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Contributed Paper

Production and Immobilization of Levansucrase †

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ABSTRACT

Escherichia coli Top-10 containing a levansucrase gene (*lsRN*) of *Bacillus licheniformis* RN-01, cultivated in 3X LB medium, produced levansucrase at 65.7 U/ml of culture medium. The purified levansucrase had a MW of 52 kDa and specific activity of 170.04 U/mg protein with 6.6 purification fold and 62.2% yield. *B. licheniformis* RN-01 levansucrase was covalently bound on chitosan beads, Sepabead EC-EP beads, and Sepabead EC-HFA beads with the immobilization efficiency of 96%, 35%, and 23%, respectively. Levansucrase immobilized on chitosan beads retained over 75% of its activity after 10 cycles of repetitive use. In contrast, levansucrase immobilized on Sepabead EC-EP or Sepabead EC-HFA lost over 60% after only 5 cycles of repetitive used. The optimum pH and temperature of the immobilized enzyme on chitosan beads (pH 4.0-6.0, 40-50°C) were significantly broader than those of the free enzyme (pH 6.0, 50°C). These results demonstrated that chitosan beads have superb characteristics for levansucrase immobilization.

Keywords: L-FOS, fructooligosaccharide, levansucrase, chitosan, *Bacillus licheniformis*, *Escherichia coli* Top-10

1. INTRODUCTION

Levansucrase (EC 2.4.1.10) is a member of the family 68 glycosylhydrolase. Levansucrase hydrolyzes sucrose, liberating glucose, and transfers fructose molecules to a growing levan fructooligosaccharide (L-FOS) chain [1, 2]. Levansucrase was diversely produced from microorganisms such as, *Acetobacter xylinum* [3], *Bacillus subtilis* [4], *Bacillus megaterium* [5], and *Zymomonas mobilis* [6].

Levan fructooligosaccharides are composed of D-fructose units joined together by a β-(2, 6) glycosidic linkage in the main chain with β-(2, 1) linkage at branch points. FOS has a broad range of utilization such as medical, cosmetic, pharmaceutical, food industry and agriculture uses [7-10]. The yield of L-FOS produced by levansucrase has been constrained by the low percent yield of

L-FOS, instability and reusing ability of the levansucrase. Consequently, immobilized levansucrase for L-FOS production is advantageous.

Immobilization of levansucrase by a covalently cross-linking technique on the surface of supporting matrices was studied in this work, since this technique provides strong binding between levansucrase and its supporting matrices. Moreover, by linking levansucrase on the surface of the supporting matrices, limitations of substrate/product transfer into or out of the supporting matrices can be eliminated.

Chitosan beads, Sepabead EC-EP beads, and Sepabead EC-HFA beads were used as supporting matrices in our experiments.

2. MATERIALS AND METHODS

Chitosan polymer from shrimp (850,000 MW) with 84% degree of deacetylation was used to generate chitosan bead, and used as supporting matrix. Chitosan beads were prepared by adding 2% (w/v) shrimp chitosan in 1% acetic acid solution to 0.5 N NaOH.

Sepabead EC-EP and Sepabead EC-HFA were obtained from Resindion, Mitsubishi Chemical Corporation, Italy.

2.1 Bacterial Strain and Plasmid

E. coli Top-10 was used as an expression host and plsRN01 containing levansucrase gene (*lsRN*) of *B. licheniformis* RN-01 (GenBank accession no. FJ171619.1) under the regulation of its putative endogenous promoter inserted into pBluscriptSK(-), was used for levansucrase production.

2.2 Production of Recombinant *B. licheniformis* RN-01 Levansucrase in *E.coli*

E. coli Top-10 harboring plsRN01 was cultured in various concentration of Luria-Bertani media, LB, supplying increasing concentrations of nitrogen and B-complex vitamins. Luria-Bertani media, 2X LB, 3X LB, 4X LB and 5X LB medium was used to culture *E. coli* Top-10 at 37°C, 250 rpm for 30 h, for levansucrase expression. After cultivation, cells were discarded by centrifugation at 8,000xg, 4°C for 10 min. Extracellular enzyme in the culturing media was collected.

2.3 Purification of Recombinant *B. licheniformis* RN-01 Levansucrase

Crude recombinant levansucrase was purified by DEAE-cellulose chromatography in 50 mM acetate buffer pH 6.0 using stepwise elution, 0.1-0.5 M NaCl. The fraction containing levansucrase activity at 0.2 M NaCl was collected. Levansucrase was further purified by Phenyl Sepharose column chromatography by initially binding levansucrase to the column using 25 mM acetate buffer pH 6.0 with 1.0 M ammonium sulfate. Levansucrase was eluted using a reversed salt gradient from 1.0 to 0 M ammonium sulfate. The fraction containing levansucrase activity was pooled and collected for further experiments.

2.4 Levansucrase Immobilization

Seven units, containing 50 mg protein of purified levansucrase were covalently linked to 0.1 g (wet weight) of the chitosan beads-supporting matrix in each immobilization experiment. Glutaraldehyde (GTA) 0.5% (v/v) was used as the cross-linking reagent. Purified levansucrase was incubated with chitosan beads then covalently linked with 0.5% GTA in 50 mM citrate buffer pH 6.0 at 4°C under static condition for 2 days. Covalently linking levansucrase on epoxide beads was carried out using Sepabead EC-EP and Sepabead EC-HFA. The 0.1 g of Sepabead EC-EP and Sepabead EC-HFA were activated with 1 ml of 1 M NaOH to

open the epoxide ring, then washed with DI water. Thereafter, the activated Sepabead EC-EP and Sepabead EC-HFA were incubated with purified 7 U of purified levansucrase, containing 50 mg proteins, in 50 mM citrate buffer pH 6.0 at 4°C, with gentle agitation for 2 days.

2.5 Levansucrase Activity Assay

The activity of levansucrase was measured by quantifying the reducing ability of free glucose liberated from the reaction by the modified DNS method [11]. One unit was defined as the amount of levansucrase that produces 1 mmole of reducing sugar (glucose equivalent) per min. Protein quantity was determined by Bradford's method [12].

2.6 Optimization of pH and Temperature on Free and Immobilized Levansucrase

The effect of pH and temperature on free and immobilized levansucrase was studied in 10 mM Britton-Robinson Universal buffer at pH 3-12 and at temperature range of 20-80°C to determine the optimum pH and the temperature, respectively. Two hundred milliunits of free and immobilized levansucrase were incubated in 1.6% (w/v) sucrose in 50 mM citrate buffer, pH 6.0 (final volume 1 ml), at 50°C for 5 min. The beads were removed then the activity of immobilized levansucrase was measured by the modified DNS method [11].

2.7 Production of L-FOS

Free and immobilized levansucrase on chitosan beads were used for L-FOS production. The reaction contained 1 U of either free or immobilized levansucrase, 20% (w/v) sucrose, 50 mM citrate buffer pH 6.0 (final volume 0.5 ml). The reaction was incubated at 50°C for 12 h in an orbital shaker, at 200 rpm.

Product was analyzed by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) and thin layer chromatography (TLC). CarboPac PA-1 used as the column for HPAEC-PAD analysis. Elution was made by gradient of 0-0.6 M NaOAc in 0.1 M NaOH with flow rate 1 ml/min, detected with a Dionex ED40 electrochemical detector ED40 with an AU working electrode and an Ag/AgCl reference electrode. The TLC analysis was run in a mobile phase containing acetic acid: butanol: distilled water at the ratio of 3: 3: 2, stained with an ethanol: sulfuric acid ratio of 9: 1 and baked in a hot air oven at 120°C for 5 min to visualize the separated products.

2.8 Operational Stability of Immobilized Levansucrase

The operational stability of the immobilized levansucrase was determined. Two hundred milliunits of immobilized levansucrase were incubated in 1.6% (w/v) sucrose and 50 mM citrate buffer, pH 6.0, at 40°C for 5 min. After each cycle, supporting matrices were washed with cold 50 mM citrate buffer, pH 6.0 for 3 times. The residual activity of levansucrase after each reaction cycle was measured by modified DNS method.

3. RESULTS AND DISCUSSION

The activity of levansucrase produced in various culture media, LB, 2X LB, 3X LB, 4X LB and 5X LB, were determined. The maximum activity was detected in the 3X, 4X and 5X LB media (65.7-67.7 U/ml of culture medium). There were no significant differences in the activity of levansucrase in 3X, 4X and 5X LB (Figure 1). These results suggest that higher levels of nutrients and/or salt in the medium can induce the cells to

produce more enzymes. Furthermore, when the cultivation time was extended to 7 days, the activity did not significantly increase compared to the second day, at every concentration of LB medium (data not shown), which suggests that the cells cease enzyme production after medium saturation.

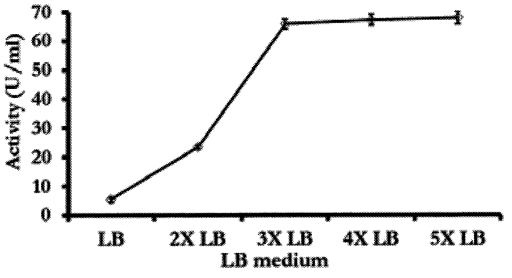

Figure 1. Effect of culture LB medium concentration on levansucrase activity produced by *Escherichia coli* Top-10.

3.1 Purification of Levansucrase

Purification of crude levansucrase was performed by DEAE-cellulose column and phenyl- sepharose chromatography. Firstly, 50 ml of crude protein was applied to the DEAE-cellulose column. Proteins were eluted with NaCl gradient from 0-0.6 M in 50 mM NaOAc buffer, pH 6.0. The chromatogram in Figure 2 shows that there were at least two protein peaks containing levansucrase activity. Both peaks were pooled and applied to a Phenyl-sepharose column. Proteins were eluted by a $(NH_4)_2SO_4$ gradient from 1.0-0 M in 50 mM NaOAc buffer, pH 6.0. The result indicated that only a single peak contained levansucrase activity as shown in Figure 3. Analysis of the purified levansucrase by SDS-PAGE showed that the purified levansucrase had a MW of about 52 kDa and revealed a high degree of apparent homogeneity (Figure 4). Levansucrase purification data is shown in Table 1. The specific activity of levansucrase fraction increased to 170 U/mg protein with a 6.6 purification fold and 62.2% yield. The results indicate that the crude levansucrase produced by *B. licheniformis* RN-01 recombinant *E.coli* was successfully purified.

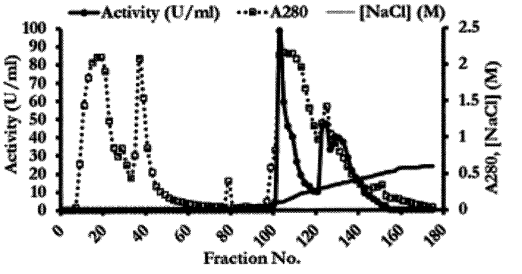

Figure 2. Levansucrase purification profile by DEAE-cellulose chromatography.

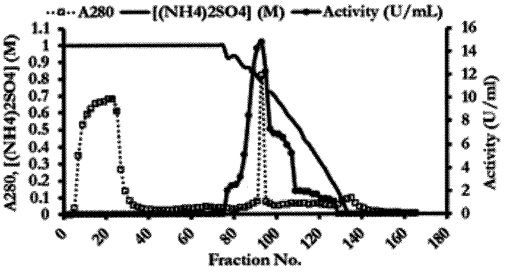

Figure 3. Levansucrase purification profile by phenyl-sepharose chromatography.

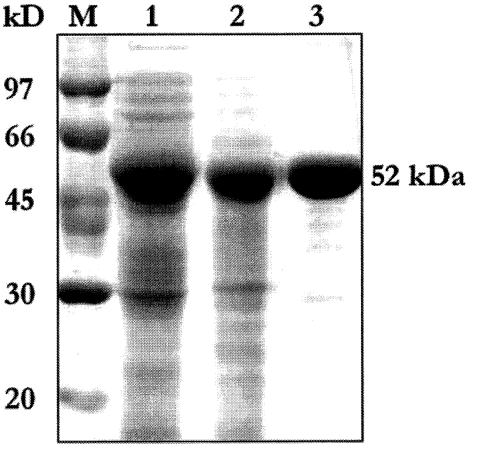

Figure 4. Analysis of proteins by 10% SDS-PAGE.
Lane M: Low MW protein marker
Lane 1: Crude protein
Lane 2: DEAE-cellulose fraction
Lane 3: DEAE-cellulose and Phenyl-sepharose fraction

Table 1. Levansucrase purification data.

	Fraction		
	Crude	DEAE	Phenyl
Volume (ml)	50	82	50
Activity (U)	2,435	2,007	1,514
Protein (mg)	94.5	27.4	8.9
Specific activity (U/mg)	25.8	73.3	170.0
%Yield	100	82.4	62.2
Fold no.	1	2.8	6.6

3.2 Levansucrase Immobilization Using Different Matrices

Levansucrase was immobilized on different supporting matrices using a covalently cross-linking method. The percentages of immobilized levansucrase on Sepabead EC-HFA, Sepabead EC-EP, and chitosan beads after immobilization were 23%, 35%, and 96%, respectively, as shown in Table 2. An approximate 50% loss in enzymatic activity occurred with levansucrase bound to Sepabead EC-HFA and Sepabead EC-EP. This loss of activity was probably due to enzyme cross-linking to itself or improper orientation of the enzyme on the supporting matrix, rendering the active site of the enzyme inaccessible.

Remarkably, 96% of levansucrase activity was retained when the enzyme was immobilized on chitosan beads. This result demonstrated that chitosan beads are a very efficient matrix for levansucrase immobilization.

This high yield indicates that chitosan beads provide not only a very effective immobilization matrix for levansucrase but also maximize its activity by ensuring proper enzyme orientation. As shown in Figure 5, a chitosan bead has a solid core, but its surface has nano-fibrous-like texture. This nano-fibrous-like structure provides a large surface area, which increases the loading capacity of chitosan beads. This structure might have also helped orientating levansucrase, by interacting with the proper surface on enzyme molecule. This allowed the active site of levansucrase to be exposed, and to remain functionally active.

Table 2. Immobilization efficiency of different supporting matrices for levansucrase.

Total activity 7U and 50 mg protein	Matrix		
	Chitosan bead	Sepabead EC-EP	Sepabead EC-HFA
Activity on matrix	96% (6.72 (U)	38% (2.66 U)	30% (2.10 U)
Activity in supernatant	2% (0.14 U) (1.15 mg)	15% (1.05 U) (7.65 mg)	18% (1.26 U) (9.17 mg)
Activity lost	2	47	52

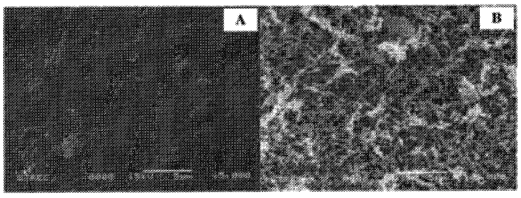

Figure 5. Scanning electron micrographs (SEM) of a shrimp chitosan bead. The cross-section of a chitosan bead is shown in panel A, at 5,000X magnification. The outer surface of chitosan bead is shown in panel B, at 5,000X magnification. Chitosan beads were prepared by the critical point drying method for SEM analysis.

3.3 Effect of pH and Temperature on Free and Immobilized Levansucrase

The optimum pH of free and immobilized levansucrase is shown in figure 6. The optimum pH of the free and all forms of immobilized levansucrase was similar, at pH 6.0. However, the immobilized levansucrase has a broader activity range. It was observed that the chitosan immobilized of levansucrase appeared to have higher activity in both the acidic range (pH 3-5) and basic range (8.0-11.0) than the other forms of the enzyme. The levansucrase immobilized on chitosan beads retained more than 70% of its activity at pH range 3.0-7.0. Increased pH stability was also observed when Sepabead EC-EP and Sepabead EC-HFA beads were used as the matrix for levansucrase immobilization, but it was lower than on chitosan beads.

The optimum temperature of the free and immobilized levansucrase was similar, at 50□ C. At low temperatures, below 35□ C, free levansucrase exhibited higher activity than immobilized levansucrase. However, at temperatures above 35□ C all forms of immobilized levansucrase were found to be more stable and showed a significant increase in enzymatic activity compared to free levansucrase (Figure 7). The levansucrase immobilized by covalently linking it to the matrix may result in an increase of the enzyme rigidity by multiple point attachment. This would lead to the increase in stability observed at higher temperatures.

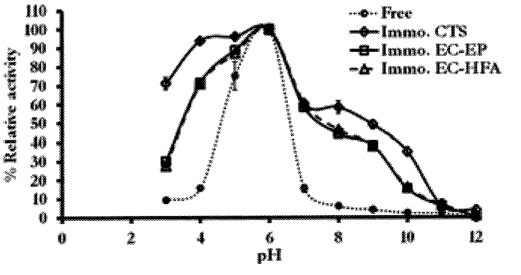

Figure 6. Effects of pH on the activity of free and immobilized levansucrase. CTS = Chitosan beads; EC-EP = Sepabead EC-EP beads; EC-HFA = Sepabead EC-HFA beads.

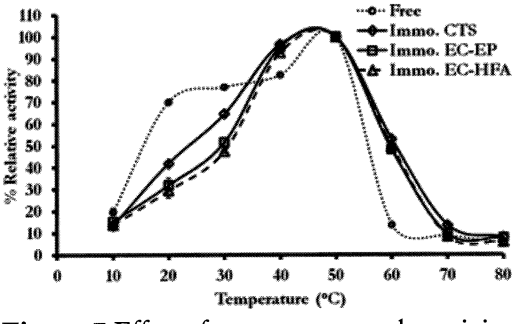

Figure 7. Effect of temperature on the activity of free and immobilized levansucrase. CTS = Chitosan beads; EC-EP = Sepabead EC-EP beads; EC-HFA = Sepabead EC-HFA beads.

3.4 Operational Stability of Immobilized Levansucrase

Levansucrase immobilized on chitosan beads retained over 75% of its activity after 10 cycles of repetitive use and over 60% activity after 17 cycles of repetitive used. In contrast, Sepabead EC-EP and Sepabead EC-HFA lost 67% and 70% of its activity after only 5 cycles of repetitive use (Figure 8). This result demonstrated that chitosan beads could help increase the stability of levansucrase after multiple usages. The porous,

fibrous surface of chitosan beads may help prevent the denaturation of the enzyme bound on the surface from shearing forces during the reaction process.

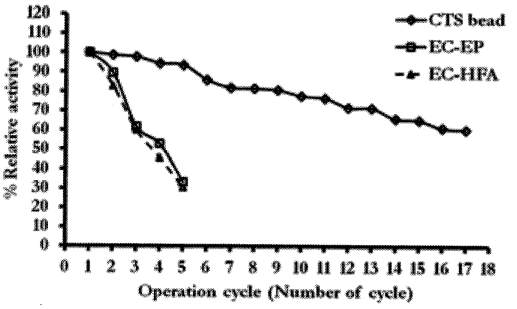

Figure 8. Operational stabilities of levansucrase immobilized on different supporting matrices. CTS = Chitosan beads; EC-EP = Sepabead EC-EP beads; EC-HFA = Sepabead EC-HFA beads.

3.5 Product Analysis by HPAEC-PAD

The levansucrase immobilized on chitosan beads was used for the production of L-FOS. The L-FOS products were detected by HPAEC-PAD as shown in Figure 9. The result demonstrated that immobilized levansucrase can synthesize L-FOS within 12 h. However, free levansucrase did not completely utilize the sucrose substrate; a large amount of sucrose is still present and only a small amount of high molecular weight L-FOS was produced (Figure 10).

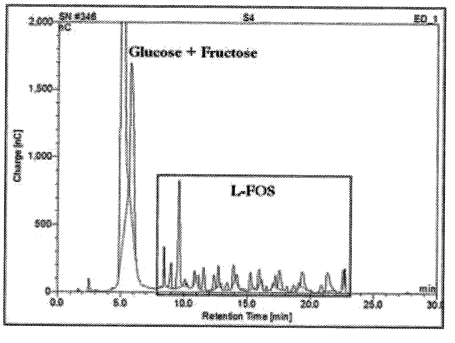

Figure 9. The L-FOS production by immobilized on chitosan beads analysis by HPAEC-PAD.

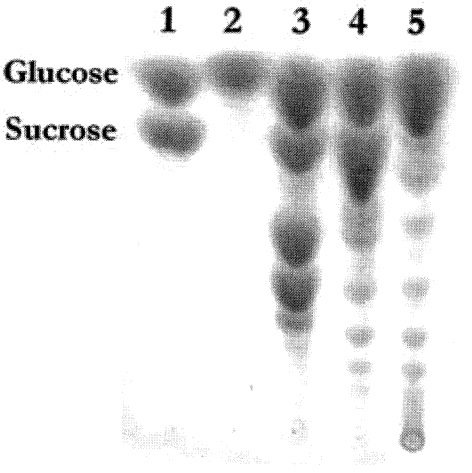

Figure 10. Product analysis by TLC; lane 1: standards glucose and sucrose, lane 2: standards fructose, lanes 3: standards oligosaccharides were prepared by partially hydrolyzed inulin (F_2, F_3, F_4 and F_5), lane 4: products from free levansucrase, lane 5: products from immobilized levansucrase on chitosan bead.

4. CONCLUSIONS

The *lsRN* gene was expressed under the regulation of its putative endogenous promoter. The highest activity of levansucrase occurred using the 3X concentration of the LB medium. We successfully purified levansucrase, resulting in 170 U/mg protein with a 6.6 purification fold and 62.2% yield. The levansucrase produced exhibited a high degree of apparent homogeneity with a MW of 52 kDa. Chitosan beads were identified as a suitable immobilization matrix for levansucrase by covalently binding. Chitosan beads have superior characteristics providing a large surface area, high immobilization yield, and enhanced the stability of levansucrase and increased reusability for the production of L-FOS. Chitosan beads had superior immobilization properties than the commercially available Sepabead EC-EP and Sepabead EC-HFA.

ACKNOWLEDGEMENTS

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