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Immobilization of *Penicillium lilacinum* dextranase to produce isomaltooligosaccharides from dextran

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Abstract

Penicillium lilacinum dextranase was immobilized covalently onto Eupergit C and was used for the production of isomaltooligosaccharides. Immobilization resulted in 90% relative activity. The immobilized enzyme showed no decrease in activity for 20 batch reactions. Optimum conditions were not affected by immobilization, and optimum pH and temperature for free and immobilized enzyme were 4.5–5.5 and 30–35 °C, respectively. Immobilized enzyme was more stable at low and high pH and high temperatures. The kinetic parameters for the free and immobilized enzyme were also determined. The immobilized enzyme could be used for the production of isomaltooligosaccharides, since the immobilization efficiency is high (90%) and immobilized enzyme retains its activity for 20 days without any decrease.
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Keywords: Immobilization; *Penicillium lilacinum*; Dextranase; Eupergit C; Isomaltooligosaccharides

1. Introduction

Isomaltooligosaccharides (IMO) are α (1 → 6) linked glucooligosaccharides with degrees of polymerization (DP) ranging for commerical products, between 2 and 6 [1]. They have many biological functions such as promotion of the growth of *Bifidobacteria* in the large intestine of humans and animals and reduction of cariogenic effect of sucrose [2,3]. IMO can be produced using such carbohydrates as starch, maltose, sucrose, and dextran [4]. Starch is treated with α-amylase and neopullulanase to obtain a mixture comprised of glucose, maltose, panose, and IMO [5]. Maltose is reacted with cells of *Aureobasidium pullulans* to obtain IMO [6]. Acceptor reactions of dextransucrase or treatment of sucrose with dextransucrase and dextranase also gives rise to IMO [7,8]. These oligosaccharides can also be obtained by hydrolysis of dextran with endodextranase [9]. The hydrolysis products by fungal dextranases are isomaltose and isomaltotriose with a small amount of glucose [10].

Dextranase 50 L (*Penicillium lilacinum* dextranase) has been used together with dextransucrase to produce IMO [11,12]. Dextranase was purified, and immobilized covalently onto APTES-CPG (3-aminopropyltrietoxysilane-controlled porous glass) and used for the removal of dextran from the infected sugar juices [13]. In this work, dextranase 50 L was immobilized with high yield onto Eupergit C, and used for the production of IMO from dextran. Eupergit C having active epoxy groups, is an excellent matrix for immobilization of enzymes [14,15]. Many enzymes have been immobilized onto Eupergit C. Epoxy groups on matrix reacts 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 [15]. Immobilization of enzymes onto Eupergit C is affected by amount of Eupergit C, duration of immobilization, pH and concentration of buffer used [17–19].

2. Materials and methods

2.1. Materials

Dextranase 50 L, a commercial enzyme preparation with dextranase activity of 63 IU mL^{-1} was a gift from Novozyme (Bagsvaerd Denmark). One IU is defined as the amount of enzyme forming 1 μmol of IMO per minute from dextran (2%, w/v) at pH 5.3 at 30 °C. Dextran (M_W, 5000 Da) was obtained

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from Dextran Products Limited (Ontario, Canada). Eupergit C was a gift from Röhm GmbH & Co. KG (Darmstadt, Germany). Calcium acetate, D-maltose and α-naphthol 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 was carried out by reacting Eupergit C (200 mg) with Dextranase 50 L (0.1 mL) in phosphate buffer (5 mL, 1.0 M, pH 5.3) at 25 °C with gentle shaking (150 rpm) for 24 h. Beads (0.71 g) were obtained after filtering and washing with phosphate buffer (10 mL, 0.1 M, pH 5.3) and distilled water (10 mL) on a sintered glass filter by suction under vacuum. The immobilized enzyme was stored in a refrigerator at 4 °C until used.

2.2.2. Optimization of immobilization procedure

Optimum conditions of immobilization were determined by changing individually the conditions (pH from 5.0 to 8.0; buffer concentration from 0.5 to 2.5 M; amount of Eupergit C from 100 to 1000 mg; and duration of immobilization from 12 to 72 h).

2.2.3. Determination of activity

Dextran (10 mL, 2%, w/v) in calcium acetate buffer (25 mM, pH 5.3) was reacted with agitation at 200 rpm with free (0.1 mL) or immobilized dextranase (0.71 g) at 30 °C for 60 min in a water bath. Reaction mixture (0.2 mL) was added to distilled water (0.8 mL) and boiled for 10 min to inactivate the enzyme. The amount of IMO formed was determined by a 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 with 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 constants

K_m, V_m, optimum temperature and pH were determined by changing individually the conditions of activity assays: pH from 3.5 to 7.5; temperature from 25 to 60 °C; and dextran concentrations from 1 to 8% (w/v). Buffer solutions of calcium acetate (pH 3.5–5.5) and imidazole (pH 6–7.5) 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.

Table 1
Influence of pH on immobilization efficiency

pH	Immobilization efficiency (%)
5.0	83.5
5.3	90.0
6.0	86.9
7.0	79.4
8.0	66.7

Conditions for immobilization: *P. lilacinum* dextranase (0.1 mL) was reacted with Eupergit C (200 mg) in sodium dihydrogen phosphate buffer (5 mL, 1 M) at different pH with gentle shaking (150 rpm) at 25 °C for 24 h.

2.2.6. pH stability

Free (0.1 mL) or immobilized enzyme (0.71 g) was incubated in various buffers (3.3–7.5) at room temperature for 60 min and the remaining activity was determined using standard activity assay method was mentioned in Section 2.2.3.

2.2.7. Thermal stability

Free (0.1 mL) or immobilized enzyme (0.71 g) was incubated in calcium acetate buffer (pH 5.3) at temperatures from 25 to 60 °C for 60 min and the remaining activity was determined with the standard assay method was mentioned in Section 2.2.3.

2.2.8. Operational and storage stability

Operational stability test was carried out by repeated 20 batch experiments using the method for activity determination. Storage stability was tested for 20 days by determining the activity every other day with activity assay method and then keeping the immobilized enzyme in a refrigerator at 4 °C.

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 [14]. 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 (90%) at optimum pH (5.3).

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 with Eupergit C: In our experiments, the immobilization efficiency increased from 86.3% to 90% in phosphate buffers of 0.5 and 1.0 M, respectively (Table 2).

3.1.3. Effect of amount of Eupergit C

Different amounts of support (100–1000 mg) for dextranase 50 L (0.1 ml) were tested. Table 3 shows that the highest (90%) immobilization efficiency was obtained for 200 mg Eupergit C.

Table 2
Effect of buffer concentration on immobilization efficiency

Buffer concentration (M)	Immobilization efficiency (%)
0.5	86.3
1.0	90.0
1.5	82.6
2.0	64.5
2.5	49.2

Conditions for immobilization: *P. lilacinum* dextranase (0.1 mL) was reacted with Eupergit C (200 mg) in sodium dihydrogen phosphate buffer (5 mL, 1 M, pH 5.3) at different concentrations with gentle shaking (150 rpm) at 25 °C for 24 h.

Table 3
Effect of Eupergit C amount on immobilization efficiency

Eupergit C (mg)	Immobilization efficiency (%)
100	84.7
200	90.0
300	85.4
400	76.6
600	70.4
800	56.7
1000	43.9

Conditions for immobilization: *P. lilacinum* dextranase (0.1 mL) was reacted with various amount of Eupergit C in sodium dihydrogen phosphate buffer (5 mL, 1.0 M, pH 5.3) with gentle shaking (150 rpm) at 25 °C for 24 h.

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 tertiary structure of enzyme.

3.1.4. Effect of immobilization duration

The duration of immobilization is of importance: the immobilization efficiency changed from 74.1 to 90% in 12 and 24 h, respectively (Table 4). In immobilization lasting for 24 h, there was no activity in the filtrate indicating that the entire soluble enzyme used for immobilization was bound to the Eupergit C.

3.2. Characterization of immobilized enzyme

The highest immobilization efficiency (90%) was obtained by reacting dextranase 50 L (0.1 ml) with Eupergit C (200 mg) shaking at 150 rpm at room temperature for 24 h in a phosphate buffer (5 ml, 1.0 M, pH 5.3). Under these conditions, there was no activity in the filtrate indicating that all of soluble enzyme used for the immobilization was attached to matrix. It was found that immobilization has not altered the product composition. Isomaltose and isomaltotriose were obtained from dextran using free or immobilized enzyme.

Table 4
Effect of duration of coupling to immobilization efficiency

Time (h)	Immobilization efficiency (%)
12	74.1
24	90.0
48	78.8
72	61.4

Conditions for immobilization: *P. lilacinum* dextranase (0.L ml) was reacted with Eupergit C (200 mg) in sodium dihydrogen phosphate buffer (5 mL, 1.0 M, pH 5.3) with gentle shaking (150 rpm) at 25 °C for various amount of time.

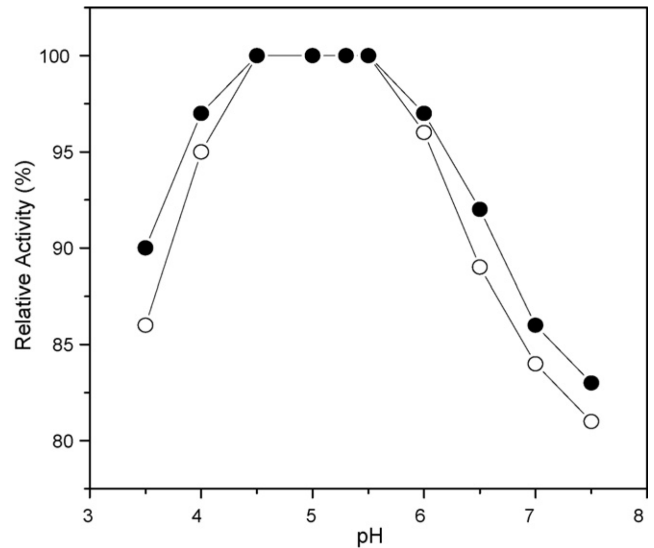

Fig. 1. Effect of pH on activity of free (○) and immobilized (●) *Penicillium lilacinum* dextranase.

3.2.1. Optimum pH and pH stability

As Fig. 1 shows, optimum pH for activity (4.5–5.5) was not affected by immobilization but immobilized enzyme was more stable in lower pH (3.5–4.0) and higher pH (6.0–7.5) ranges (Figs. 1 and 2).

3.2.2. Optimum temperature and thermal stability

Apparent optimum temperature with reaction time of 60 min ranges for both soluble or immobilized enzyme was the same (30–35 °C) but immobilization enhanced the thermal stability

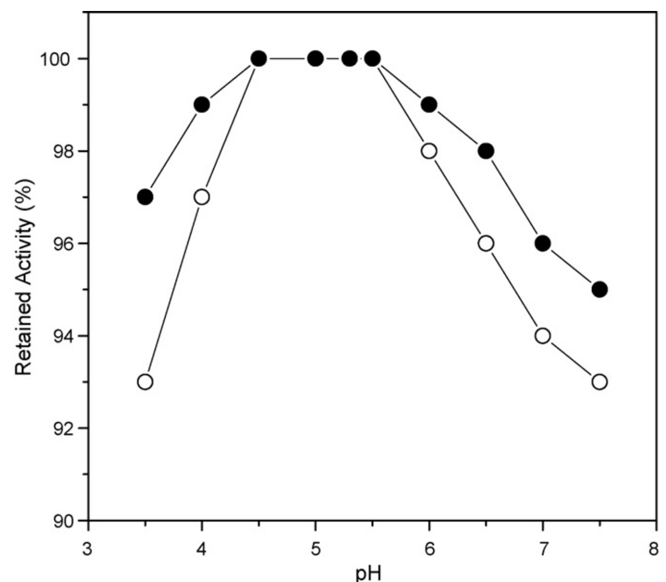

Fig. 2. Effect of pH on stability of free (○) and immobilized (●) *P. lilacinum* dextranase.

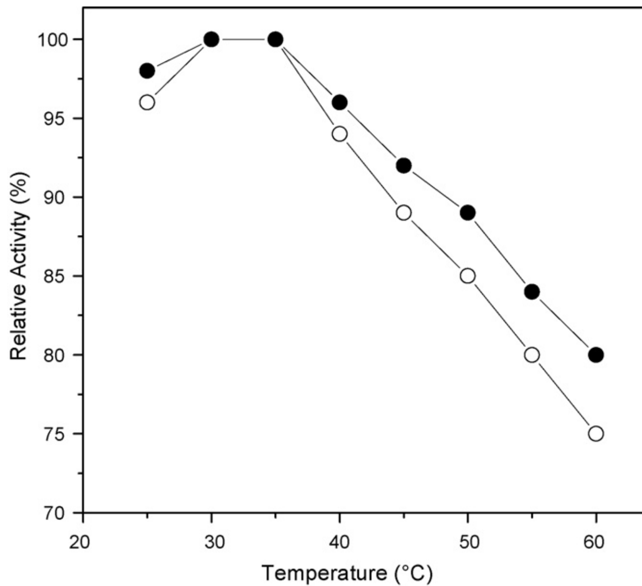

Fig. 3. Effect of temperature on activity of free (◯) and immobilized (●) *P.lilacinum* dextranase.

of the enzyme (Figs. 3 and 4). Soluble enzyme lost about 6 and 16% of its activity at 55 and 60 °C, respectively, whereas immobilized enzyme retained 99 and 94% of its full activity at same temperatures (Fig. 4).

3.2.3. Kinetic constants

Kinetic parameters were also determined with Lineweaver–Burk plot (Fig. 5). The K_m values for dextran of soluble or immobilized enzyme are 13.1 and 15.7 g dextran L^{-1}, respectively. V_{max} for free and immobilized enzyme are 4.4 and 4.0 g IMO L^{-1} min^{-1}, respectively.

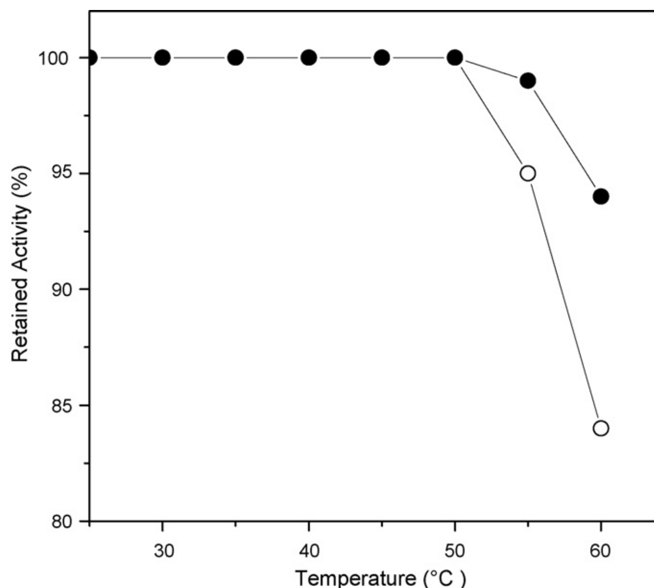

Fig. 4. Effect of temperature on stability of free (◯) and immobilized (●) *P. lilacinum* dextranase.

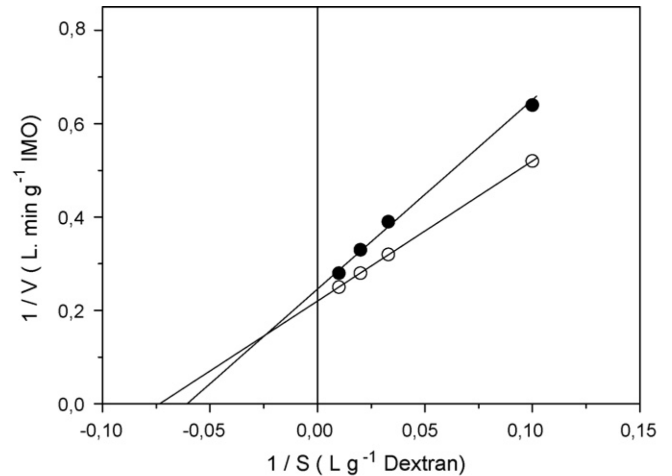

Fig. 5. Lineweaver–Burk plots of free (◯) and immobilized (●) *P. lilacinum* dextranase.

3.2.4. Operational and storage stabilities

The immobilized enzyme has operational and storage stabilities. The immobilized enzyme retained its activity during 20 consecutive batch reactions, each lasting for 60 min at 30 °C, It was also found that the immobilized enzyme retains its activity for 20 days of storage in a refrigerator during which activity was determined every other day using standard activity assay.

4. Conclusion

Since the immobilization efficiency is quite high (90%) and the immobilized enzyme has retained its activity without decrease for 20 days, the Eupergit C immobilized *P. lilacinum* dextranase could be used for the industrial production of IMO from dextran.

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