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Immobilization of Pectinex Ultra SP-L to produce fructooligosaccharides

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Abstract

Pectinex Ultra SP-L, a commercial enzyme preparation containing fructosyltransferase activity, was immobilized covalently onto Eupergit C and was used for the production of fructooligosaccharides. Immobilization resulted in 96% relative activity. The immobilized enzyme showed no decrease in activity for 20 batch reactions. Optimum conditions were not affected by immobilization, and the optimum pH and temperature for free and immobilized enzyme were 5.5–6.5 and 65 °C, respectively. Immobilized enzyme was more stable at high pH and temperatures. The amount of fructooligosaccharides produced from 60% (w/v) sucrose solution using the free and immobilized enzyme was determined to be approximately 57% of the total sugar in the reaction mixtures. The kinetic parameters for free and immobilized enzyme were also determined. The immobilized Pectinex Ultra SP-L could be used for the production of fructooligosaccharides since the immobilization efficiency is quite high (96%) and immobilized enzyme retains its activity for 20 days without any decrease.

Keywords: Eupergit C; Immobilization; Fructosyltransferase; Fructooligosaccharides; Pectinex Ultra SP-L

1. Indroduction

Fructooligosaccharides, found naturally in such foods as onions, bananas, artichokes, and tomatoes, have biofunctions. They increase the number of *Bifidobacteria*, occur in the human large intestine, contributing many healthful benefits such as immune system activation, resistance to some infections, synthesis of B-complex vitamins, and calcium absorbtion [1]. Fructooligosaccharides are added in pig and chicken food to improve growth [2]. In humans, they could be used to treat breast cancer, diarrhea, and constipation [3]. In addition, incidence of otitis media could be reduced by daily intake of fructooligosacchharides [4]. These oligosaccharides are calorie free and noncariogenic. The reaction leading to the formation of fructooligosaccharides from sucrose could be catalyzed by either immobilized fungal cells having fructosyltransferase activity or immobilized enzymes for the industrial production. Cells of *Aspergillus niger* and *Aureobasidum pullulans* were entrapped in alginate and used for fructooligosaccharide production [5,6]. Porous glass, alginate, ion-exchange resin, ceramic membrane, and methacrylamide-based reactive polymeric beads containing oxirane groups have been used for immobilization of fructosyltransferases [5,7–9]. Commerical enzyme preparation called Pectinex Ultra SP-L derived from *Aspergillus aculeatus* has several enzymes including pectinase, cellulase, β-galactosidase, and fructosyltransferase [10]. This enzyme preparation has generally been used in fruit juice production [11]. The enzyme preparation has also been used for the production of fructooligosacharides from sucrose [11,12]. In this work, Pectinex Ultra SP-L was immobilized with high yield onto Eupergit C, and used for the production of fructooligosaccharides. Eupergit C, epoxy-activated acrylic beads, is quite stable and has good mechanical and chemical properties. Enzymes are coupled to Eupergit C covalently via their amino, mercapto, or hydroxyl groups [13,14].

2. Materials and methods

2.1. Materials

Pectinex Ultra SP-L, a commerical enzyme preparation, was kindly provided by Novo Nordisk. It had 269 IU/mL

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fructosyltransferase activity. One IU is defined as the amount of enzyme forming 1 μmol glucose per min from sucrose (60% w/v) at pH 5.5 at 60 °C. Eupergit C was a gift from Röhm GmbH & Co. KG, Darmstadt, Germany. Glucose, fructose, and α-naphthol were from Sigma. Calcium acetate and sodium dihydrogen phosphate were purchased from Carlo Erba. Calcium chloride, sulphuric acid, sucrose, sodium azide and imidazole were obtained from Merck. Fructooligosaccharide standards were kindly provided by Meiji Seika Kaisha, Ltd., Japan.

2.2. Methods

2.2.1. Immobilization procedure

Immobilization of fructosyltransferase was carried out by reaction of Eupergit C (400 mg) with Pectinex Ultra SP-L (0.2 mL) in phosphate buffer (5 mL, 1 M, pH 8) at 25 °C with gentle shaking (150 rpm) for 96 h. After immobilization the beads were filtered and washed with distilled water (15 mL) and phosphate buffer (15 mL, 0.1 M, pH 5.5) on a sintered glass filter by suction under vacuum. The wet weight of beads obtained was 1.43 g.

2.2.2. Optimization of immobilization procedure

Optimum conditions for immobilization were determined by changing individually the conditions (pH from 5 to 9; buffer concentration from 0.025 to 2.5 M; duration of immobilization from 12 to 96 h; and amount of Eupergit C from 100 to 1000 mg).

2.2.3. Determination of activity

Sucrose solution (10 mL, 60% w/v) in calcium acetate buffer (25 mM, pH 5.5) was reacted with agitation at 255 rpm with immobilized (1.43 g) or free enzyme (0.2 mL) at 60 °C for 60 min in a water bath. Reaction mixture (50 μL) was added to distilled water (1950 μL) and boiled for 10 min to inactivate the enzyme. The amount of glucose formed was determined by a TLC-imaging densitometer technique using a Bio-rad GS 670 densitometer [15].

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.

2.2.5. Determination of optimum temperature, pH, and kinetic parameters

Km, Vm, optimum temperature and pH were determined by changing individually the conditions of activity assay (pH from 4.5 to 8.0; temperature from 35 to 75 °C; and sucrose concentrations from 1 to 10% (w/v). Buffer solutions of calcium acetate (pH 4.5–5.5) and imidazole (pH 6–8) were used. Initial velocities for kinetic parameters were determined by carrying out the reactions for 1 min. Km and Vm were calculated from Lineweaver–Burk plots.

2.2.6. pH stability

Free (0.2 mL) or immobilized enzyme (1.43 g) was incubated in various buffers (4.5–8.0) at room temperature for 1 h and the remaining activity was determined under standart assay conditions.

2.2.7. Thermal stability

Free (0.2 mL) or immobilized enzyme (1.43 g) was incubated in calcium acetate buffer (25 mM, pH 5.5) at temperatures from 35 to 75 °C for 1 h and then the remaining activity was determined using the standart assay method.

2.2.8. Operational stability of the immobilized Pectinex Ultra SP-L

The operational stability was tested by repeated batch experiments using the method for activity determination.

2.2.9. Production of fructooligosaccharides

Free (0.2 mL) or immobilized enzyme (1.43 g) was reacted with 10 mL of 60% (w/v) sucrose in 25 mM calcium acetate buffer (pH = 5.5) at 60 °C for 24 h and the remaining sucrose 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 the immobilization procedure

Eupergit C having active epoxy group, is an excellent matrix for immobilization of enzymes [13,14]. Many enzymes have been immobilized onto Eupergit C. Epoxy groups on the matrix react with amino, sulfhydryl and hydroxyl groups of biomolecules depending on pH of the 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 12–72 h [14]. Immobilization of enzymes onto Eupergit C is affected by amount of Eupergit, duration of immobilization, pH and concentration of buffer used [16,17].

3.2. Effect of the amount of Eupergit C on the immobilization efficiency

Different amounts of support (100–1000 mg) for Pectinex Ultra SP L (200 μL) were tested. Table 1 shows that the immobilization efficiency, defined as the ratio of activity of immobilized enzyme to the activity of soluble enzyme used in immobilization, was very high (96%) for 400 mg Eupergit C

Table 1
Effect of Eupergit C amount on immobilization efficiency

Eupergit C (mg)	Immobilization efficiency (%)
100	68
200	88
300	92
400	96
600	90
800	83
1000	69

Conditions for immobilization: Pectinex Ultra SP-L (0.2 mL) was reacted with various amounts of Eupergit C in phosphate buffer (5 mL, 1 M, pH 8) with gentle shaking (150 rpm) at 25 °C for 96 h.

at optimum conditions. There was no activity in the filtrate indicating that all the soluble enzyme used for immobilization was bound to the Eupergit. Usage of higher amounts of Eupergit C yielded low immobilization efficiency possibly due to multiple attachments and reaction with groups associated with active site and those responsible for the tertiary structure of the enzyme. High immobilization yield (100%) was also obtained by Chiang and Lee [5] for the immobilization of fructosyltransferase from *A. niger* onto methacrylamide-based polymeric beads carrying epoxy groups.

3.3. Effect of pH of the immobilization buffer

The epoxy groups on Eupergit C can react with various reactive groups of enzymes in a wide pH range (0–12) [13]. Table 2 shows the influence of pH on immobilization. Although optimum pH for both soluble and immobilized enzyme was 5.5–6.5, the highest immobilization efficiency was obtained at pH 8.

3.4. Effect of molarity of the immobilization buffer

Buffer concentration and salts such as sodium sulphate also influence the immobilization efficiency considerably in immobilization using Eupergit C: in our experiments, the immobilization efficiency changed from 14 to 80% in phosphate buffers of 25 mM and 1 M, respectively (Table 3).

3.5. Effect of immobilization time

The duration of immobilization is of importance: the immobilization efficiency increased from 80 to 96% in 24 and 96 h, respectively (Table 4).

Table 2
Influence of pH on immobilization efficiency

pH	Immobilization efficiency (%)
5	74
6	75
7	78
8	80
9	70

Conditions for immobilization: Pectinex Ultra SP-L (0.2 mL) was reacted with Eupergit C (0.4 g) in phosphate buffer (5 mL, 1 M) at different pH with gentle shaking (150 rpm) at 25 °C for 24 h.

Table 3
Effect of buffer concentration on immobilization efficiency

Buffer concentration (M)	Immobilization efficiency (%)
0.025	14
0.25	20
0.5	68
1.0	80
1.5	58
2.0	28
2.5	19

Conditions for immobilization: Pectinex Ultra SP-L (0.2 mL) was reacted with Eupergit C (0.4 g) in phosphate buffer (5 mL, pH 8) at different concentrations with gentle shaking (150 rpm) at 25 °C for 24 h.

Table 4
Effect of duration of coupling on immobilization efficiency

Time (h)	Immobilization efficiency (%)
12	67
24	80
48	89
72	93
96	96

Conditions for immobilization: Pectinex Ultra SP-L (0.2 mL) was reacted with Eupergit C (0.4 g) in phosphate buffer (5 mL, 1 M, pH 8) with gentle shaking (150 rpm) at 25 °C for various amount of time.

3.6. Characterization of immobilized enzyme

The highest immobilization efficiency (96%) was obtained by reacting Pectinex Ultra SP-L (0.2 mL) with Eupergit C (400 mg) stirring at 150 rpm at room temperature for 96 h in phosphate buffer (1 M, pH 8). Under these conditions, there was no activity in the filtrate indicating that all the soluble enzyme used for the immobilization was attached to the matrix. High immobilization efficiency (96%) indicates that almost all the enzyme covalently attached to matrix was active. The immobilized enzyme was characterized: as Fig. 1 shows, immobilization has not changed the optimum pH for activity (5.5–6.5) but immobilized enzyme was more stable at pH in range of 4.5–5.5. pH-stability experiments showed that immobilized enzyme was more stable in pH ranges of 4.5–5.5

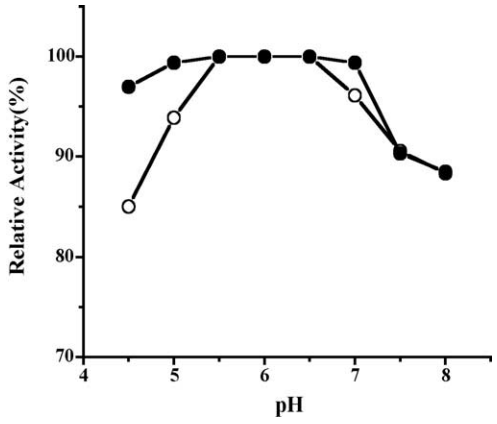

Fig. 1. Effect of pH on the activity of free (◯) and immobilized (●) Pectinex Ultra SP-L.

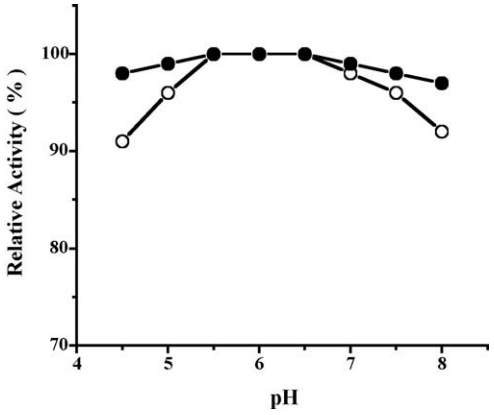

Fig. 2. Effect of pH on stability of free (◯) and immobilized (●) Pectinex Ultra SP-L.

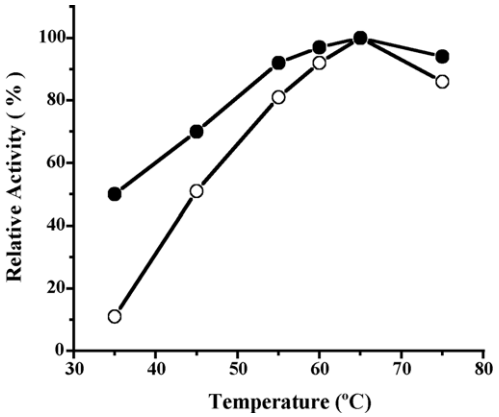

Fig. 3. Effect of temperature on the activity of free (◯) and immobilized (●) Pectinex Ultra SP-L.

and 6.5–8 (Fig. 2). Optimum temperature for both soluble and immobilized enzyme was 65 °C but immobilization enhanced the thermal stability of the enzyme (Figs. 3 and 4). Free enzyme was fully inactived at 75 °C, whereas immobilized enzyme retained aproximately 30% of its activity (Fig. 4). Kinetic parameters were also studied using Lineweaver–Burk plot (Fig. 5). The Km values for sucrose of free and immobilized enzyme are 47 and 91 g sucrose/L, respectively. Vm for free and immobilized enzyme are found to be 86 and 82 (g glucose/L)/min, respectively. Both free and immobilized enzymes were used for the production of fructooligosaccharides and found that the product compositions were almost the same. The product contained sucrose (11.9%), fructose (2.1%), glucose (28.8%), 1-kestose (23.5%), nystose (27.2%), and fructofuranosyl nystose (6.5%). The amounts of fructooligosaccharides produced by free and immobilized enzyme are 57% of the total carbohydrates in the reaction mixture. This result is comparable to those obtained by immobilized fructosyltransferases from different sources [9,18]. Sucrose consumption during the fructooligosaccharides production by free and immobilized enzyme was similar (Fig. 6). In both cases, most of the sucrose was consumed within 4 h, but all the substrate was not converted to products even in 24 h reaction due to the formation of by-product glucose, which competes with sucrose for the substrate binding site [19,20]. 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 retained its activity for 20 days of operation, determined by activity assays carried out each day. Since the

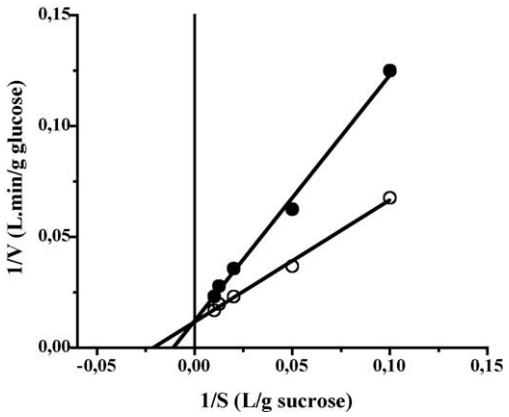

Fig. 5. Lineweaver–Burk plots of free (◯) and immobilized (●) Pectinex Ultra SP-L.

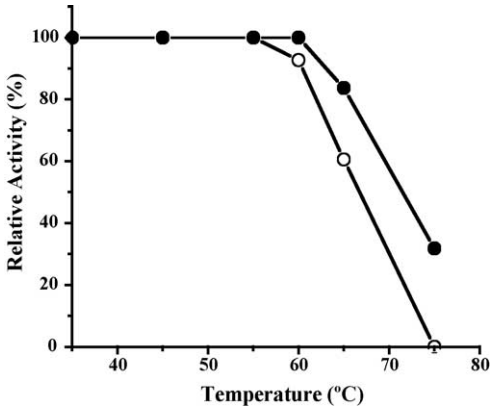

Fig. 4. Thermal stability of free (◯) and immobilized (●) Pectinex Ultra SP-L.

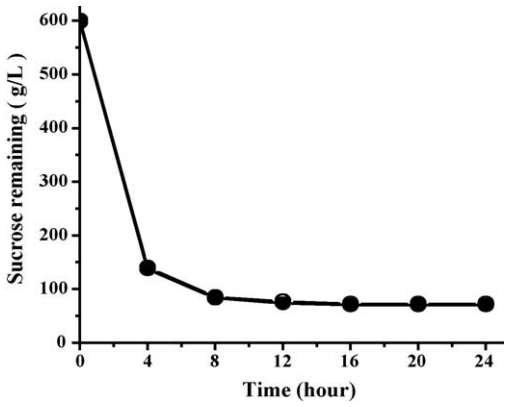

Fig. 6. Sucrose consumption during the fructooligosaccharides production by free (◯) and immobilized (●) Pectinex Ultra SP-L.

immobilization efficiency is quite high (96%), and the immobilized enzyme has retained its activity without decrease for 20 days, the Eupergit C immobilized Pectinex Ultra-SP could be used for the industrial production of fructooligosaccharides from sucrose.

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