Songklanakarin J. Sci. Technol.
38 (3), 295-303, May - Jun. 2016

http://www.sjst.psu.ac.th

Original Article

Levan-type fructooligosaccharide production using *Bacillus licheniformis* RN-01 levansucrase Y246S immobilized on chitosan beads

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Received: 11 August 2015; Accepted: 30 November 2015

Abstract

Bacillus licheniformis RN-01 levansucrase Y246S (LsRN-Y246S) was immobilized by covalently linking onto chitosan, Sepabead EC-EP, and Sepabead EC-HFA, beads. The stability of immobilized LsRN-Y246S was found to be the highest with chitosan beads, retaining more than 70% activity after 13 weeks storage at 4 °C, and 68% activity after 12 hours incubation at 40°C. LsRN-Y246S immobilized on chitosan beads withstands sucrose concentrations up to 70% (w/v), retaining over 85% of its activity, significantly better than LsRN-Y246S immobilized on others supporting matrices. LsRN-Y246S immobilized on chitosan showed a 2.4 fold increase in activity in the presence of Mn^{2+}, and gave slight protection against deactivation by of Cu^{2+}, Zn^{2+}, Fe^{3+}, SDS and EDTA. A maximum of 8.36 g and an average of 7.35 g LFOS yield at least up to DP 11 can be produced from 25 g of sucrose, during five production cycles. We have demonstrated that LFOS can be effectively produced by chitosan immobilized LsRN-Y246S and purified.

Keywords: LFOS, levansucrase, *Bacillus licheniformis*, immobilization, chitosan

1. Introduction

Fructooligosaccharides (FOSs) are indigestible for most mammalians (Oku *et al*., 1984); therefore they can be used as prebiotics (Gibson *et al*., 1995; Fabio *et al*., 2001). FOSs have numerous other potential applications in the food industry, and have been shown to be a cholesterol and triacylglycerol lowering agent (Yamamoto *et al*., 1999). Levan type fructooligosaccharide (LFOS) is a type of fructan composed of D-fructofuranosyl residues linked together by β-(2, 6) glycosidic linkage, with β-(2, 1) linkage at branch points. It is produced by either microbial fermentation or enzymatic synthesis from sucrose by levansucrase.

Levansucrase (sucrose 6-fructosyltransferase, EC 2.4.1.10), a member of the family 68 glycosylhydrolase, hydrolyzes sucrose liberating glucose and transferring the fructosyl residue to the growing LFOS chain (Chambert *et al*., 1976; 1991). Levansucrase is produced by a wide variety of bacteria such as *Acetobacter xylinum* (Tajima *et al*., 1998), *Bacillus subtilis* (Chambert *et al*., 1974), *Bacillus megaterium*

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(Homann *et al*., 2007), *Gluconacetobacter diazotrophicus* (Hernandez *et al*., 1995), *Lactobacillus reuteri* (Van Geel-Schutten *et al*., 1999), *Leuconostoc mesenteroides* (Kang *et al*., 2005), *Pseudomonas syringae pv. Phaseolicola* (Hettwer *et al*., 1998), *Rahnella aquatilis* (Ohtsuka *et al*., 1992) and *Zymomonas mobilis* (Song *et al*., 1993).

The conversion yield of sucrose to LFOS by enzymatic synthesis is higher than that of microbial fermentation (Yun, 1996). However, enzymatic production of LFOS has been constrained by instability, poor recyclability and reusability of the enzyme giving low yields of LFOS. Therefore production of LFOS was previously accomplished via fermentation process.

Therefore, immobilized enzyme for LFOS production is of our interest because it can be used repeatedly and continuously, and it is more suitable than free enzyme. Various immobilized enzymes have been used for oligosaccharides production: galacto-oligosaccharides, inulo-oligosaccharides, isomalto-oligosaccharides and manno-oligosaccharides produced by immobilized *b*-galactosidase from *Bullera singularis* on chitosan beads (Shin *et al*., 1998), immobilized endoinulinase from *Pseudomonas sp.* on anion exchange resin (Yun *et al*., 1997), immobilized dextransucrase from *Leuconostoc mesenteroides* on alginate fiber (Tanriseven *et al*., 2002) and immobilized mannanase from *Penicillium occitanis* on chitin (Blibech *et al*., 2011).

Levansucrase has been previously immobilized using various techniques and supporting matrices such as covalently linking on activated amino silica, spherosil (Parlot *et al*., 1984) or chitosan (Esawy *et al*., 2008), adsorbed on honeycomb shaped ceramic (Iizuka *et al*., 1993), titanium-activated magnetite (Jang *et al*., 2001), wool (El-Refai *et al*., 2009) and entrapped in calcium alginate (Chambert *et al*., 1993). The immobilized levansucrase showed improved stability when compared with free levansucrase, but with low immobilization efficiency and poor performance of the immobilized enzyme. Furthermore, levansucrase produces mostly high molecular weight polymer instead of LFOS.

Previous, production of lower MW levan was achieved by using levansucrase immobilized on titatium-actived magnetite, which renders the enzyme to produce slightly lower MW products (Jang *et al*., 2001). Orientating the enzyme by using a levansucrase/chitin-binding domain fusion protein immobilized on chitin beads was also reported to enhance levan production (Chiang *et al*., 2009). Our previous work, using chitosan beads as a supporting matrix instead of chitin, demonstrated that levansucrase can retain over 90% of its initial activity. This result suggested that chitosan matrix may help orientating levansucrase prior being covalently crosslinked without the necessity of an additional binding domain (Sangmanee *et al*., 2015). Therefore, in this work we have combined the enzyme immobilization technique with *Bacillus licheniformis* RN-01 levansucrase mutant Y246S (LsRN-Y246S), a LFOS producing mutant enzyme resulted from an error prone PCR mutagenesis (data not shown), for LFOS production. LsRN-Y246S was immobilized on the surface of the matrix by a covalently crosslinking technique, to provide strong binding between levansucrase and the supporting matrix, and to eliminate the limitation of substrate/product transfer into or out of the supporting matrix. Chitosan beads, Sepabead EC-EP beads, and Sepabead EC-HFA beads were employed as supporting matrices. We have identified a suitable matrix that can enhance the stability of levansucrase during prolong storage and in its working condition, as well as retaining its activity at high sucrose concentration. LFOS was produced from immobilized LsRN-Y246S and purified.

2. Materials and Methods

2.1 Supporting matrices

Three different supporting matrices were used in this work. Shrimp chitosan polymer 850,000 MW with 84% degree of deacetlylation, was dissolved to 2% (w/v) in 1% (v/v) acetic acid. Beads were produced by dropping chitosan solution into 0.5 N NaOH with gentle stirring. Chitosan beads produced were then washed in DI water until pH became neutral.

Sepabead EC-EP beads, with epoxide functional group, and Sepabead EC-HFA beads, with amino epoxide functional group, were obtained from Resindion, Mitsubishi Chemical Corporation, Italy. The epoxide Sepabead EC-EP and epoxide Sepabead EC-HFA beads were incubated with 1 M NaOH at room temperature for 24 hours on an orbital shaker to activate the epoxide group and then washed with DI water until pH became neutral.

2.2 Bacterial strains and plasmid

E. coli Top-10 was used as an expression host and plsRN01-Y246S containing a levansucrase gene (*lsRN*) of *B. licheniformis* RN-01 (GenBank accession no. FJ171619.1) with the mutation Y246S under the regulation of its putative endogenous promoter was used for levansucrase production.

2.3 Production of levansucrase

Bacillus licheniformis RN-01 levansucrase mutant Y246S (LsRN-Y246S) expression, the *E. coli* Top-10 containing plsRN01-Y246S was cultured in LB 3X medium; 3% (w/v) casein tryptic peptone, 1.5% (w/v) yeast extract, and 1.5% (w/v) sodium chloride, supplemented with 5 mM $CaCl_2$ at 37°C in an orbital shaker at 250 rpm for 30 hours. After cultivation, cells were removed by centrifugation, 8,000 x g, at 4°C for 10 minutes.

2.4 Purification of levansucrase

Crude recombinant LsRN-Y246S was purified by DEAE-cellulose and Phenyl Sepharose column chromatography, respectively. The enzyme was initially bound to the

DEAE-cellulose column in 50 mM acetate buffer pH 6.0 then it was eluted using stepwise elution with 50 mM acetate buffer pH 6.0 containing 0.1-0.5 M NaCl, at 0.1 molar increments. The fraction containing levansucrase activity at 0.2 M NaCl was collected. LsRN-Y246S was further purified by Phenyl Sepharose column chromatography by initially binding it to the column using 25 mM acetate buffer pH 6.0 with 1.0 M ammonium sulfate. LsRN-Y246S was eluted using a reversed salt gradient from 1.0 to 0 M ammonium sulfate. The fractions containing levansucrase activity were collected and pooled.

2.5 Covalently linking levansucrase on chitosan beads

Seven units containing 50 mg of purified LsRN-Y246S were incubated with chitosan beads, 0.1 g (wet weight), then covalently linked by adding glutaraldehyde (GTA) solution to the final concentration of 0.5% in 50 mM citrate buffer, pH 6.0 at 4°C. The reaction was incubated under static condition for 2 days. The chitosan beads with immobilized LsRN-Y246S were separated from unbounded LsRN-Y246S then washed with ice-cold 50 mM citrate buffer pH 6.0, three times. The unbound enzyme remaining in the supernatant was assayed and used to calculate the immobilization efficiency. The immobilized LsRN-Y246S was stored at 4°C for further experiments.

2.6 Covalently linking levansucrase on Sepabead EC-EP and Sepabead EC-HFA

In separate preparations, 0.1 g of Sepabead EC-EP or Sepabead EC-HFA were activated with 1 M NaOH to open the epoxide ring, before being incubated with 7 units containing 50 mg of purified LsRN-Y246S in 50 mM citrate buffer, pH 6.0 at 4°C with gentle agitation for 2 days. Sepabead beads with immobilized LsRN-Y246S were separated from the unbound LsRN-Y246S and washed with ice-cold 50 mM citrate buffer, pH 6.0, three times. The unbound enzyme remaining in the supernatant was assayed and used to calculate the immobilization efficiency. The immobilized LsRN-Y246S on Sepabead EC-EP and Sepabead EC-HFA beads were stored at 4°C for further experiments.

2.7 Levansucrase activity determination

The activity of free and immobilized LsRN-Y246S was measured by determining the amount of the reducing sugar (glucose and fructose) liberated from the reaction by a modified DNS method (Miller, 1959). One levansucrase unit was defined as the amount of levansucrase that produces 1 mmol of reducing sugar (glucose equivalent) per minute.

2.8 Product quantitation

The amounts of sugars (LFOS, sucrose, glucose and fructose) synthesized by levansucrase were measured by HPLC equipped with a SugarPak column and a refractive index detector, using 50 mg/L Na_2EDTA as the mobile phase with a flow rate of 0.5 mL/min and injection volume of 20 mL. By measuring the sugar contents at the beginning (initial) and at the end (final) of the reaction, LFOS production can be determined.

$$LFOS_{produced} = Sucrose_{intial} - (Sucrose_{final} + Glucose_{final} + Fructose_{final})$$

2.9 Levansucrase stability

Free and immobilized LsRN-Y246S were pre-incubated in 50 mM citrate buffer, pH 6.0 at 40°C for 0-12 hours. The residual activities of the free and immobilized LsRN-Y246S from each time point was assayed by modified DNS method. The residual activity at each time point was plotted against time. The residual activity at different time points were determined and expressed as relative activity against the initial activity and plotted against time.

2.10 Levansucrase storage stability

Free and immobilized LsRN-Y246S were stored in 50 mM citrate buffer, pH 6.0 at 4°C for 3 months. The residual activity of the levansucrase was assayed weekly by modified DNS method. The residual activity at each time point was plotted against storage time.

2.11 Effect of sucrose concentration on the activity of free and immobilized levansucrase

Two hundred milliunits of each free and immobilized LsRN-Y246S were incubated with 10-70% (w/v) sucrose in 50 mM citrate buffer, pH 6.0, at 40°C for 5 minutes. The activity of levansucrase was measured by modified DNS method. The relative activity of each of the free or immobilized enzymes at different sucrose concentration was plotted against sucrose concentration. The maximum activity of each enzyme form was set as 100%

2.12 Effect of metal ions and chemical reagents on the activity of free and immobilized levansucrase

Two hundred milliunits of each free and immobilized LsRN-Y246S were incubated with 1.6 % (w/v) sucrose in 50 mM citrate buffer, pH 6.0 supplemented with 5 mM of one of the metal ions, Na^+, K^+, Co^{2+}, Ca^{2+}, Mn^{2+}, Cu^{2+}, Zn^{2+}, or Fe^{3+}, or chemical reagents, EDTA or SDS, then incubated at 40°C for 5 minutes. The activity of levansucrase was measured by modified DNS method.

2.13 Effect of ionic strength on the activity of free and immobilized levansucrase

Two hundred milliunits of each free and immobilized levansucrase were incubated with 1.6% (w/v) sucrose and

50 mM citrate buffer, pH 6.0, at 40°C for 5 minutes in the presence of 0-3 M NaCl. The activity of levansucrase at different NaCl concentration was measured by modified DNS method. The relative activity of each of the free or immobilized enzymes at different NaCl concentration was plotted against NaCl concentration. The activity at 0 M NaCl was set as 100%.

2.14 Production kinetics of LFOS by levansucrase immobilized on chitosan beads

Five hundred units of immobilized LsRN-Y246S on chitosan beads in 50 mL of 50% (w/v) sucrose with 50 mM citrate buffer pH 6.0, was incubated at 40°C for 12 hours in an orbital shaker at 150 rpm. LFOS concentration was determined every 60 minutes and the finial yield was quantitated.

2.15 Recyclability of immobilized levansucrase for the production of LFOS

Five hundred units of immobilized LsRN-Y246S on chitosan beads in 50 mL of 50% (w/v) sucrose (25 g of sucrose) with 50 mM citrate buffer pH 6.0, was incubated at 40°C for 12 hours in an orbital shaker at 150 rpm. At the end of the cycle, the supernatant was collected and the products were determined. The immobilized levansucrase was washed with ice-cold 50 mM citrate buffer, pH 6.0 and used repeatedly for five cycles. The products from each cycle were quantitated and the yield was calculated.

2.16 Product analysis

LFOS product was analyzed by HPAEC-PAD equipped with a CarboPac PA-1 column. Elution was performed by a gradient of 0-0.6 M NaOAc in 0.1 M NaOH with 1 mL/min flow rate, and a Dionex ED40 electrochemical detector with an Au working electrode and an Ag/AgCl reference electrode. The product was also analyzed by TLC using a mobile phase containing acetic acid:butanol:distilled water, 3:3:2, the TLC plate was subjected to three consecutive runs before developing the spots using ethanol:sulphuric acid, 9:1.

2.17 Product purification

LFOS product was purified by Biogel P2 column chromatography (3.2x112 cm) at 50°C using DI water as mobile phase, eluting at 27 mL/hour. The separated products were analyzed by TLC.

3. Results and Discussion

3.1 Levansucrase stability of free and immobilized levansucrase

To compare the stability of free versus immobilized enzyme, free and immobilized LsRN-Y246S were subjected to prolonged incubation at its optimum reaction condition at temperature of 40°C. The results in Figure 1A clearly demonstrated that immobilized LsRN-Y246S retained higher activity than the free LsRN-Y246S throughout the 12-hour incubation period. Free LsRN-Y246S rapidly lost more than 50% of its activity within the first two hours of incubation (Figure 1A). LsRN-Y246S immobilized on chitosan beads showed higher stability than LsRN-Y246S immobilized on Sepabead EC-EP and Sepabead EC-HFA beads, both of which showed similar stability profiles.

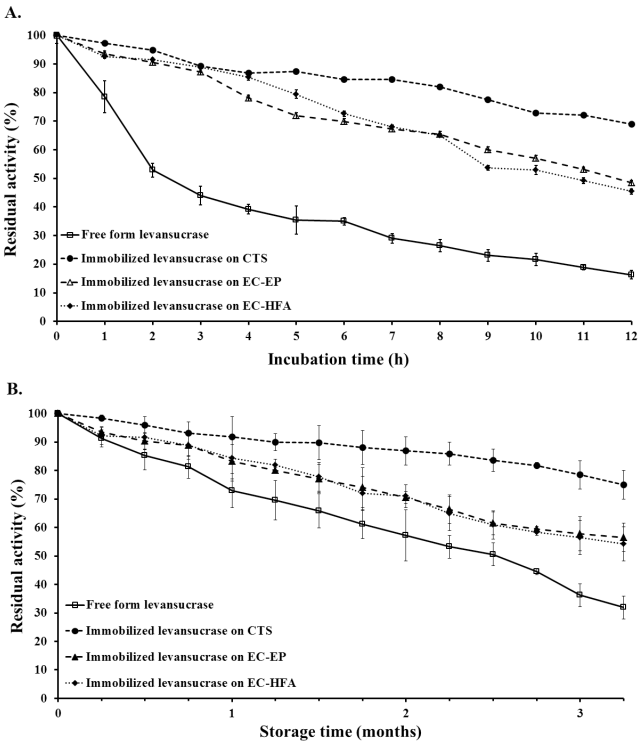

Figure 1. Levansucrase stability. Levansucrase in the reaction condition (A), and stability during storage (B) was determined. Free or immobilized LsRN-Y246S was incubated in 50 mM citrate buffer, pH 6.0 at 40°C for 0-12 hours or at at 4°C for 3 months.

Chitosan beads are hydrogel beads with fibrous-like surface with high porosity (Sangmanee *et al*., 2015) where Sepabead EC-EP and Sepabead EC-HFA beads are porous polymethylacrylate beads, with epoxide and amino epoxide functional group, respectively. LsRN-Y246S can be cross-linked directly to Sepabead EC-EP and Sepabead EC-HFA beads through the reaction of the epoxide group on the bead surface and the amino group of exposed lysine residues on the surface of the enzyme. For chitosan beads, glutaraldehyde was used as a crosslinking agent, linking the amino group of chitosan matrix with the amino group of exposed lysine residues on the surface of the enzyme. Immobilizing levansucrase by covalently linking it to a fibrous matrix with multiple attachment points, such as chitosan, might have

helped to stabilize the overall structure of the enzyme better than the single or a few attachment points provided by Sepabead EC-EP and Sepabead EC-HFA beads. Our earlier result has shown that LsRN-Y246S immobilized on chitosan beads retained over 90% of its activity (Sangmanee *et al*., 2015), suggesting that chitosan may also help to orientate the enzyme molecule, keeping its active site exposed, through site specific interactions. These interactions may also play a role in the enzyme stabilization.

3.2 Levansucrase storage stability

Upon prolong storage of immobilized enzyme in its storage condition, 50 mM citrate buffer, pH 6.0 at 4°C, for 13 weeks, LsRN-Y246S immobilized on chitosan beads had the best stability, retaining over 90% of its activity up to 7 weeks of prolong storage (Figure 1B). It lost only 25% of its activity after 13 weeks. LsRN-Y246S immobilized on Sepabead EC-EP and Sepabead EC-HFA beads retained 54% of its initial activity after 13 weeks, both had similar rate of activity lost. Free enzyme, on the other hand, lost 68% of its activity after 13 weeks. These results suggested that immobilization of LsRN-Y246S can stabilize the enzyme and reduce the rate of its activity lost by 2.7 folds, comparing free enzyme with chitosan beads immobilized enzyme.

3.3 Effect of sucrose concentration on free and immobilized levansucrase

As shown in Figure 2, free levansucrase activity increased as the sucrose concentration increases, reaching its maximum activity at 40% (w/v) sucrose then decreased once sucrose concentration exceeded 40%. It had only half of its activity left at 70% sucrose. This reduction in activity of the enzyme may result from the high viscosity that reduced the mass exchange rate of the system or the loss of enzymatic activity. However, when LsRN-Y246S was immobilized on chitosan beads, the optimum sucrose concentration increased to 50% and the enzyme retained 89% of its activity at 70% sucrose. Immobilizing the enzyme on Sepabead EC-EP and Sepabead EC-HFA beads did not further increase the optimum sucrose concentration when compared to the free enzyme, and could only slightly protect the enzymatic activity above 40% sucrose. These results further implies that the reduction in enzymatic activity at sucrose concentrations above 40% was more likely a result of the high sucrose concentration disrupting the structure or affecting the activity of the enzyme rather than the reduced mass exchange rate of the reaction. Since LsRN-Y246S immobilized on chitosan beads was found to have higher activity at concentrations above 40% despite the much lower mass exchange rate of immobilized enzyme versus free enzyme. This result observed on chitosan beads was better than the previously reported levan production, using titanium-activated magnetite and chitin beads immobilized recombinant *Zymomonas mobilis* levansucrase, where the optimum sucrose concentration was only at 20% and 30% respectively (Jang *et al*., 2001; Chiang *et al*., 2009). The ability of the immobilized enzyme to tolerate higher concentration of sucrose is beneficial for LFOS production, since higher amounts of substrate can be applied and further condensation steps can be kept to a minimum.

3.4 Effect of metal ions and chemical reagents on the activity of free and immobilized levansucrase

Metal ions can promote or inhibit the activity of enzymes; therefore their effects were evaluated on free and immobilized LsRN-Y246S. EDTA and SDS effect on the enzymatic activity was also studied. Metal ions, Na^{+}, K^{+}, Mg^{2+}, and Co^{2+}, at 5 mM concentration did not affect the activity of both free and immobilized LsRN-Y246S. Calcium ion slightly activated the activity of LsRN-Y246S of both free and immobilized enzyme. Interestingly, Mn^{2+} activated the activity of free LsRN-Y246S and chitosan beads immobilized LsRN-Y246S by 1.6 and 2.4 fold, respectively, while having little effect on LsRN-Y246S immobilized on Sepabead EC-EP and Sepabead EC-HFA beads (Figure 3).

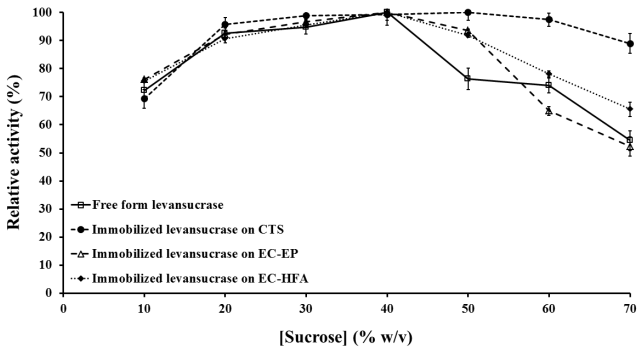

Figure 2. Effect of sucrose concentration on the activity of free and immobilized levansucrase. The activity of free and immobilized LsRN-Y246S was determined at sucrose concentrations from 10 to 70% (w/v), as described in Section Materials and Methods.

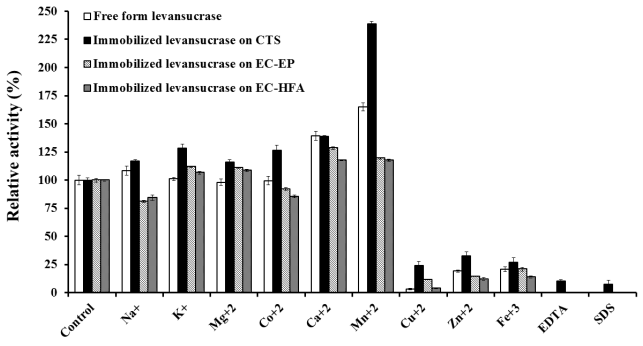

Figure 3. Effect of metal ions and chemical reagents on the activity of free and immobilized levansucrase. The effect of metal ions, EDTA and SDS on the activity of free and immobilized LsRN-Y246S was determined, as described in Section Materials and Methods.

However, Cu^{2+}, Zn^{2+}, and Fe^{3+} had strong inhibitory effect on the activity of all levansucrase forms. LsRN-Y246S immobilized on chitosan beads had less inhibitory effect from these metal ions. This inhibition by metal ions was also reported for immobilized levansucrase from *B. subtilis* and *B. circulans* (Esawy *et al*., 2008; El-Refai *et al*., 2009). The ionic surfactant SDS and chelating agent EDTA strongly inhibited the activity of LsRN-Y246S. It should be noted that immobilization of LsRN-Y246S on chitosan beads gave slight protection from the inactivation by Cu^{2+}, Zn^{2+}, Fe^{3+}, SDS and EDTA, as trace activity was detected.

3.5 Effect of ionic strength on the activity of free and immobilized levansucrase

Previous result has shown that immobilizing LsRN-Y246S on chitosan beads can help to increase its stability at high sucrose concentration. Ionic strength can also affect protein folding and the activity of levansucrase, therefore free and immobilized enzyme were analyzed in the presence of 0.5 to 3.0 M NaCl, results are shown in Figure 4. Interestingly, as the ionic strength increases, the activity of free LsRN-Y246S increased and maximized at 2.0 M NaCl with 1.6 fold increase. Further increase in ionic strength reduced the activity of the free enzyme down to its initial level. On the other hand, ionic strength affected immobilized LsRN-Y246S differently on different immobilization matrices. On chitosan beads the activity was slightly enhanced, approximately 10%, from 0-1.5 M NaCl, and then slowly decreased to 55% at 3.0 M NaCl. LsRN-Y246S immobilized on Sepabead EC-EP and EC-HFA beads had the highest activity in the absence of NaCl. Their activity gradually dropped as the NaCl concentration increases, to 50% at 3.0 M NaCl. These results demonstrated that chitosan beads continues to be the best supporting matrix in our experiments retaining the activity of LsRN-Y246S at low ionic strength, 0-1.5 M NaCl. Though, at 2.0 M NaCl the activity of chitosan beads immobilized enzyme was lower than that of the free enzyme, it was still higher than the activity of LsRN-Y246S immobilized on Sepabead EC-EP and EC-HFA beads (data not shown).

3.6 Production of LFOS by immobilized levansucrase and product purification

LsRN-Y246S immobilized on chitosan beads was used for LFOS production. As shown in Figure 5, the rate of LFOS production was steady in the first 5 hours then reaches equilibrium at 5-8 hours, giving the maximum LFOS yield of 8.36 g from 25 g of sucrose starting material, 33.4% yield, and an average of 7.35 g, 29.4% yield, of LFOS can be produced from the first five production cycles. Interestingly, hydrolysis reaction ceased after three hours of incubation, as shown by the constant fructose content in the reaction, indicating that after 3 hours mainly transglycosylation reaction occurs.

Analysis of LFOS products by HPAEC-PAD with CarboPac PA-1 column demonstrated that immobilized LsRN-Y246S on chitosan beads produces low to medium chain LFOS with a degree of polymerisation (DP) of at least upto 11 (Figure 6A and B).

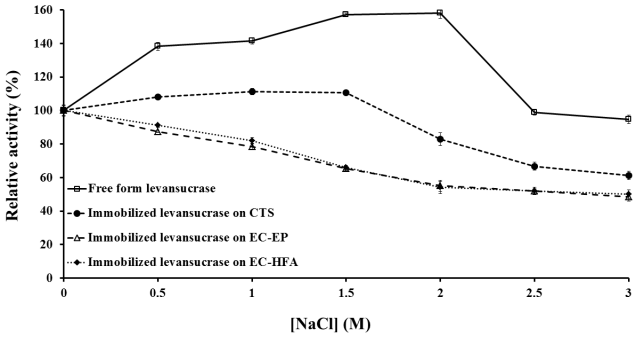

Figure 4. Effect of ionic strength on the activity of free and immobilized levansucrase. Free or immobilized LsRN-Y246S were incubated in reactions, supplemented with NaCl to the finial concentration of 0 to 3 M, as described in Section Materials and Methods.

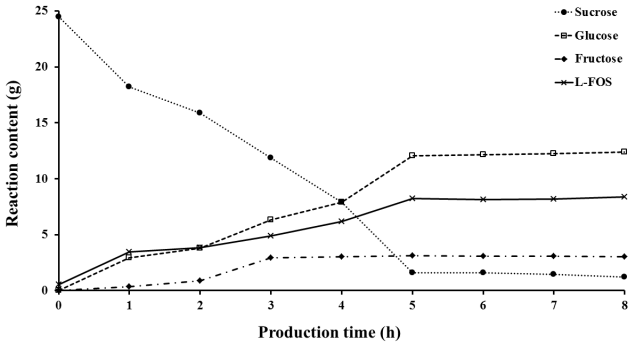

Figure 5. Production kinetics of LFOS by immobilized LsRN-Y246S on chitosan beads. Immobilized LsRN-Y246S on chitosan bead was used for LFOS production, as described in Section Materials and Methods.

Previously LFOS DP1–DP4 was successfully purified by using a single activated charcoal column (Nobre *et al*., 2012). However, LFOS products from our work has DP up to 11 that did not resolve well using an activated charcoal column, therefore, a Biogel P2 size exclusion column chromatography was applied. This procedure could separate high purity LFOS ranging from DP1 (MW. 180.16) to DP9 (MW. 1477.28) (Figure 6B).

3.7 Recyclability of immobilized levansucrase for the production of LFOS

To determine the ability of immobilized enzyme to be repeatedly used, LsRN-Y246S immobilized on chitosan beads was subjected to five consecutive production cycles. The LFOS yield and sugar composition in the reaction were analyzed at the end of each production cycle, results are shown in Figure 7. After five repetitive cycles, LsRN-Y246S

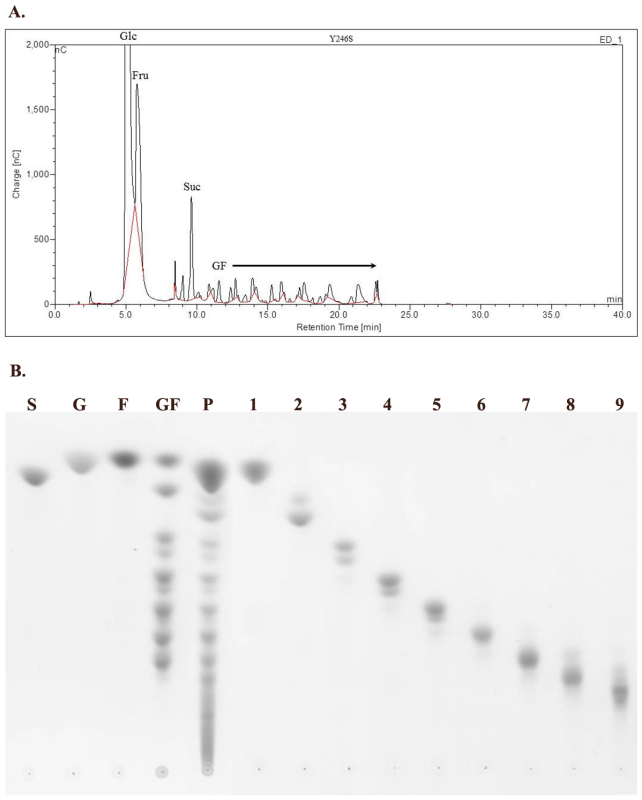

Figure 6. LFOS production, purification, and product analysis. The synthesized L-FOS products by immobilized LsRN-Y246S on chitosan bead were analyzed by HPAEC-PAD equipped with a CarboPac PA-1 column, as described in Section Materials and Methods (A). Crude L-FOS products were then purified by Biogel P2 column and the purified products were analyzed by TLC, as described in Section Materials and Methods (B). Lanes; S: sucrose standard, G: glucose standard, F: fructose standard, GF: GF standard, P: LFOS product, 1-9: purified product with DP1-DP9, respectively.

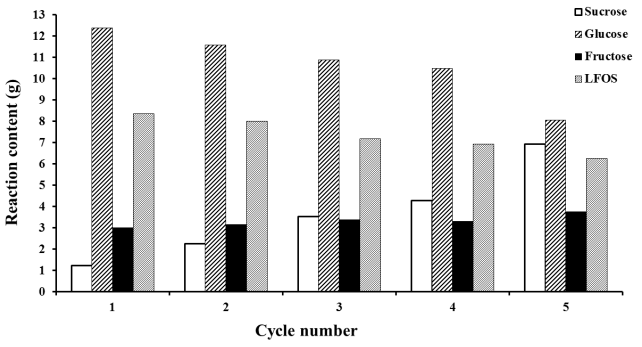

Figure 7. Recyclability of immobilized levansucrase for L-FOS production. Immobilized LsRN-Y246S on chitosan beads were subjected to five consecutive production cycles, as described in Section Materials and Methods. The amount in grams of sucrose, LFOS, fructose and glucose was determined at the end of each cycle during the 5 production cycle.

immobilized on chitosan beads can retain 75% LFOS production, with the initial yield of 8.36 g to 6.26 g in the fifth cycle. An average yield from five production cycle was 7.35 g, 29.4% yield. This result was better than that of the titanium-activated magnetite immobilized levansucrase, previously reported, that could retain only 61% of its activity after five production cycles (Jang *et al*., 2001) suggesting that chitosan beads could better stabilize the enzyme during LFOS production. This reduction in the enzymatic activity may be resulted partly from the shearing force caused by collision of the beads during production, since the enzyme was bound on the surface of the supporting matrix.

4. Conclusions

Immobilized LsRN-Y246S by covalent linking the enzyme onto the prepared chitosan beads were found to have better properties than using the commercially available Sepabead EC-EP and Sepabead EC-HFA beads. Immobilization of levansucrase on chitosan beads gave higher stability both under the reaction condition and in long-term storage. The chitosan beads can be repeatedly used and can retain up to 75% of its activity after five consecutive production cycles. LFOS products, DP1-DP9, were successfully purified by Biogel P2 size exclusion column chromatography. This work demonstrated for the first time that LFOS can be produced effectively using chitosan beads immobilized LsRN-Y246S. This provides a better alternative over the fermentation process previously reported. LFOS produced by this technique have high purity and can be readily purified. Higher concentration of sucrose, up to 70% (w/v), can also be used with this technique producing higher yields.

Acknowledgements

This work was supported by the 90th anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund) and the Rachadaphiseksomphot Endowment Fund Part of the "Strengthen CU's Researcher's Project", Chulalongkorn University, Thailand.

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