commercial enzyme preparation was kindly provided by Novozyme (Bagsvaerd, Denmark). It had

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8.4 IU/mL β-galactosidase activity. One IU is defined as the amount of enzyme forming 1 μmol of 6′-galactosyl lactose per minute from lactose (5%, w/v) at pH 4.5 at 60 °C. Eupergit C was a gift from Röhm GmbH & Co. KG (Darmstadt, Germany). Lactose, α-naphthol, calcium acetate were from Sigma (St. Louis MO, USA). Sulphuric acid, sodium dihydrogen phosphate, sodium azide, ethanol, imidazole and acetonitrile were obtained from Merck (Darmstadt, Germany). TLC plates were purchased from Whatman (New York, USA).

2.2. Methods

2.2.1. Immobilization procedure

Immobilization of β-galactosidase was carried out by reaction of Eupergit C (200 mg) with Pectinex Ultra SP-L (200 μL) in phosphate buffer (5 mL, 1.5 M, pH 4.5) at 25 °C with gentle shaking (150 rpm) for 120 h. After immobilization, beads were filtered and washed with phosphate buffer (10 mL, 0.1 M, pH 4.5) and with distilled water (10 mL) on a sintered glass filter by suction under vacuum. The wet weigh of beads obtained was 0.71 g. The obtained biocatalyst was used immediately to produce GOS from lactose. The immobilized enzyme was stored in phosphate buffer (100 mM, pH 4.5) at a refrigerator (+4 °C). It was washed with distilled water before using.

2.2.2. Optimization of immobilization procedure

Optimum conditions of immobilization were determined by changing individually the conditions (pH from 4.5 to 7.5; buffer concentration from 25 to 2.5 M; amount of Eupergit C from 100 to 1000 mg; and duration of immobilization from 12 to 120 h).

2.2.3. Determination of activity

Lactose solution (10 mL, 5% w/v) in calcium acetate buffer (25 mM, pH 4.5) was reacted with agitation at 200 rpm with free (200 μL) or immobilized (0.71 g) enzyme at 60 °C for 60 min in a water bath. An aliquot (200 μL) from reaction mixture was added to boiling distilled water (800 μL) and boiled for 10 min to inactivate the enzyme. The amount of 6′-galactosyl lactose formed was determined by TLC-imaging densitometry technique using a Bio-Rad GS 670 densitometer [16].

2.2.4. Analysis of reaction mixture

Thin layer chromatography (TLC) was used for quantitative analysis of carbohydrates. TLC was carried out using three ascents of solvent system of acetonitrile/water (85:15, v/v) on Whatman K5 silica plates. Carbohydrates on TLC plates were visualized by dipping the plates into 5% (v/v) sulphuric acid in ethanol containing 0.5% (w/v) α-naphthol, followed by heating on a hot plate at 110 °C for 10 min. TLC densitometer was used for quantitative determination of carbohydrates. Lactose was used as standard for determining the amount of 6′-galactosyl lactose [17].

2.2.5. Determination of optimum temperature, pH and kinetic parameters

K_m, V_m, optimum temperature and pH were determined by changing individually the conditions of activity assays: pH from 3.0 to 7.0; temperature from 35 to 70 °C; and lactose concentrations from 4 to 20% (w/v); time 60 min. Buffer solutions of calcium acetate (pH 3.0–5.5) and imidazole (pH 6–7.0) were used. Initial velocities for kinetic parameters were determined by carrying out the reactions for 3 min. K_m and V_m were calculated from Lineweaver–Burk plots.

2.2.6. pH stability

Free (200 μL) or immobilized (0.71 g) enzyme was incubated in various buffers (pH 3.0–7.0) at room temperature for 1 h and the remaining activity was determined under standard assay conditions. The buffers used were calcium acetate (pH 3.0–5.5) and imidazole (pH 6–7.0).

2.2.7. Thermal stability

Free (200 μL) or immobilized (0.71 g) enzyme was incubated in calcium acetate buffer (4.5) at temperatures from 35 to 80 °C for 1 h and the remaining activity was determined using the standard assay method.

2.2.8. Operational stability of immobilized Pectinex Ultra SP-L

Operational stability was tested by repeated batch experiments using the method for activity determination. The beads were washed with plenty of distilled water after each reaction.

2.2.9. Production of galactooligosaccharides

Free (200 μL) or immobilized (0.71 g) enzyme was reacted with 10 mL of 30% (w/v) lactose in 25 mM calcium acetate buffer (pH 4.5) at 60 °C for 24 h and remaining lactose concentration was determined with 4 h intervals. The product compositions for free and immobilized enzyme catalyzed reactions were determined in 24 h reaction.

3. Results and discussion

3.1. Optimization of immobilization procedure

3.1.1. Effect of pH of the immobilization buffer on the immobilization efficiency

Although epoxy groups on Eupergit C can react with various reactive groups of enzymes in a wide pH range (1–12), immobilization of many enzymes resulted in the highest yield at their optimal pH range [13]. Table 1 shows that the immobilization efficiency, defined as the ratio of activity of immobilized enzyme

Table 1
Influence of pH on immobilization efficiency

pH	Immobilization efficiency (%)
4.5	107
5.5	98
6.5	94
7.5	90

Conditions for immobilization: Pectinex Ultra SP-L (200 μL) was reacted with Eupergit C (200 mg) in phosphate buffer (5 mL, 1 M) at different pH with gentle shaking (150 rpm) at 25 °C for 24 h.

Table 2
Effect of buffer concentration on immobilization efficiency

Buffer concentration (M)	Immobilization efficiency (%)
0.025	63
0.25	73
0.5	84
1.0	107
1.5	109
2.0	99
2.5	89

Conditions for immobilization: Pectinex Ultra SP-L (200 μL) was reacted with Eupergit C (200 mg) in phosphate buffer (5 mL, 1 M pH 4.5) at different concentrations with gentle shaking (150 rpm) at 25 °C for 24 h.

to the activity of soluble enzyme used in immobilization, was very high (107%) at optimum pH (4.5).

3.1.2. Effect of molarity of the immobilization buffer

Buffer concentrations and salts such as ammonium sulphate also influence the immobilization efficiency considerably in immobilization using Eupergit C [18]. In our experiments, the immobilization efficiency increased from 63 to 109% in phosphate buffers of 25 mM and 1.5 M, respectively (Table 2).

3.1.3. Effect of amount of Eupergit C

Different amounts of support (100–1000 mg) for Pectinex Ultra SP-L (200 μL) were tested. Table 3 shows that the highest immobilization efficiency was obtained for 200 mg Eupergit C. Usage of higher amounts of Eupergit C yielded low immobilization efficiency possible due to multiple attachments and reaction with groups associated with active site and those responsible for tertiary structure of enzyme [13].

3.1.4. Effect of immobilization time

The duration of immobilization is also important [14]: the immobilization efficiency changed from 109 to 124% in 12 and 120 h, respectively (Table 4). There was no activity in the filtrate indicating that all the soluble enzyme used for immobilization was bound to the Eupergit C. Since no activity determined in the filtrate after immobilization at optimum conditions, all of protein content (16.2 mg/mL) of Pectinex Ultra SP-L were bounded onto Eupergit C. Therefore, the bounded protein was 16.2 mg/g solid support.

Table 3
Effect of Eupergit C amount on immobilization efficiency

Eupergit C (mg)	Immobilization efficiency (%)
100	94
200	109
300	103
400	99
600	91
800	81
1000	68

Conditions for immobilization: Pectinex Ultra SP-L (200 μL) was reacted with various amount of Eupergit C in phosphate buffer (5 mL, 1.5 M pH 4.5) with gentle shaking (150 rpm) at 25 °C for 24 h.

Table 4
Effect of duration of coupling immobilization efficiency

Time (h)	Immobilization efficiency (%)
24	109
48	116
72	120
96	123
120	124

Conditions for immobilization: Pectinex Ultra SP-L (200 μL) was reacted with Eupergit C (200 mg) in phosphate buffer (5 mL, 1.5 M pH 4.5) with gentle shaking (150 rpm) at 25 °C for various amount of time.

3.2. Characterization of immobilized enzyme

The highest activity yield (124%) was obtained by reacting Pectinex Ultra SP-L (200 μL) with Eupergit C (200 mg) stirring at 150 rpm at room temperature for 120 h in a phosphate buffer (1.5 M, pH 4.5). Under these conditions, there was no activity in the filtrate indicating that all of soluble enzyme used for the immobilization was attached to matrix without losing any activity. To our knowledge, this is the highest activity yield obtained in the literature. Sheu et al. [7] reported a maximum activity yield of 5.3%. Albayrak and Yang [9], reported a maximum activity yield of 90–95%. The immobilized enzyme was characterized: as Fig. 1 shows, optimum pH for activity (4.0–5.0) was unaffected by immobilization but immobilized enzyme was more stable in lower pH (3.0–3.5) and higher pH (5.5–7.0) ranges (Figs. 1 and 2). Optimum temperature ranges for both soluble and immobilized enzyme was the same (55–60 °C) but immobilization enhanced the thermal stability of the enzyme (Figs. 3 and 4). Soluble enzyme was fully inactivated at 70 °C, whereas immobilized enzyme was fully inactivated at 80 °C (Fig. 4). Kinetic parameters were also determined using Lineweaver–Burk plot (Fig. 5). The K_m values for lactose of soluble and immobilized enzyme are 161 and 313 g lactose L^{-1}, respectively. V_m values for lactose of soluble and immobilized enzyme are 6.2 g and 7.7 g 6′-galactosyl lactose L^{-1} min^{-1}, respectively.

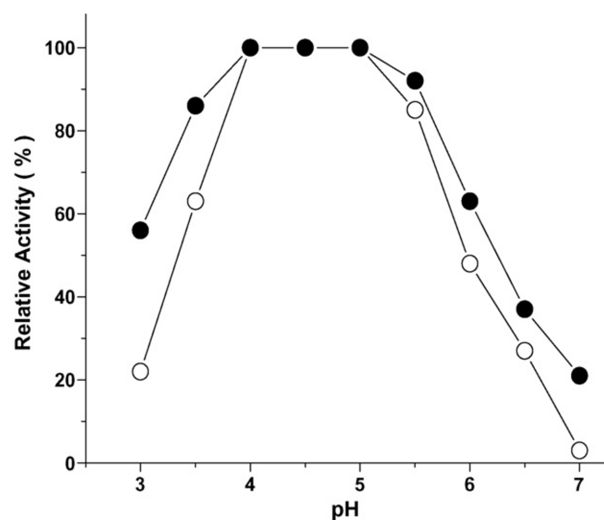

Fig. 1. Effect of pH on activity of free (○) and immobilized (●) β-galactosidase.

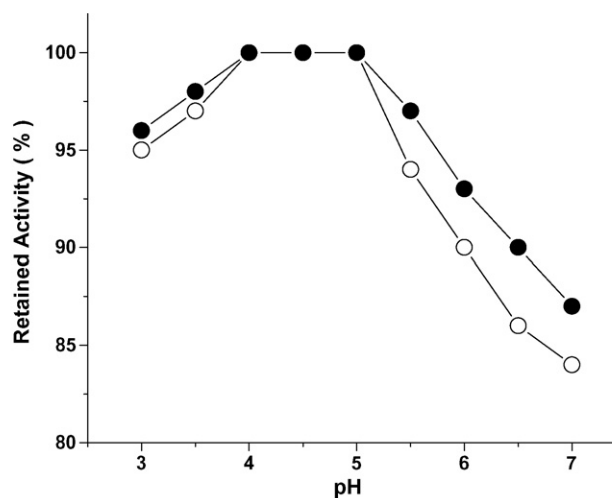

Fig. 2. Effect of pH on stability of free (◯) and immobilized (●) β-galactosidase.

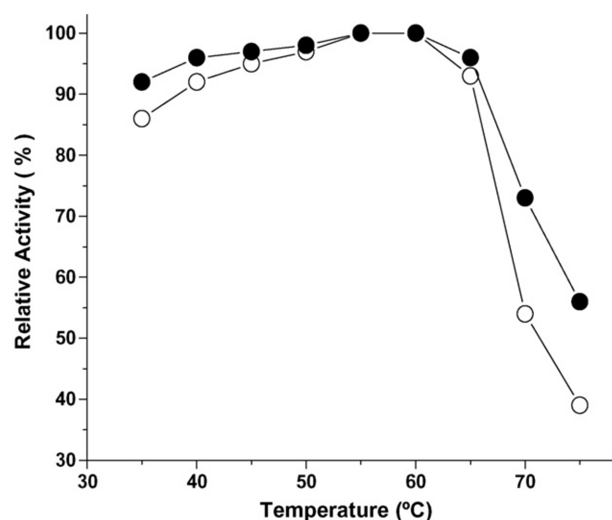

Fig. 3. Effect of temperature on activity of free (◯) and immobilized (●) β-galactosidase.

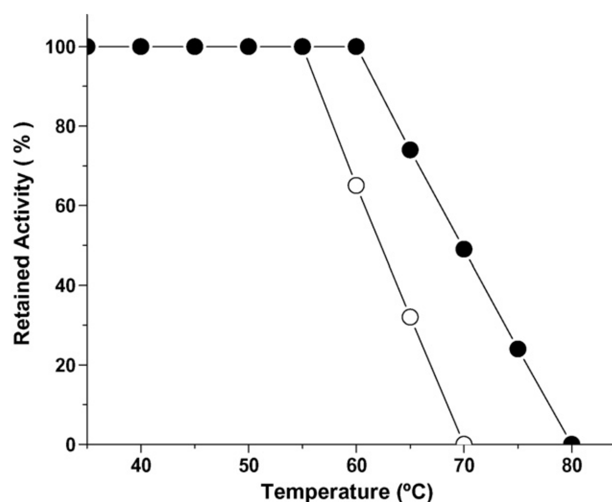

Fig. 4. Effect of temperature on stability of free (◯) and immobilized (●) β-galactosidase.

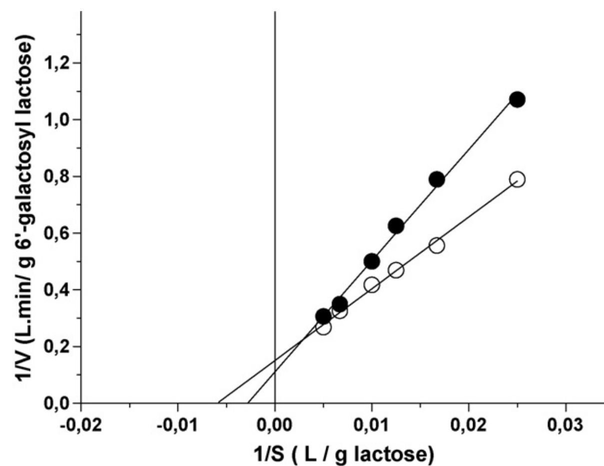

Fig. 5. Lineweaver–Burk plots of free (◯) and immobilized (●) β-galactosidase.

Both free and immobilized enzyme were used for the production of galactooligosaccharides and found that product composition was altered by immobilization. The product obtained from lactose (30%, w/v) by 24 h reaction of free enzyme contained lactose (68.2%, w/v), 6′-galactosyl lactose (12.8%, w/v), glucose (11.8%, w/v) and galactose (7.2%, w/v), whereas the product obtained from lactose (30%, w/v) by the reaction of immobilized enzyme contained lactose (61.4%, w/v), 6′-galactosyl lactose (15.8%, w/v), glucose (14.2%, w/v) and galactose (8.6%, w/v). In both cases, most of the lactose was not consumed in 24 h reaction due to the formation of by-products galactose and glucose, which compete with lactose for the substrate-binding site [8]. The immobilization increased the amount of 6′-galactosyl lactose by 24%. The enzyme catalyzes both hydrolysis and transgalactosylation reactions. Transgalactosylation is favored at high substrate concentrations and low water content. The increase in the amount of the product could be due to the hydrophobic character of the immobilization matrix, which provides low water content for the enzyme. We have used the immobilized enzyme in 20 batch reactions, each lasting for 1 h at 60 °C, and observed that there was no decrease in activity. It was also found that the immobilized enzyme retains its activity for 20 days of operation, determined by activity assays carried out each day. Since the immobilization efficiency is quite high (124%) and immobilized enzyme retains its activity without decrease for 20 days, the Eupergit C immobilized *A. aculeatus* β-galactosidase could be used for the production of 6′-galactosyl lactose from lactose.

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