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Co-immobilization of levansucrase and endo-levanase on Immobead 150P support for fructooligosaccharide production

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ABSTRACT: Fructooligosaccharides (FOS) are commonly used as alternative sweeteners in healthy food and beverages due to their zero-calorie content and biologically beneficial activity. In this study, levan-type FOS were synthesized using co-immobilized levansucrase and endo-levanase. The enzymes were co-immobilized on Immobead 150P, which was activated with ethylenediamine and glutaraldehyde. The effects of immobilization time and enzyme concentration on immobilization efficiency and enzyme activity were investigated. Biochemical characterization revealed that the optimal pH and temperature for catalysis by both enzymes remained unchanged after immobilization. Moreover, the thermostability of both enzymes was significantly improved. FOS synthesis by the immobilized enzymes was examined using sucrose syrup as a substrate. The results showed that the immobilized enzymes could synthesize FOS with a yield of ∼45% across a broad range of pH (pH 3–7) and temperature (30–40 °C), indicating their potential for industrial applications.

KEYWORDS: levan, inulin, co-immobilization, glutaraldehyde, fructosyltransferase

INTRODUCTION

Fructooligosaccharides (FOS) are widely used as sweeteners in food and beverage industries. FOS not only provide sweetness but also contain no calories, making them an excellent alternative sweetener for healthy foods [1]. Additionally, because they are indigestible in the gastrointestinal tract, FOS serve as effective prebiotics by promoting the growth of beneficial bacteria in the colon [2, 3]. In addition, FOS also demonstrate several bioactivities such as antioxidant, immunological stimulation [4], and anticancer [5].

FOS could be divided into 2 groups based on glycosidic linkage: inulin FOS (β(2-1)) and levan FOS (β(2-6)). Inulin FOS could be obtained from plants (such as onion, garlic, chicory, and artichoke) or synthesized by inulosucrase [6]. Levan FOS could be synthesized by levansucrase [7]. However, both inulosucrase and levansucrase mainly synthesize high molecular weight fructans rather than short oligosaccharides [8]. Since the biological activity of FOS is better than the polymer, due to higher bioavailability [5], various strategies for improving the yield of oligosaccharides were developed. The biological activity of FOS depend on their degree of polymerization (DP). For example, FOS with a DP of 2–8 can promote the growth of the probiotic *Bifidobacterium* [9]. Meanwhile, FOS with a DP of 8–13 have shown stronger antioxidant activity than inulin [10]. Additionally, FOS with a DP of 2–8 exhibits intestinal anti-inflammatory effects, suggesting their potential benefits in treating inflammatory bowel disease under appropriate conditions [11]. To improve the yield of oligosaccharides, rational protein engineering at the oligosaccharide-binding site was performed [3, 12]. However, most mutants capable of increasing oligosaccharide yield usually have higher hydrolysis activity. On the other hand, oligosaccharides could be synthesized using the coupling reaction of transferase and endo-typed glycoside hydrolase. For example, dextransucrase-dextranase co-immobilized on agarose-epoxy support produced oligosaccharides with a DP of up to 5 [13], levansucrase-inulinase synthesized oligolevan with a DP of up to 6 [14], combined cross-linked enzyme aggregates of cyclodextrin glucanotransferase-maltogenic amylase produced maltooligosaccharides with a yield of up to 490 mg/g starch [15], and levansucrase-levanase synthesized FOS with a DP ranging from 2 to 10 [16]. Unlike the use of engineered enzymes, the product profile in a coupling reaction system could be controlled by adjusting the ratio of the enzymes used.

Enzyme immobilization is a widely used technique to enhance enzyme properties such as stability and specificity [17–21]. Enzymes can be attached to a support via covalent bonds or non-covalent interactions. In addition, immobilized enzymes could be used and reused to increase stability in continuous processes, contributing to significant reduction of production costs. Furthermore, two or more enzymes can be co-immobilized on the same support, enabling synthesis through coupled reactions [22]. This technique makes enzyme immobilization a valuable technique for industrial biotechnology.

Immobead 150P is a commercial support of

methacrylate polymers, and it has a spherical shape with an average particle size of 0.15–0.30 mm. Additionally, Immobead 150P contains epoxy functional group which facilitate multipoint covalent immobilization of proteins. Various chemicals have been employed to modify epoxy groups in the support to assess alternative techniques of immobilization. Many studies reported the benefits of Immobead 150P in immobilized enzymes. For example, the immobilization of lipase increased thermostability of the enzyme by 30 times [23]; the immobilized β-galactosidase doubled the free enzyme's half-life [24]; formate dehydrogenases showed 1.32 times higher activity when immobilized [25]; immobilized α-amylase on Immobead 150P had a better reusability than immobilized on ReliZyme EP403/M and ReliZyme HFA403/M [26]; and the immobilized laccase showed a significant increase in pH stability [27]. However, to the best of our knowledge, immobilization of levansucrase and endo-levanase on Immobead 150P has not been reported.

The immobilization of fructosyltransferases, such as inulosucrase and levansucrase, for FOS synthesis has been reported [22, 28, 29]. Although the coupled enzyme system of levansucrase and levanase has been previously reported, the co-immobilization of these enzymes on Immobead 150P has not yet been reported. In this study, recombinant levansucrase from *Erwinia tasmaniensis* [5] and endo-levanase from *Bacillus subtilis* [30] were co-immobilized on Immobead 150P using glutaraldehyde as a cross-linker. The biochemical characteristics of the immobilized enzymes were investigated and compared with those of the soluble enzymes. Finally, the effectiveness of the immobilized enzymes was further evaluated based on their ability to produce FOS in batch reaction.

MATERIALS AND METHODS

Chemicals

Immobead 150P was purchased from Sigma-Aldrich (Saint Louis, MO, US). Ampicillin and Isopropyl β-D-1-thiogalactopyranoside (IPTG) were obtained from Gold Biotechnology (Saint Louis, MO, US). Silica gel 60 F254 aluminum TLC plates were purchased from Merck (Rahway, NJ, US). Glutaraldehyde and ethylenediamine were sourced from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Tryptone and yeast extract were acquired from HiMedia (Maharashtra, India).

Expression and purification of enzymes

Levansucrase from *E. tasmaniensis* (EtLS) and endo-levansucrase from *B. subtilis* HM7 were expressed and purified using previous reported protocol [5, 30]. In brief, the recombinant plasmids were transformed into *Escherichia coli* BL21 (DE3). The recombinant cells were cultured on LB agar containing 0.1 mg/ml Ampicillin overnight. After that, the cells were picked up, put into the LB broth, and cultured at 37 °C until the OD600 reaching 0.6–0.8. Then, 0.5 mM IPTG was added into the culture, and the temperature was shifted to 16 °C for endo-levanase, or 20 °C for levansucrase expression. After 18–20 h of shaking, the cells were harvested by centrifugation, resuspended in 25 mM phosphate buffer (pH 7.4), and lysed by ultrasonic homogenizer. The crude extract was collected by centrifugation at 12,000×g. The enzymes were purified by TOYOPEARL™ AF-Chelate-650 M column (Tosoh bioscience, Tokyo, Japan).

Activity assay

The activity of levansucrase and levanase were determined by DNS assay [31]. Levansucrase was incubated with 500 mM sucrose in 50 mM acetate buffer pH 5, while levanase was incubated with 2% (w/v) levan in 50 mM acetate buffer pH 5.0. The activities of both enzymes were evaluated at 40 °C. The amount of reducing sugar released in the reactions were determined using glucose as external standard. One unit of enzyme (U) was defined as the amount of enzyme required to released 1 μmol of reducing sugar per min.

Enzyme immobilization

Ten grams of Immobead 150P were resuspended in 1 M ethylenediamine solution (pH 10) for 24 h with gentle shaking at room temperature. After that, the modified beads were washed with deionized water, resuspended in 2% (v/v) glutaraldehyde solution for 8 h, and then washed with deionized water. The obtained glutaraldehyde-activtated beads were incubated with enzymes at 4 °C. The immobilized enzymes were washed with 25 mM acetate buffer (pH 5.0) and kept at 4 °C until further uses. Immobilized efficiency (%) was defined as the ratio of immobilized activity to the activity of soluble enzymes added for immobilization. Fig. 1 shows the schematic representation of the modification of Immobead 150P support for enzyme immobilization.

Biochemical characterization of enzymes

Effects of pH and temperature on the catalytic activity of free and immobilized enzymes was evaluated. The effect of pH was measured by enzyme activity at 30 °C using Britton-Robinson universal buffer (pH 3–7) [32], while effect of temperature was assessed using 50 mM acetate buffer (pH 5.0) over a temperature range of 20–50 °C. The operational stability of the enzymes was evaluated by preincubating them in 50 mM acetate buffer (pH 5.0) at 30 °C, and then measuring the remaining activity at various time intervals relative to the initial enzyme activity. For storage stability, the enzymes were stored at 4 °C for 4 weeks.

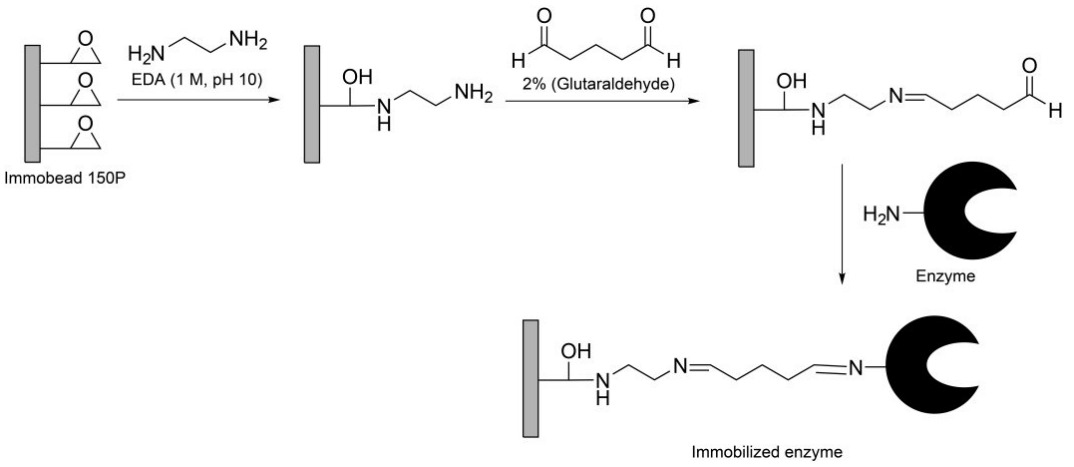

Fig. 1 Schematic representation immobilization of enzymes onto the modified Immobead 150P support (EDA, ethylenediamine).

FOS production and analysis

Ten milligrams of immobilized enzyme were added to 0.5 ml of a substrate solution containing 5% (w/v) sucrose and 50 mM acetate buffer at pH 5.0. The reaction mixture was incubated at 30 °C for 24 h. After incubation, the immobilized enzyme was separated from the reaction mixture by centrifugation. The total amount of monosaccharides was determined using the DNS assay, while the glucose concentration was measured using the glucose oxidase assay. The amount of fructose was calculated by subtracting the glucose concentration from the total monosaccharide content. The acetone precipitation method was used to quantify the levan polymer. The reaction mixture was mixed with an equal volume of acetone and centrifuged at 10,000×g for 10 min. The amounts of monosaccharides and polysaccharides were subtracted from the initial amount of sucrose to quantify the FOS synthesized in the reaction. The size distribution of FOS was analyzed by TLC using silica gel 60 F254 aluminum plates. Sugars were separated using a solvent system consisting of acetic acid, acetonitrile, 1-butanol, and water in a ratio of 1:2:1:1 and detected using orcinol staining.

RESULTS AND DISCUSSION

Immobilization of levansucrase and endo-levanase on Immobead 150P

Immobead 150P is a commercial carrier commonly used for protein immobilization. The epoxy groups on the bead's surface can be modified using various chemicals, depending on the selected immobilization technique. The homology models of levansucrase and endo-levanase revealed that both enzymes contain lysine residues on their surfaces, which can readily react with the aldehyde groups of glutaraldehyde, but not

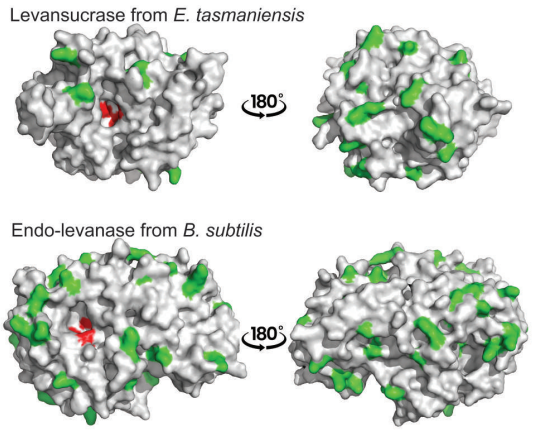

Fig. 2 Homology modeling of enzymes by means of Swiss-model using PDB: 6RV5 and 4FFF as templates for levansucrase and endo-levanase, respectively. Green, lys residues; Red, three catalytic residues.

in their active sites (Fig. 2). Therefore, in this study, Immobead 150P was modified using glutaraldehyde. Immobilization of protein on Immobead 150P usually involves two steps: the first is activation of the beads using ethylenediamine and glutaraldehyde, and the second is covalent attachment of the proteins to the activated beads. For this co-immobilized enzyme system, levansucrase should be considered the key enzyme since it produces the substrate levan for the second enzyme, endo-levanase. Therefore, the conditions for levansucrase immobilization on glutaraldehyde-activated Immobead 150P were initially optimized. First, the effect of immobilization time was investigated at 4 °C. As a result, the immobilized activity and immobilization efficiency increased with incubation

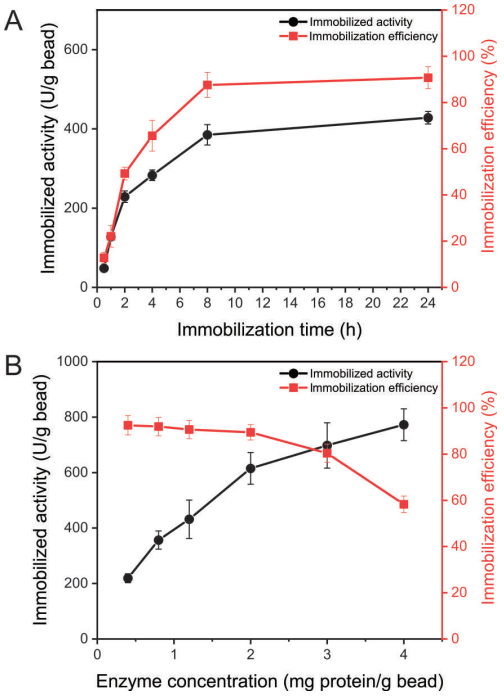

Fig. 3 Effects of immobilization time, (A); and protein concentration, (B); on immobilization efficiency and immobilized activity of co-immobilized enzymes.

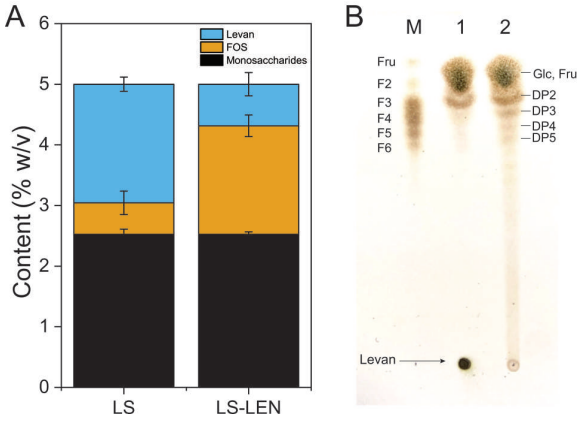

Fig. 4 FOS synthesis by immobilized levansucrase (LS) and co-immobilized enzyme (LS-LEN). (A), Analysis of sugar contents in reaction mixture; (B), TLC analysis of reaction mixture from immobilized levansucrase (lane 1) and co-immobilized enzyme (lane 2).

time and reached the optimal at 8 h. The increased activity of immobilized enzyme after 8 h of incubation was not significant (Fig. 3A). At 8 h of incubation, the immobilized levansucrase expressed 385 U/g bead with the immobilization efficiency of 87.6%. The longer incubation period between the enzyme and the support facilitated the more efficient establishment of the covalent bonds, resulting in a greater yield of immobilized enzyme [28].

After that, the effect of various enzyme concentrations on immobilization efficiency was evaluated using the enzyme concentration range of 0.4–4.0 mg protein/g bead (Fig. 3B). The result showed that the activity of immobilized enzyme rapidly increased when the enzyme loaded increased from 0.4 to 2.0 mg protein/g bead. After that, the activity of immobilized enzyme slightly increased. Hence, the immobilization efficiency largely decreased when the amount of levansucrase loaded was higher than 2.0 mg protein/g bead. This is possibly due to the saturation of the activated supports with the enzyme [28]. These phenomena were reported in various immobilized enzymes including inulosucrase [28], levansucrase [29], and alcohol dehydrogenase [33]. Therefore, the selected concentration of levansucrase was 2.0 mg protein/g bead, and the immobilized efficiency was 89.4% with immobilized activity of 615 U/g bead.

Subsequently, the co-immobilized enzyme was prepared by incubating the endo-levanase with the pre-immobilized levansucrase beads. The selected concentration of endo-levanase was 10.0 mg protein/g bead. The unreacted aldehyde groups on the beads underwent a reaction with the amino groups of endo-levanase, resulting in the formation of co-immobilized enzymes. After the unattached proteins had been removed, the activity of the co-immobilized enzyme was assessed using TLC. The results showed that the co-immobilized enzyme synthesized a higher amount of FOS than the immobilized levansucrase alone, suggesting their cooperation in FOS synthesis (Fig. 4). In comparison to previous research, the co-immobilized enzyme produced FOS with a pattern comparable to that of the mixed free enzyme [30]. Additionally, both systems synthesized FOS with a comparable yield (∼45%), suggesting that immobilization of enzymes on Immobead 150P was effective.

Biochemical characteristics of immobilized enzyme

An immobilization process might affect biochemical characteristics and activity of enzymes since it generates unusual microenvironments as well as changes in protein conformation. Therefore, the effects of pH and temperature on activities of both enzymes before and after immobilization were compared. The results showed that immobilized levansucrase catalyzed the reaction the highest at pH 5–6 and 50 °C, while immobilized endo-levanase showed the highest activity at pH 5.0 and 40 °C. The results of both enzymes were comparable to the free enzymes (Fig. 5). These findings indicated that the biochemical properties of both enzymes were not affected by immobilization. Many studies revealed that immobilization might or might not affect the biochemical properties of the free en-

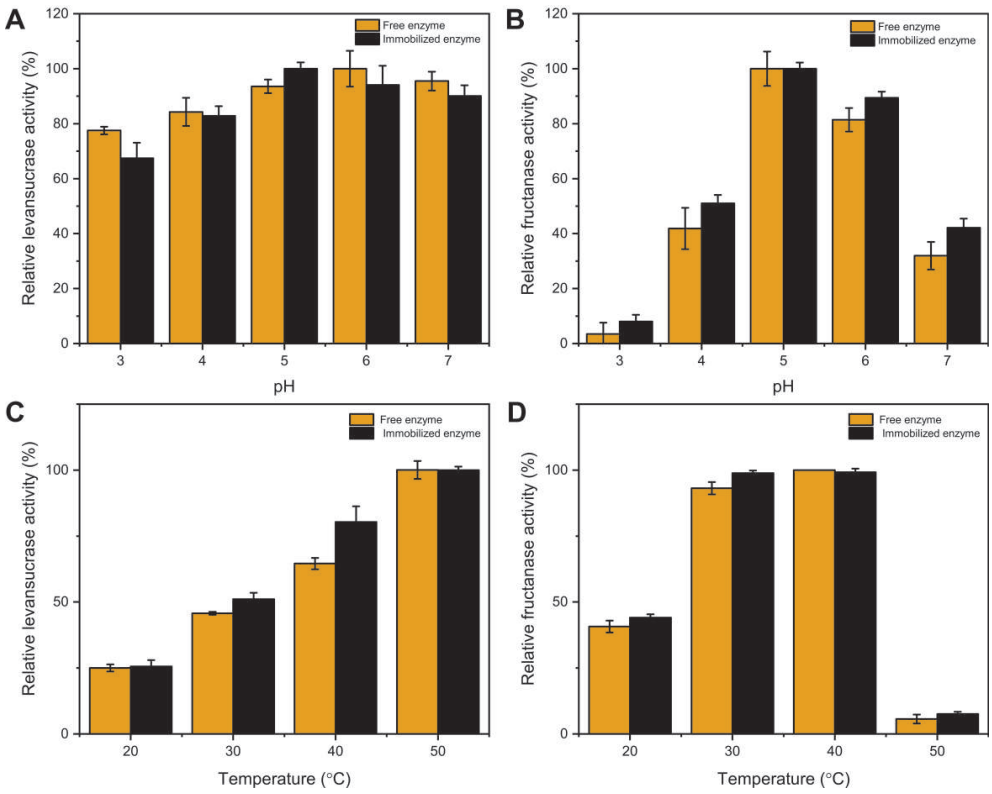

Fig. 5 Effects of pH and temperature on activities of free and immobilized enzymes. (A), Effect of pH on levansucrase activity; (B), effect of pH on endo-levanase activity; (C), effect of temperature on levansucrase activity; and (D), effect of temperature on endo-levanase activity.

zyme. For example, the optimum pH for catalytic activity of inulosucrase shifted from 5 to 4 upon immobilization as cross-linked enzyme aggregates (CLEAs) [34], whereas the optimum pH of β-galactosidase from *B. licheniformis* remained unchanged [35]. Due to both levansucrase and endo-levanase having an optimum pH for catalysis at 5.0, this pH was selected for further stability analysis and FOS synthesis.

Operational and storage stability

Enzyme immobilization preserves enzyme activity by creating a microenvironment that prevents its deactivation. This study examined the stability of both levansucrase and endo-levanase enzymes at 30 °C due to their strong catalytic activity at this temperature. The results clearly showed that after 24 h of incubation, immobilized levansucrase and endo-levanase retained 83% and 70% of their initial activity, respectively, compared with only 26% and 17% of their respective free forms (Fig. 6A). Since these enzymes contain many lysine residues (free amino groups) on their surfaces, they can form covalent bonds with the aldehyde groups on the activated beads. The multiple covalent bonds between the enzymes and the support helped maintain the enzymes' native structures.

Furthermore, the stability of the immobilized enzymes during long-term storage was examined at 4 °C. The results clearly showed that both enzymes immobilized on the beads retained about 90% of their initial activity after 4 weeks of storage (Fig. 6B). This result suggested their potentials for industrial applications.

FOS synthesis from sucrose syrup

The activity of enzymes was largely dependent on environmental factors, including pH and temperature. Therefore, the effect of these factors on the efficiency of immobilized enzyme to synthesize FOS were investigated. As shown in Fig. 7A, the immobilized enzymes produced the highest yield of FOS at 40 °C, correspondent to the optimum pH of levanase. TLC analysis revealed that the immobilized enzyme could produce a broad spectrum of FOS. The immobilized enzyme tended to produce longer chains of FOS at lower temperature (Fig. 7B). For the effect of pH, the yield of FOS was slightly affected by the reaction pH (Fig. 7C,D). The optimum pH for FOS synthesis was pH 5, corresponding to the optimum pH for endo-levanase activity. Altogether, the immobilized enzyme could synthesize FOS at broad ranges of pH and temperature.

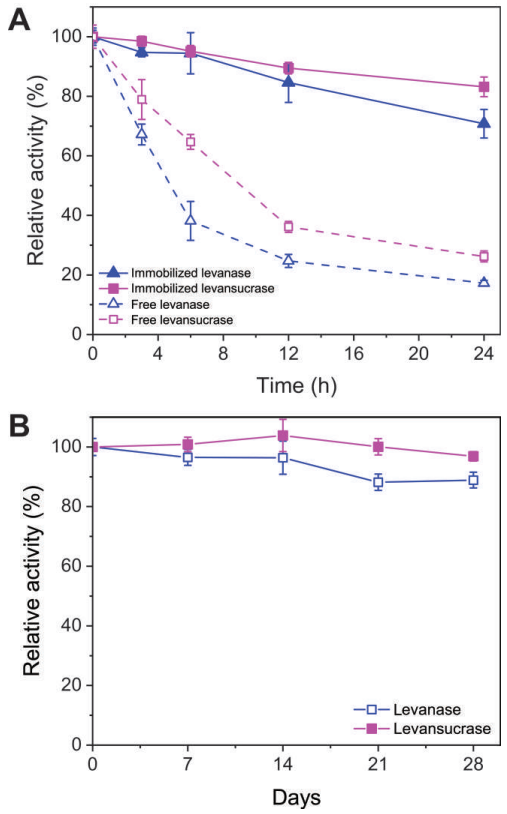

Fig. 6 Stability of free and immobilized enzymes. (A), Operational stability of enzymes examined at 30 °C, pH 5.0; (B), storage stability of immobilized enzymes stored at 4 °C for 28 days.

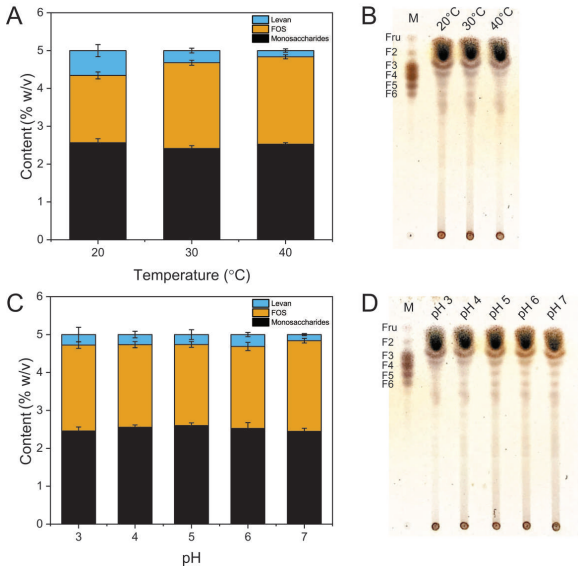

Fig. 7 FOS synthesis by co-immobilized enzymes. (A, B), Effect of temperature on FOS synthesis; (C, D), effect of pH on FOS synthesis.

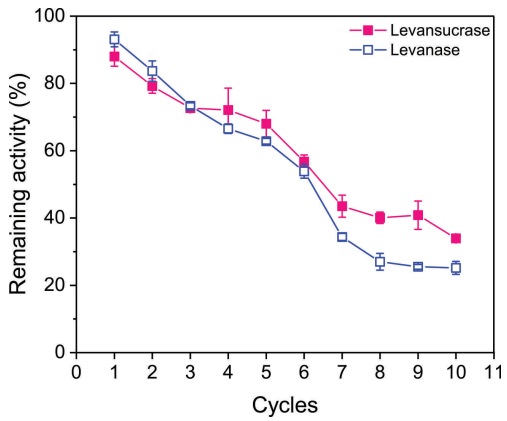

Fig. 8 Reusability of immobilized enzyme for FOS synthesis.

Reusability of immobilized enzyme

An advantage of using immobilized enzymes in industrial applications is their ability to be reused. In this study, the reusability of the co-immobilized enzymes was assessed using a sequence of batch reactions. The synthesis of FOS was conducted using 50 g/l sucrose as a substrate. After each batch, the remaining activity of the immobilized enzymes was evaluated. As shown in Fig. 8, the activity of the immobilized biocatalyst gradually decreased over the first five cycles. After the fifth cycle, the immobilized levansucrase and levanase enzymes retained approximately 68% and 63% of their initial activities, respectively. However, after the sixth cycle, the activity of both enzymes significantly decreased. These findings suggested that the immobilized enzymes could be reused effectively for at least five reaction cycles. The reusability of CLEAs is highly dependent on the enzyme type. In some cases, CLEAs exhibit a significant loss of activity—over 50%—within the first few reuse cycles. For instance, combi-CLEAs derived from levansucrase and inulosucrase showed a reduction of more than 50% in catalytic activity after only three cycles [22]. Similarly, CLEAs prepared from inulosucrase alone retained only 50% of their initial activity after five cycles [34]. In contrast, the CLEA system described in this study could maintain a higher operational stability, with a 50% activity loss observed only after seven reuse cycles.

CONCLUSION

In this study, levansucrase and endo-levanase were co-immobilized on Immobead 150P using glutaraldehyde as a cross-linker. After immobilization, the enzymes exhibited significantly higher operational stability. This co-immobilized enzyme system produced a higher yield of FOS compared with the use of a single enzyme. Additionally, the immobilized enzymes were able to synthesize FOS over a broad range of

pH and temperature. The FOS synthesized by this enzyme system displayed a wide spectrum of degrees of polymerization, with an average yield of 45%. Overall, this novel immobilized enzyme system showed the properties that could be advantageous for FOS synthesis in industrial applications.

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