การผลิตฟรุกโตออลิโกแซ็กคาไรด์ชนิดลีแวนโดยลีแวนซูเครสแบบตรึง

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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PRODUCTION OF LEVAN-TYPE FRUCTOOLIGOSACCHARIDE BY IMMOBILIZED LEVANSUCRASE

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A Dissertation Submitted in Partial Fulfillment of the Requirements
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สุรวุฒิ แสงมณี : การผลิตฟรุกโตออลิโกแซ็กคาไรด์ชนิดลีแวนโดยลีแวนซูเครสแบบตรึง. (PRODUCTION OF LEVAN-TYPE FRUCTOOLIGOSACCHARIDE BY IMMOBILIZED LEVANSUCRASE) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร.รัฐ พิชญางกูร, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร.ชวลิต งามจรัสศรีวิชัย, ดร.กมลทิพย์ ขัตติยะวงศ์, 138 หน้า.

ลีแวนซูเครสผลิตฟรุกโตออลิโกแซ็กคาไรด์ชนิดลีแวน (L-FOS) โดยย่อยสลายซูโครสด้วยน้ำ จากนั้นเคลื่อนย้ายฟรุกโตสไปต่อกับซูโครสเพื่อสร้างสาย L-FOS และปลดปล่อยกลูโคส ยีนลีแวนซูเครส (*ls*RN) จาก *Bacillus licheniformis* แสดงออกใน *Escherichia coli* Top-10 โดยเลี้ยงในอาหารเหลว LB ที่มีความเข้มข้น 3 เท่า ประสิทธิภาพในการตรึงลีแวนซูเครสด้วยวิธีโควาเลนต์กับเม็ดไคโตซาน ไคติน/ไคโตซานที่กำจัดหมู่แอซิทิลบางส่วน ไคโตซานอนุภาคเล็ก อีพ็อกไซด์ Sepabeads EC-EP และอะมิโนอีพ็อกไซด์ Sepabeads EC-HFA เท่ากับ 96%, 94%, 11%, 38% และ 30% ตามลำดับ ลีแวนซูเครสที่ตรึงบนเม็ดไคโตซานสูญเสียแอกทิวิตีน้อยกว่า 25% หลังจากใช้งานไป 10 รอบ / ครั้ง ในทางกลับกันลีแวนซูเครสที่ตรึงบน Sepabeads EC-EP หรือ Sepabeads EC-HFA สูญเสียแอกทิวิตีมากกว่า 60% หลังจากใช้งาน 5 รอบ / ครั้ง pH และอุณหภูมิที่เหมาะสมต่อการทำงานของลีแวนซูเครสที่ตรึงบนเม็ดไคโตซาน เท่ากับ 4.0-6.0 และ 40-50 °C ตามลำดับ ซึ่งมีช่วงกว้างกว่าลีแวนซูเครสอิสระ ซึ่งมี pH และอุณหภูมิที่เหมาะสมต่อการทำงาน เท่ากับ 6.0 และ 40 °C ตามลำดับ ลีแวนซูเครสที่ตรึงบนเม็ดไคโตซานสามารถทนต่อความเข้มข้นของซูโครสได้สูงถึง 50% (w/v) โดยที่ยังคงแอกทิวิตีอยู่ถึง 95% ซึ่งดีกว่าลีแวนซูเครสที่ตรึงบน Sepabeads EC-EP, Sepabeads EC-HFA และลีแวนซูเครสอิสระอย่างมีนัยสำคัญ ลีแวนซูเครสที่ตรึงบนเม็ดไคโตซานสามารถลดการยับยั้งการทำงานจาก Cu^{2+}, Zn^{2+}, Fe^{3+}, SDS และ EDTA ได้บ้างเล็กน้อย และลีแวนซูเครสที่ตรึงบนเม็ดไคโตซานมีแอกทิวิตีสูงขึ้น 2 เท่าเมื่อเติม Mn^{2+} ความเสถียรในการทำงานของลีแวนซูเครสแบบตรึงพบได้สูงสุดเมื่อตรึงบนเม็ดไคโตซานโดยคงแอกทิวิตี 70% หลังจากชั่วโมงที่ 12 เมื่อบ่มในซิเตรทบัฟเฟอร์ pH 6.0 ที่อุณหภูมิ 50 °C ซึ่งผลการทดลองนี้แสดงให้เห็นว่าเม็ดไคโตซานมีคุณสมบัติที่ดีเยี่ยมในการนำไปใช้ตรึงลีแวนซูเครสเพื่อใช้ผลิต L-FOS ลีแวนซูเครสจาก N251Y ผลิต L-FOS ได้ 7.83 กรัม ในรอบการผลิตแรก และผลิตได้ 5.22 กรัม ในการผลิตรอบที่ 5 ลีแวนซูเครสจาก Y246S ผลิต L-FOS ได้ 8.36 กรัม ในรอบการผลิตแรก และผลิตได้ 6.26 กรัม ในการผลิตรอบที่ 5 และลีแวนซูเครสจาก Y246W ผลิต L-FOS ได้ 8.14 กรัม ในรอบการผลิตแรก และผลิตได้ 4.56 กรัม ในการผลิตรอบที่ 5 ในการผลิต L-FOS ปริมาณสูงโดยใช้ลีแวนซูเครสที่ตรึงบนเม็ดไคโตซานด้วยระบบปฏิกรณ์แบบ packed-bed ซึ่งมีแอกทิวิตีของลีแวนซูเครสที่ตรึงบนเม็ดไคโตซานเท่ากับ 1,890 ยูนิต ใช้สารละลายน้ำตาลซูโครสปริมาณ 250 กรัม (ความเข้มข้น 50% (w/v)) เป็นสารตั้งต้นในการผลิต โดยผ่านเข้าสู่ระบบปฏิกรณ์แบบ packed-bed จากด้านล่างไหลขึ้นสู่ด้านบนด้วยอัตรา 10 มิลลิลิตร/นาที ควบคุมอุณหภูมิที่ 40 °C พบว่าสามารถผลิต L-FOS ที่มีขนาดโมเลกุลสายกลางและโมเลกุลสายยาว โดยใช้เวลาในการผลิต 12 ชั่วโมง ได้ปริมาณ 118.39 และ 130.45 ตามลำดับ เมื่อผลิตด้วยลีแวนซูเครสจาก Y246S และ Y246W ที่ตรึงบนเม็ดไคโตซาน ตามลำดับ ส่วนลีแวนซูเครสจาก N251Y ที่ตรึงบนเม็ดไคโตซาน ผลิต L-FOS ที่มีขนาดโมเลกุลสายสั้นได้ปริมาณ 102.93 กรัม โดยใช้เวลาในการผลิต 24 ชั่วโมง ผลิตภัณฑ์ L-FOS แบบผสมสามารถแยกแต่ละขนาดของ L-FOS ให้มีความบริสุทธิ์ได้ตั้งแต่สายโมเลกุลที่ DP1 ถึง DP9 โดยวิธีโครมาโทรกราฟี โดยใช้ Biogel P2

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2556 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

5273867423 : MAJOR BIOTECHNOLOGY
KEYWORDS: L-FOS / LEVANSUCRASE / BACILLUS LICHENIFORMIS / IMMOBILIZATION / CHITOSAN / FIXED-BED REACTOR

SURAWUT SANGMANEE: PRODUCTION OF LEVAN-TYPE FRUCTOOLIGOSACCHARIDE BY IMMOBILIZED LEVANSUCRASE. ADVISOR: ASST. PROF. PROF. RATH PICHYANGKURA, Ph.D., CO-ADVISOR: ASST. PROF. CHAWALIT NGAMCHARUSSRIVICHAI, Ph.D., KAMONTIP KUTTIYAWONG, Ph.D., 138 pp.

Levansucrase produces levan-type fructooligosaccharide (L-FOS) by hydrolyzing sucrose, and then transfers the fructofuranosyl residue to sucrose or the growing L-FOS chain, liberating glucose. Levansucrase gene (*ls*RN) of *Bacillus licheniformis* RN-01 was expressed in *Escherichia coli* Top-10, using in 3X LB medium. The efficiency of levansucrase (LsRN) when covalently bound on chitosan bead, partially deacetylated chitin/chitosan, fine particle chitosan, epoxide Sepabeads EC-EP bead, and amino epoxide Sepabeads EC-HFA bead was 96%, 94%, 11%, 38%, and 30%, respectively. Levansucrase immobilized on CTS beads lost less than 25% of its activity after 10 cycles of repeated use. In contrast, levansucrase immobilized on Sepabead EC-EP or Sepabead EC-HFA lost over 60% after only 5 cycles of repeated use. The optimum pH and temperature of the immobilized enzyme on CTS beads were broader than that of free enzyme, pH 6.0 at 40 $^{\circ}$C compared to pH 4.0-6.0, 40-50 $^{\circ}$C, respectively. Levansucrase immobilized on CTS beads could tolerate sucrose concentration up to 50% (w/v) while retaining over 95% of its activity, which is significantly better than levansucrase immobilized on Sepabead EC-EP and EC-HFA and free enzyme. Immobilization of levansucrase on CTS bead could slightly protect levansucrase from the inactivation by Cu^{2+}, Zn^{2+} and Fe^{3+}, as well as SDS and EDTA. Interestingly, immobilizing levansucrase on CTS beads further elevate the enhancement of levansucrase activity by Mn^{2+} more than 2 folds. The stability of immobilized levansucrase activity was found to be highest on CTS beads, retaining approximately 70% activity after 12 h incubation in 50 mM citrate buffer, pH 6.0, at 50 $^{\circ}$C. L-FOS products were produced by LsRN-N251Y for 7.83 g at the first cycle and decreased to 5.22 g at fifth cycle. LsRN-Y246S produced L-FOS for 8.36 g and 6.26 g for the first and fifth cycle, respectively. The 8.14 g and 4.56 g of L-FOS at the first and fifth cycle respectively were produced from LsRN-Y246W. Scale up production of L-FOS was performed, using packed-bed immobilized levansucrase reactor. The packed-bed column contained 1,890 U of immobilized levansucrase. A 250 g (50% (w/v)) of sucrose solution was feed into the column with an upward flow direction with a flow rate of 10 mL/min, at 40 $^{\circ}$C. The medium chain and long chain of L-FOS products produced at 12 h about 118.39 g and 130.45 g from LsRN-Y246S and Y246W, respectively. LsRN-N251Y produced short chain L-FOS products, 102.93 g at 24 h. L-FOS products were successfully fractionated into DP1 –DP9 by Biogel P2 column chromatography.

Field of Study: Biotechnology Student's Signature .....

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LIST OF ABBREVIATIONS

A	Absorbance
BSA	Bovine serum albumin
^{o}C	Degree of Celsius
CTS	Chitosan
d	Day
Da	Dalton
DEAE	Diethylaminoethyl
DNA	Deoxyribonucleic acid
DNS	Dinitrosalicylic acid
EDTA	Ethylenediaminetetraacetic acid
et al.	Et. Alii (latin), and others
etc.	Et cetera (latin), other things
FOS	Fructooligosaccharide
g	Gram
GTA	Glutaraldehyde
h	Hour
HPAEC-PAD	High performance anion exchange chromatography with pulsed amperometric detection
HPLC	High performance liquid chromatography
I-FOS	Inulin type fructooligosaccharide
kb	Kilobase(s)
kDa	Kilodalton(s)
kΩ	Kilo-ohm
kv	Kilovolt
LB	Luria-Bertani
L	Litre
L-FOS	Levan type fructooligosaccharide
Ls	Levansucrase
LsRN	Levansucrase from *Bacillus licheniformis*

M	Molar
mg	Milligram
min	Minute
mL	Milliliter
mM	Milli molar
mo	Month
mol	Mole
mU	Milli unit
MW	Molecular weight
µF	Microfarad
µg	Microgram
µL	Microliter
PAGE	Poly acrylamide gel electrophoresis
PDCC	Partially deacetylated chitin/chitosan
pH	Power of hydrogen
rpm	Revolution per minute
RT	Room temperature
s	Second
SD	Standard deviation
SDS	Sodium dodecyl sulfate
sp.	Specie
spp.	Species
TLC	Thin layer chromatography
UV	Ultraviolet
v/v	Volume per volume
wk	Week
wt	Weight
w/v	Weight per volume

CHAPTER I

INTRODUCTION

Nowadays, there is an increasing consumer demand for healthier and calorie controlled foods, for instance alternative sweeteners. Fructooligosaccharide (FOS) is one of the oligosaccharides that have interestingly reported since the 1980s [1], [2] and [3]. FOS has an important primarily because of their functional properties rather than sweetness. FOS is a subunit of fructans, which can be produced from microbes or plants enzyme via transfructosylation activity, using sucrose as a substrate.

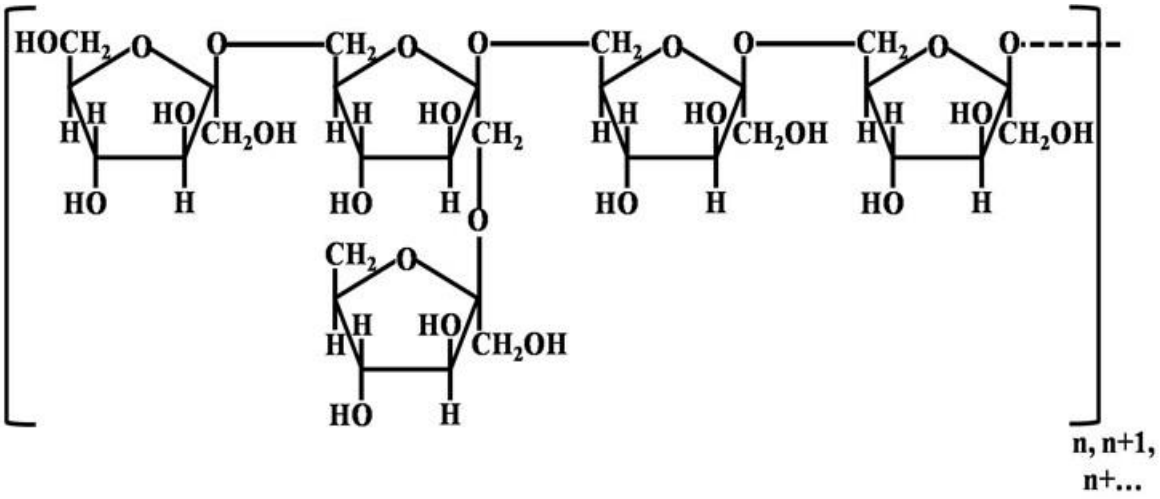
Fructans

Fructans are highly distributed biopolymer in nature. Fructans are composed of D-fructofuranosyl residues linked together by β-(2, 6) and β-(2, 1) glycosidic linkages. Fructans can be divided into two types which distinguishable by the type of their glycosidic linkage. D-fructofuranosyl residues linked in the main chain by β-(2, 6) glycosidic linkages and linked by β-(2, 1) glycosidic linkages at branching points, called levan mainly found in microbe. In contrast, inulin has the D-fructofuranosyl residues were linked by β-(2, 1) glycosidic linkages at the main chain and linked at branching points by β-(2, 6) glycosidic linkages (Figure 1.1) [4]. Inulin was mainly isolated from vegetables and plants [5]. However, levan can also be found in plants with shorter size (DP 10 – DP 200) than microbial levan. Microbial levan had molecular weights up to several million Daltons, with multiple branches.

Fructooligosaccharides

Fructooligosaccharides **(**FOSs) are an oligosaccharide composed of D-fructofuranosyl residues linked together by β-(2, 6) and β-(2, 1) glycosidic linkages. FOSs can be divided into two types which are distinguishable by the type of fructan. D-fructofuranosyl residues linked a main chain by β-(2, 6) glycosidic linkages and linked by β-(2, 1) with glycosidic linkages at branching points was used to describe a levan type fructooligosaccharide (L-FOS). In divergence, when linked D- fructofuranosyl

(A)



(B)

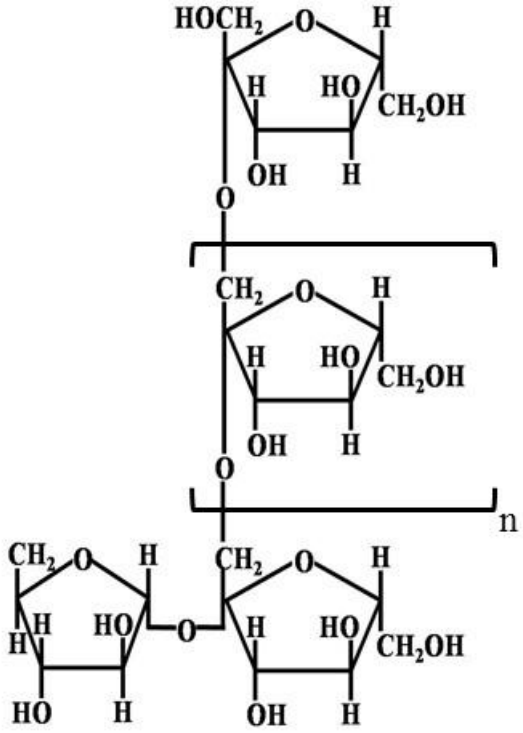


Figure 1.1 Structure of fructans;

A: Levan B: Inulin

residues by β-(2, 1) glycosidic linkages at the main chain and linked at branching points with β-(2, 6) glycosidic linkages was used to describe an inulin type fructooligosaccharide (I-FOS).

FOSs can be produced via transglycosylation by fructosyltransferase using sucrose as a substrate. Previously there are many reports of fructosyltransferase production from microbes and plants such as *Aspergillus oryzae* [6], *Aspergillus niger* [7], *Microbacterium laevaniformans* [8] *Bacillus subtilis* [9] and *Arctium lappa* [10].

Sources of FOSs

FOSs can be derived from various higher plants from reserve carbohydrates or cultures of several microbes in sucrose medium. Reserve carbohydrates in plants were mainly source of I-FOS such as sugar beet leaves (*Beta vulgaris* L.) [11], Jerusalem artichoke (*Helianthus tuberosus*) [12], onion bulb (*Allium cepa*) [13], asparagus root (*Asparagus officinalis*) [14] and also founded agave (*Agave Americana*) [15].

The L-FOS can be produced by aerobic submerged fermentation of some microbial strains. Although fermentation parameters of aeration, agitation, pH and temperature should be established for each microorganism, the general conditions for producing fructosyltransferase by growing cultures of organisms were well demonstrated. During cultivation of microbials in medium containing sucrose, the synthesis of L-FOS was observed. L-FOS was biosynthesized during cultivation *Aspergillus oryzae*, *Claviceps purpurea*, *Fusarium oxysporum*, respectively, in medium containing sucrose [16], [17] and [18]. *Aspergillus niger* enzyme fully characterized this enzyme and developed it into an industrial production of L-FOS syrup [7]. *Aureobasidium sp*. was investigated for L-FOS production process [19].

L-FOS is indigestible and has many potential applications. Rath Pichyangkura research group also screened, cloned and mutated many levansucrase (Ls) from bacterial organism. Different Ls derivatives could produce various molecular weight of L-FOS. Therefore, in this dissertation, L-FOS was produced by these Ls derivatives.

Application of L-FOS and levan

There are numbers interesting functionalities and applications of L-FOS and levan.

Medical and pharmaceutical applications

L-FOS and levan were reported tumor cells though the modification in cell membrane, which changes to inhibit cell permeability [20] and [21], as well as radioprotective and antibacterial activities [22]. Levan derivatives can also inhibit smooth muscle cell proliferation, as excipients in making tablets, and as agents to transit water into gels. Sulfated, phosphated and acetylated levans have also been suggested as anti-AIDS agents [23].

Water-soluble saccharide, including L-FOS and levan, can be used in a wide variety of applications in the pharmaceutical industry. They play a role in the formulation of solid, liquid, semisolid and even controlled release dosage forms [24]. The viscosity of levan varies with its DP and degree of branching, which relates to the number of side chains attached to one fructose unit in the main fructose chain. In these respect levan can be used in pharmaceutical formulations in various ways. Low molecular-weight, less branched levan usually provides a low viscosity, and can be used as a tablet binder in immediate-release dosage forms, while levans of medium- and high-viscosity grade are used in controlled-release matrix formulations. Levan has also been suggested as a possible substitute for blood expanders [25].

The antiviral activity of levan has been reported. Levan produced by different isolates of *B. subtilis* were antiviral activity tested on HPAI and H5N1, respiratory virus and adenovirus type 40, enteric virus. Levan with molecular weight of 43.5 and 71.9 kDa showed antiviral activity by decreased the infectivity of adenovirus type 40 to 50 and 60 % of the control, respectively. Levan with molecular weight of 40.9, 71.9 and 77.8 kDa showed the effects on HPAI and H5N1 infectivity. However, antiviral activities were detected only when pre-mixed the polymer with the virus one hour before infection of the virus into the test cells. Levan had no effect on the virus when inoculated simultaneously with the virus just after mixing or after one hour of infection [26].

The effect of levan on oxidative stress and hyperglycemia in alloxan-induced diabetic rats was evaluated. The oral administration of levan in diabetic rats resulted in an increase in glycogen level and a decrease in glucose level in plasma [27]. The polysaccharide administration also caused the significant decrease in hepatic and renal indices toxicity. The study demonstrates that levan is efficient in inhibiting hyperglycemia and oxidative stress induced by diabetes and suggests that levan supplemented to diet may be helpful in preventing diabetes [28].

Food and feed applications

Novel applications of L-FOS and levan have been suggested, particularly in food and feed. L-FOS and levan may act as a prebiotic to change the intestinal microflora, thereby offering beneficial effects when present in the human diet. Levan and its partially hydrolyzed products are fermented by intestinal bacteria including bifidobacteria and *Lactobacillus* sp. [29], [30] and [31]. Fructo-heptose was also used as a carbon source for selective intestinal microflora, including *Bifidobacterium adolescentis*, *Lactobacillus acidophilus*, and *Eubacterium limosum*, whereas *Clostridium perfringens*, *E. coli* and *Staphylococcus aureus* did not utilize levan [32].

Cholesterol- and triacylglycerol-lowering effects of levan have been reported and may be applied to develop levan as health foods or nutraceuticals. High molecular weight levan and its effect on the lipid metabolism in rats fed with cholesterol-free diets were studied. Although body weight gain and changes in morphology of the digestive tract, the serum triacylglycerol and glucose concentrations were not affected by feeding levan diets for 4 wk. Serum cholesterol level decreased to 83% or 59% by feeding a 1% or 5% levan supplemented diet, respectively. The hypocholesterolemic effect was accompanied by a significant increase in fecal excretion of sterols and lipids [30]. Moreover, it was reported that the supplementation of 1-10 percent levan in high-fat diet-induced obese rats could decrease adiposity and postprandial lipidamia (transient abnormally high concentration of lipid in the blood occurring after the ingestion of foods with a large content of fat) [33].

L-FOS and levan has been reported as prebiotic in milked-based fermented products. The saccharides were delivered to the GI tract and stimulated beneficial probiotic bacterial growth. A supplementation of milk by high molecular weight levan

from *Z. mobilis* stimulated the growth of starter ABT-5, higher level of acidification 10-14 percentage over the control test was observed [34]. When levan type fructooligosaccharides (L-FOSs) were tested as glucose substitutes on the commercial strain *Bifidobacterium lactis* Bb 12, they also exhibited an increase in cell count and acidification power [35]. *Bifidobacterium adolescentis*, *Bifidobacterium longum*, *Bifidobacterium breve*, and *Bifidobacterium pseudocatenulatum* were also *in vitro* studied for their capability of metabolizing low Mw levan prepared by acid hydrolysis. Growth, decrease in pH, formation of short- chain fatty acids (lactate, acetate, formate) and degradation of low Mw levan were markedly different among species. In those reports, *B. adolescentis* showed the best growth, produced the highest amounts of organic acids and metabolized both short- and long-chain oligosaccharides [31].

L-FOS and levan have been reported for effect to boosting mineral absorption. The intakes of minerals were not different among test groups. However fecal excretion of Mg (79.62% for control, 90.09% for high molecular weight levan and 94.23% for L-FOS) and Fe (46.62% for control, 82.64% for high molecular weight levan and 81.58% for L-FOS) were significantly different, indicating the apparent absorption is affected by the experimental administration of levan. Unlike Mg and Fe, Zn was significantly absorbed after the administration of low molecular weight levan by 1.5 fold of the control [36].

L-FOS and levan have been shown to have immuno-stimulating properties. The effects of dietary levan on the survival of *Cyprinus carpio* juveniles were studied. Fishes were fed with feed containing levan, at concentrations ranging from 0.1 to 1.0%. One hundred percent survival was obtained with 0.5% levan in the feed. But increasing the concentration to 1% probably increased the antigenic load, leading to immuno-suppression and thus reducing the protection efficiency [37].

Industrial applications

One of the striking consequences of the densely branched structure of levan is its effectiveness in resisting interpenetration by other polymers, leading to macroscopic phase-separation [38] and [39]. Microbial levans display phase-separation phenomena with pectin, locust bean gum, and PEG. Solutions of levan and locust bean gum showed a substantial reduction in viscosity, similar to the mixture levan/pectin. Levan/locust

bean gum phases can be separated into discrete phases in mixed solutions in which the lower one consisted predominantly of the denser polymer, the levan phase [38]. Levan has been utilized as an environmentally friendly adhesive. By the relatively high tensile strength and shear strength, levan is a competitor with many petrochemical based adhesives. The water soluble levan-based adhesive is useful for temporary bonds and certain indoor applications, and the cross-linked levan-based adhesive is utilized as a water resistance adhesive for a long term purpose. Levan was used in the wood adhesive industry, and in biodegradable plastic production. Levan was developed to be used as water resistant film for food preservation and for shale stabilization in the oil drilling industry. The adhesive strength, film-forming ability, and non-toxicity of levan are its selling properties, and are comparable to petrochemical derivatives in many applications. Levan has been used as a cryoprotectant for freeze-preservation of animal cells, fish and the delicate texture of frozen desserts.

Agricultural Applications

L-FOS and levan were introduced in plants to promote their agronomic performance in temperature zones, as well as their natural storage capacities [40]. Transgenic tobacco plants expressing levansucrase genes from *B. subtilis* [41] and *Z. mobilis* [42] showed an increased tolerance to drought and cold stresses. Transgenic plants accumulating fructan have been suggested as novel nutritional feed for ruminants. Recently, microbial levan produced enzymatically was developed as an animal feed and also as a soil conditioner to improve the germination of various seeds [25].

L-FOS and levan biosynthesis

L-FOS and levan biosynthesis requires the involvement of an extracellular enzyme levansucrase, which shows specificity for sucrose. Levansucrase (Ls, sucrose: α-D-glucosyl-(1→2)-(2→6)-β-D-fructan 6-β-D-fructosyltransferase, EC 2.4.1.10), a member of family 68 glycosylhydrolase, hydrolyses sucrose liberating glucose then transfers fructose molecule to a growing various acceptor molecules chain. The Ls catalyzes the following reactions:

1. Polymerization reaction

$$(\text{Sucrose})_n \rightarrow (\text{Glucose})_n + \text{Levan} + \text{Oligosaccharides}$$

2. Hydrolysis reaction

$$\text{Sucrose} + H_2O \rightarrow \text{Fructose} + \text{Glucose}$$

$$(\text{Levan})_n + H_2O \rightarrow (\text{Levan})_{n-1} + \text{Fructose}$$

3. Acceptor reaction

$$\text{Sucrose} + \text{Acceptor molecular} \rightarrow \text{Fructosyl-acceptor} + \text{Glucose}$$

4. Exchange reaction

$$\text{Sucrose} + [^{14}C]\ \text{Glucose} \rightarrow \text{Fructose-}[^{14}C]\ \text{Glucose} + \text{Glucose}$$

5. Disproportionation reaction

$$(\text{Levan})_m + (\text{Levan})_n \rightarrow (\text{Levan})_{m-1} + (\text{Levan})_{n+1}$$

Ls concomitantly catalyzes hydrolysis and polymerization reactions (Reaction 1), resulting in liberating glucose then transfers fructose molecule to a growing various acceptor molecules chain.

When water acts as an acceptor, free fructose is generated from sucrose and levan/L-FOS (Reaction 2). Reaction 2, occurs when Ls catalyzed reactions refer to above. However, a rate is slower when comparing to sugar acceptor. The acceptor molecule chains (levan/L-FOS chains) grows by a stepwise repeated transfer from donor molecules. Reaction 3, occurs in the presence of another acceptor molecule in the reaction. The enzyme transfers the fructosyl moiety of sucrose specifically to the hydroxyl group in the acceptor. Certain compounds containing hydroxyl groups, such as alcohol and oligosaccharides, can act as fructosyl acceptors to yield a non-reducing sugar compound and a series of oligosaccharides. Reaction 4 is considered as an analogous of Reactions 2 and 3, but differs in the regeneration of $[^{14}C]$ sucrose. The Ls also catalyzes reaction 5, a disproportionation reaction. The enzyme transfers the L-FOS from a donor molecule to an acceptor, i.e. levan and L-FOS. The above five reactions compete with one another, yielding a specific major product with some minor products but they are predominantly controlled by environmental factors such as type and concentration of donor and acceptor, ionic strength and temperature [43].

Ls diversely produced from microorganisms are mainly found in bacteria. Bacterial Ls was classified from similarity of amino acid, which divided into 2 types. The first type of bacterial Ls from gram positive bacteria, the relatively high similarity (>50%) in amino acid sequence when compared with sequences of gram negative bacteria. However, low similarity (<30%) exists among the genes from two different groups [43]. Most of the enzyme characteristics and levan products of bacterial Ls are different depending on sources of enzymes. However, some properties are found shared among Ls, i.e. the enzyme has high activity at pH 5-6, and is stable at pH 4-7. There were no activities below pH 3 and above pH 9. High molecular weight levan is far more processively synthesized at low temperature than at room temperature or at higher temperature, although sucrose hydrolysis activity is quite small at low temperature [9] and [44]. The bacterial Ls such as, *B. subtilis* [45], *Gluconacetobacter diazotrophicus* [46] and [47], *Z. mobilis* [48], *Acetobacter xylinum* [49], *P. syringae pv. Phaseolicola* [44], *R. aquatilis* [48], *L. reuteri* [50], *Leuconostoc mesenteroides* [51] and *Bacillus megaterium* [52] were previously reported.

Industrial production of L-FOS

L-FOS can be produced by microbial fermentation or enzymatic synthesis. The conditions for producing L-FOS by growing cultures of bacteria vary according to the microorganisms used, but yields of levan production are fairly low. This is due to the utilization of sucrose as energy source, the formation of byproducts, and the low level of Ls production. In theory, the yield of L-FOS production by Ls is not more than 50% (w/w) when sucrose is used as a substrate.

Recently developments of L-FOS production in industry have been made possible for the large-scale production. It appears that the industrial processes for the production of L-FOS can be divided into three techniques: (1) batch system using microbial fermentation, (2) batch system using soluble enzyme and (3) continuous system using immobilized enzyme or whole cells.

The conversion of sucrose to L-FOS by enzymatic synthesis is higher than that of microbial fermentation, however the enzymatic production has been strictly limited due to the low percent yield of L-FOS, instability and recycling or reusability of the enzyme. Therefore, immobilized enzyme is more favorable for L-FOS production.

Enzyme immobilization

Immobilized enzyme is currently the object of considerable interest. This is due to the expected advantage aloft of soluble enzymes. Immobilized enzyme is defined as enzyme restrained in/on supporting matrices while retaining its catalytic properties, which can be used repeatedly and continuously. The immobilized enzymes have proven extraordinarily valuable, it has allowed enzymes to be easily reused multiple times with longer half-life and less degradation and has provided a straightforward method of controlling reaction rate as well as reaction start and stop time. It has also helped to prevent contamination of the substrate with enzyme, which decreases purification costs. These benefits of immobilized enzymes have made them highly applicable to a range of evolving biotechnologies.

Enzymes can be attached to supporting matrices via interactions ranging from reversible physical adsorption and ionic linkages to stable covalent bonds. The classification of various approaches to immobilizing enzymes are divided into two categories, which are irreversible and reversible methods, as shown in Figure 1.2 and 1.3 [53]. The strength of binding is usually inversely related to the ease in which it can be reversed. These two conflicting objectives regarding stability and reversibility are difficult to be fulfilled simultaneously. The traditional approach is to make the bond as strong as possible and sacrifice reversibility.

The concept of irreversible immobilization means that once the biocatalyst is attached to the support it cannot be detached without destroying either the biological activity of the enzyme or the support.

Covalent binding

Immobilizations of enzymes based on the formation of covalent bonds are among the most widely used method. An advantage of these methods is because of the stable nature of the bonds formed between enzyme and matrix, the enzyme is not released into the solution upon use. However, in order to achieve high levels of bound activity, the amino acid residues essential for catalytic activity must not be involved in the covalent linkage to the support; this may prove a difficult requirement to fulfill in some cases. A simple procedure that sometimes improves the activity yield is to carry out the coupling reaction in the presence of substrate analogs [54].

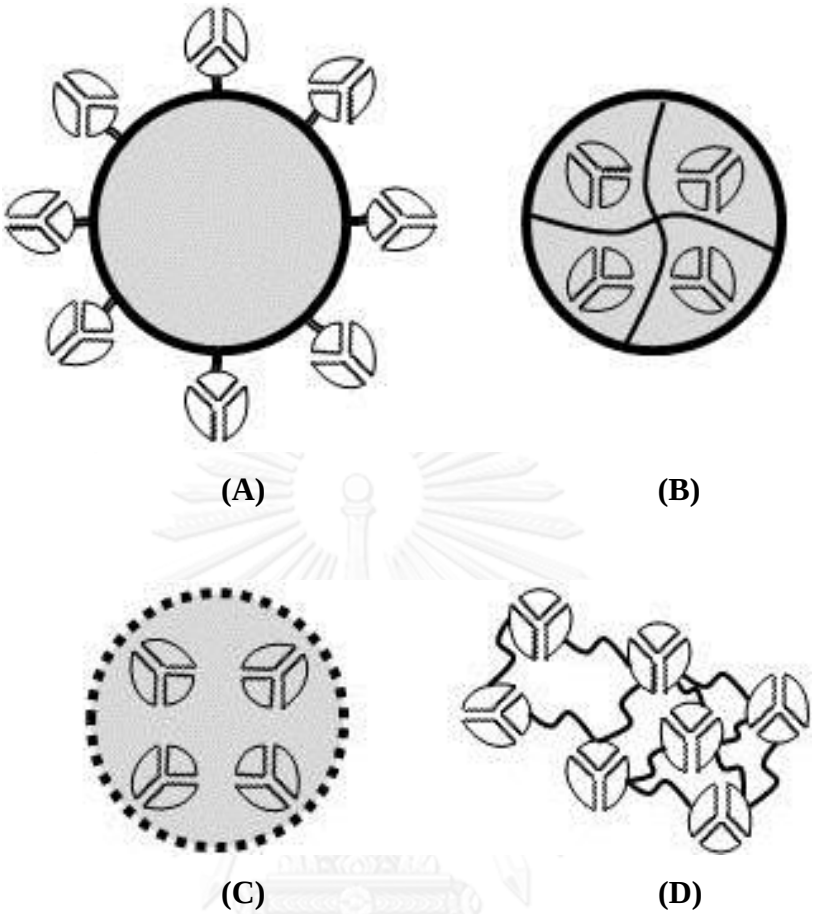


Figure 1.2 **Irreversible immobilization of enzyme**

A: covalent binding B: entrapment

C: encapsulation D: cross linking

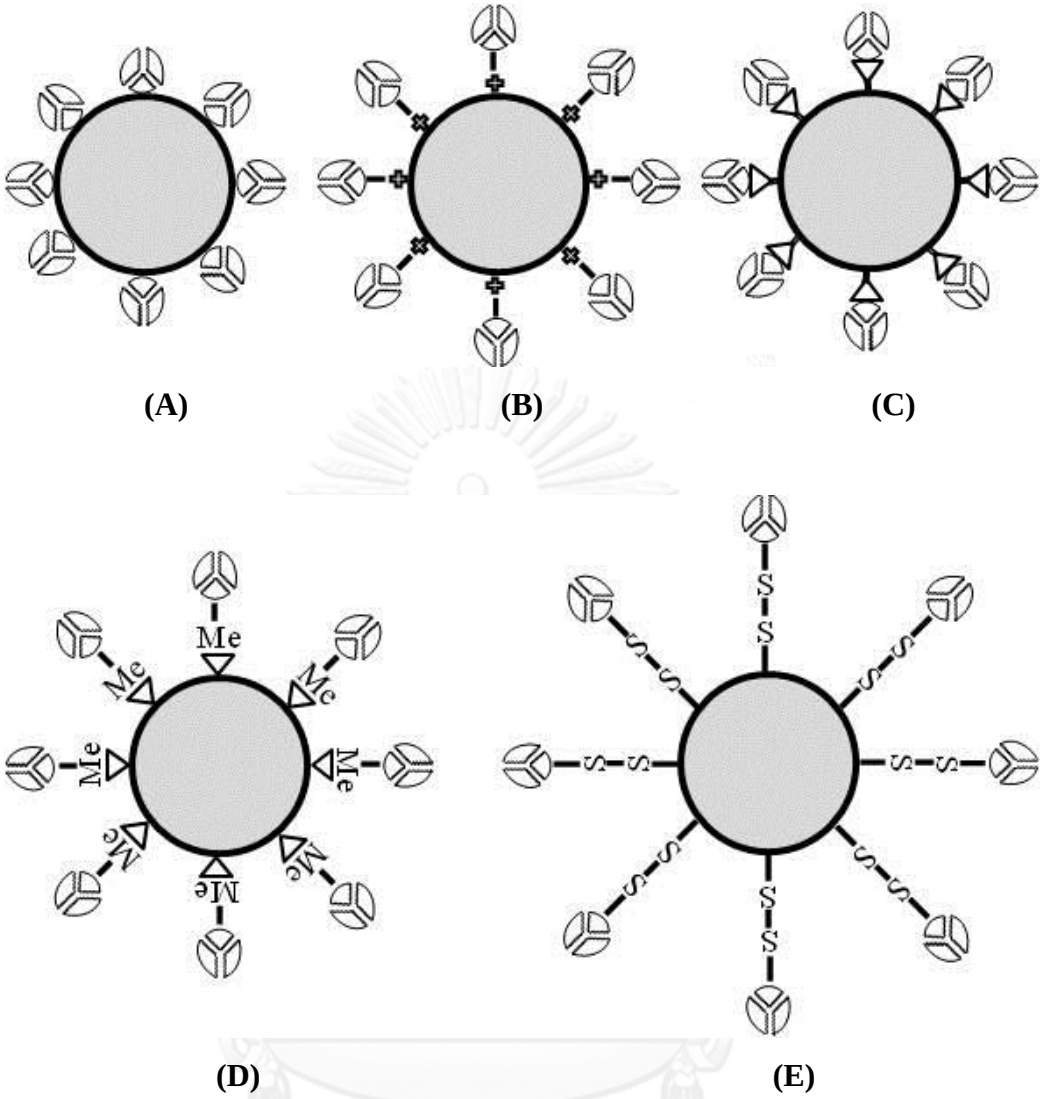


Figure 1.3 Reversible immobilization of enzyme

A: adsorption B: ionic binding C: affinity binding
D: metal binding or chelation E: disulfide bond

Covalent methods for immobilization are employed when there is a strict requirement for the absence of the enzyme in the product.

A wide variety of reactions have been developed depending on the functional groups available on the matrix. Coupling methods in general can be divided in two main classes: (1) activation of the matrix by addition of a reactive functional group to a polymer and (2) modification of the polymer backbone to produce an activated group. The activation processes are generally designed to generate electrophilic groups on the support in the coupling step, which react with the strong nucleophiles on the proteins.

The basic principles controlling the course of covalent coupling to the matrices are analogous to those used for the chemical modification of proteins. There are many commercially available supports for immobilization, the best choice in each case requires the consideration of some relevant properties of the catalyst and the intended use. However, it is usually necessary to try more than one approach and then adapt a method to the specific circumstances [55] .

Entrapment

The entrapment method is based on the occlusion of an enzyme within a polymeric network that allows the substrate and products to pass through but retains the enzyme. This method differs from the coupling methods described above, in that the enzyme is not bound to the matrix or membrane. There are different approaches to entrapping enzymes such as gel or fiber entrapping and micro-encapsulation. The practical use of these methods is limited by mass transfer limitations through membranes or gels.

Methods of Reversible Immobilization Because of the type of the enzyme-support binding, reversibly immobilized enzymes can be detached from the support under gentle conditions. The use of reversible methods for enzyme immobilization is highly attractive, mostly for economic reasons because when the enzymatic activity decays the support can be regenerated and re-loaded with fresh enzyme. Indeed, the cost of the support is often a primary factor in the overall cost of immobilized catalyst. The reversible immobilization of enzymes is particularly important for immobilizing labile enzymes and for applications in bio-analytical systems [53]

Adsorption

Nonspecific adsorption

The simplest immobilization method is nonspecific adsorption, which is mainly based on physical adsorption or ionic binding [56]. In physical adsorption the enzymes are attached to the matrix through hydrogen bonding, van der Waals forces, or hydrophobic interactions; whereas in ionic bonding the enzymes are bound through salt linkages. The nature of the forces involved in non-covalent immobilization results in a process can be reversed by changing the conditions that influence the strength of the interaction. Immobilization by adsorption is a mild, easy to perform process, and usually preserves the catalytic activity of the enzyme. Such methods are therefore economically attractive, but may suffer from problems such as enzyme leakage from matrix when the interactions are relatively weak.

Ionic binding

An obvious approach for the reversible immobilization of enzymes is to base the protein–ligand interactions on principles used in chromatography. For example, one of the first applications of chromatographic principles in the reversible immobilization of enzymes was the use of ion-exchangers [56]. The method is simple and reversible but, in general, it is difficult to find conditions under which the enzyme remains both strongly bound and fully active. More recently, the use of immobilized polymeric-ionic ligands has allowed for modulation of protein– matrix interactions and has thus optimized the properties of the derivative.

Hydrophobic adsorption

Hydrophobic interactions do not the formation of chemical bonds but rather an entropically driven interaction that takes place. Hydrophobic adsorption has been used as a chromatographic principle for more than three decades. It relies on well-known experimental variables such as pH, salt concentration, and temperature [57]. The strength of interaction relies on both the hydrophobicity of the adsorbent and the protein. The hydrophobicity of the adsorbent can be regulated by the degree of substitution of the support and by the size of the hydrophobic ligand molecule. The successful reversible immobilization of β-amylase and amyloglucosidase to hexyl-

agarose carriers has been reported [58]. Several other examples of strong reversible binding to hydrophobic adsorbents have also been reported [59].

Affinity binding

The principle of affinity between complementary biomolecules has been applied to enzyme immobilization. The remarkable selectivity of the interaction is a major benefit of the method. However, the procedure often requires the covalent binding of a costly affinity ligand to the matrix [60].

Type of supporting matrices

The characteristics of supporting matrices are of paramount importance in determining the performance of the enzyme immobilization. Ideal the supporting matrices properties including physical resistance to compression, hydorphilicity, inertness toward enzymes ease of derivatization, biocompatibility, resistance to microbial attack, and availability at low cost. The supporting matrices can be divided as inorganic and organic.

The organic supporting matrices can be subdivided into natural and synthetic polymers [61]. The physical characteristics of the supporting matrices (such as mean particle diameter, swelling behavior, mechanical strength, and compression behavior) will be of major importance for the performance of the immobilized systems and will determine the type of reactor used under technical conditions. In particular, pore parameters and particle size determine the total surface area and thus critically affect the capacity for binding of enzymes. Nonporous supporting matrices show few diffusional limitations but have a low loading capacity. Therefore, porous supporting matrices are generally preferred because the high surface area allows a higher enzyme loading and the immobilized enzyme receives greater protection from the environment. Porous supporting matrices should have a controlled pore distribution in order to optimize capacity and flow properties.

In spite of many advantages of inorganic carriers (e.g., high stability against physical, chemical, and microbial degradation), most of the industrial applications are performed with organic matrices. The hydrophilic character is one of the most important factors determining the level of activity of an immobilized enzyme [62]. In

addition to its high porosity, this leads to a high capacity for proteins. The enzymes can be attached to the supporting matrices via interactions ranging from reversible physical adsorption and ionic linkages to stable covalent bonds.

Several researchers have studied the various supporting matrices to improve the efficiency of immobilization processes. The organic and inorganic supporting matrices such as chitin, chitosan, agarose, alginate, epoxy supporter and other natural polymer have a potential for used as supporting matrices.

Chitosan

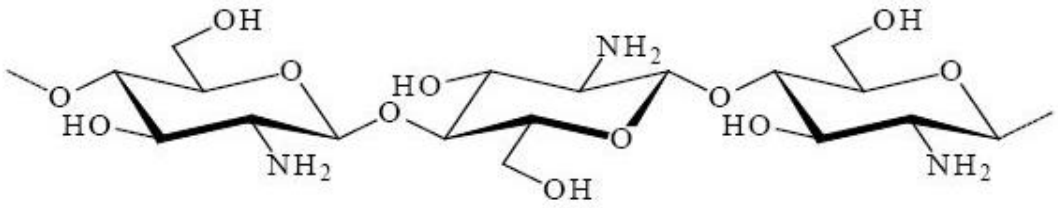
Chitosan is the *N*-deacetylated derivative of chitin, although this *N*-deacetylation is almost never complete, the structure of chitin and chitosan as shown in Figure 1.4. A sharp nomenclature with respect to the degree of *N*-deacetylation has not been defined between chitin and chitosan [63] and [64]. Chitosan, the deacetylated product of chitin, is soluble in dilute acids such as acetic acid, formic acid, etc. Recently, the gel forming ability of chitosan in *N*-methylmor pholine *N*-oxide and its application in controlled release formulations has been reported [65], [66] and [67].

Apocella [68] and Ida [69] have been studied simple coacervation of chitosan in the product ion of chitosan beads. By using a compressed air nozzle, chitosan solution is blown into NaOH or NaOH–methanol solution to form coacervate drops. Varying the exclusion rate of the chitosan solution or the nozzle diameter can control the diameter of the droplets. The porosity and strength of the beads correspond to the concentration of the chitosan acid solution, the degree of *N*-deacetylation of chitosan, and the type and concentration of coacervation agents used. The chitosan beads described above have been applied in various fields such as, enzymatic immobilization, chromatographic support, adsorbent of metal ions, or lipoprotein and cell cultures. It was confirmed that the porous surfaces of the chitosan beads form a good cell culture carrier. Hayashi and Ikada [70] immobilized protease onto porous chitosan beads with a spacer and found that the immobilized protease had higher pH, and thermal storage stability, and exhibited higher activity towards the small ester substrate *N*-benzyl-L-arginine ethyl ester. In addition, Chandy and Sharma [71] investigated the possibilities of using chitosan beads as a carrier for the cancer chemotherapeutic adriamycin. Recently, Chandy and Sharma [72] prepared chitosan

micro-beads for oral sustained delivery of nefedipine, Ampicillin and various steroids by adding these drugs to chitosan and then entering a simple coacervation process. These coacervate beads can be hardened by crosslinking with glutaraldehyde or epoxychloropropane to produce microcapsules containing rotundine. The release profiles of the drugs from all these chitosan delivery systems were monitored and showed, in general, higher release rates at pH 1–2 than at pH 7.2–7.4. The effect of the amount of drug loaded, the molecular weight of chitosan and the crosslinking agent on the drug-delivery profiles have been reported.

Recently, a chitosan hydrogel was investigated for supporting matrix. Chitosan hydrogel is highly swollen, hydrophilic polymer networks that can absorb large amounts of water and drastically increase in volume. It is well known that the physicochemical properties of the hydrogel depend not only on the molecular structure, the gel structure, and the degree of crosslinking, but also on the content and state of the water in the hydrogel. Hydrogels have been widely used in controlled-release systems [73] and [74]. Recently, hydrogels which swell and contract in response to external pH [75] and [76] have been explored. The pH-sensitive hydrogels have potential use in site-specific delivery of drugs to specific regions of the gastrointestinal tract (GI) and have been prepared for low molecular weight and protein drug delivery [77]. It is known that the release of drugs from hydrogels depends on their structure or their chemical properties in response to pH [78]. These polymers, in certain cases, are expected to reside in the body for a longer period and respond to local environmental stimuli to modulate drug release [79]. Sometimes the polymers used are biodegradable to obtain a desirable device to control drug release [68]. Thus, to be able to design hydrogels for a particular application, it is important to know the nature of the systems in their environmental conditions. Some recent advances in controlled-release formulations using gels of chitin and chitosan are presented here.

(A)



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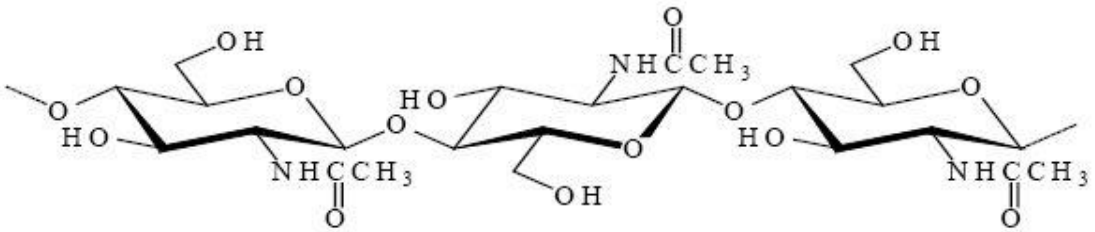


(C)

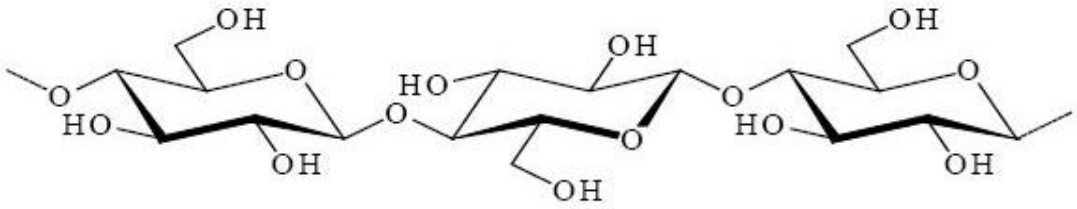


Figure 1.4 Chemical structures of (A): chitosan, (B): chitin and (C): cellulose

Epoxy supporting matrices

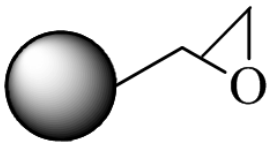
In present, the commercial epoxy supporting matrices have been interested to be used for immobilization of various enzymes. The commercial epoxy supporting matrices Sepabeads EC-EP and Sepabeads EC-HFA are composed of rigid methacrylic polymer matrix with different porosity degree, as shown in Figure 1.5. The enzyme can be immobilized through several methods, such as interaction with the carrier and chemical binding by which the enzyme is adsorbed on the carrier under the suitable conditions via interaction with the functional groups distributed on the carrier surface. The interaction is reversible and the enzyme can be de-adsorbed changing the condition. In some cases the adsorption can be quite strong.

Chemical binding as a reversible immobilization can become irreversible by means of a cross-linking step: the enzyme is adsorbed onto the carrier and then crosslinked by using, for example, glutaraldehyde. The crosslinked enzyme molecules cover the carrier link a net. The alternate method to obtain an irreversible immobilization is the covalent binding between the enzyme and the functional groups of the carrier. This immobilization can be performed using carriers with oxyrane groups or with an amino functional group. In case of amino groups, a pre-activation step with a bifunctional coupling agent, such as Glutaraldehyde can be performed.

Torres reported the immobilization of β-galactosidase from *A. oryzae* on Epoxy Sepabeads EC-EP and HFA. These supporting matrices have a layer of epoxy groups over a layer of ethylenediamine that is covalently bound to the support. For these supporting matrices, immobilization may be performed at moderately low ionic strength, which occurs very rapidly, and it is not necessary to use a hydrophobic support [80].

Sepabeads-EP has been utilized to immobilize-stabilize the enzyme penicillin G acylase (PGA) from *E. coli* via multipoint covalent attachment. Sepabeads supports have been shown to be able to give very high levels of stabilization when properly used.

(A)



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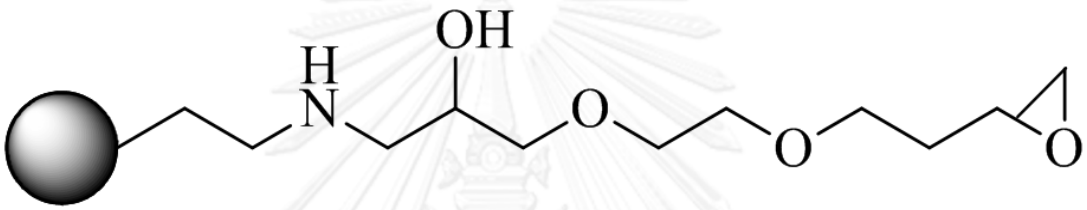


Figure 1.5 Chemical structures of epoxide beads

(A): Sepabeads EC-EP (epoxy)

(B): Sepabeads EC-HFA (amino-epoxy)

Previously, various immobilized enzymes have been used for oligosaccharides production. The production of galacto-oligosaccharides by immobilized *β*-galactosidase from *Bullera singularis* on chitosan beads was reported [81]. Galacto-oligosaccharides (GalOS) were continuously produced using lactose and immobilized *β*-galactosidase from *Bullera singularis* ATCC 24193 in a packed bed reactor. 55% (w/w) oligosaccharides were obtained continuously with a productivity of 4.4 g/L.h from 100 g/L lactose solution during a 15 day operation. Batch productivity was 6.5 g GalOS/L.h from 300 g/L of lactose.

Yun [82] reported for inulo-oligosaccharides produced by immobilized endoinulinase from *Pseudomonas sp.* on anion exchange resin. Continuous production of inulo-oligosaccharides from pure inulin was conducted using an immobilized endoinulinase reactor. The optimal operating conditions of the reactor for maximizing the productivity were as follows: 50 g/L of inulin feed concentration, flow rate as superficial space velocity 1.1 g/h, and temperature of 55 °C. The enzyme reactor was run for 15 day at 55 °C achieving an oligosaccharide yield of 83% without any significant loss of initial enzyme activity, during which the volumetric productivity was 55 g/L.h and half-life of the immobilized enzyme indicated 35 d.

Mannanase was immobilized on chitin with glutaraldehyde by cross-linking reaction. The immobilization conditions and the characterization of immobilized enzyme were carried out. The immobilization yield and the mannanase activity recovery were 94.81% and 72.17%, respectively. The optimal mannanase activity shifted to lower pH after immobilization. The main hydrolysis products yielded from locust bean gum were mannotriose and mannotetraose. The resulting manno-oligosaccharides could be used as a special nutrient for lactic bacteria. Manno-oligosaccharides and immobilized mannanase form *Penicillium occitanis* on chitin [83].

The immobilization of Ls using different supporting matrix for levan production have been previously reported. The Ls from *Bacillus subtilis* NRC33a was immobilized on different carriers using different immobilisation methods including physical adsorption, covalent binding, ionic binding and entrapment [84]. The immobilized enzyme prepared by covalent binding on chitosan through 3% glutaraldehyde had the highest immobilization yield (81.51%). The immobilized Ls on chitosan bead showed

a slight increase in activity compared with the free Ls above 35 °C. The thermal stability of the immobilized Ls on chitosan bead was significantly improved in comparison to the free Ls. The deactivation energy of the immobilized Ls on chitosan bead was lower than that of the free Ls. The half-life of the immobilized Ls on chitosan bead was retained 51.13% after 14 repeated use.

The Ls from *Bacillus circulans* was immobilized on wool adopting the physical adsorption technique. The results revealed that the immobilized *B. circulans* Ls on wool showed resistance against thermal and chemical denaturation, increased tolerance to pH in a wide range and could be used in the production of L-FOS and levan. This provides a good efficiency for frucosylation to synthesized fructan polymers from sucrose [85].

Design and operation of immobilized enzyme reactor

Several important factors have been determined the choice of reactor for a particular process. In general, the choice depends on the cost of a predetermined productivity within the product specifications. This must be inclusive of the costs associated with substrate, downstream processing, labor, depreciation, overheads and process development, in addition to the more obvious costs concerned with building and running the reactor. Types of reactor will be described this following, and as shown in Figure 1.6.

Batch reactor

Batch reactors generally consist of a tank containing a stirrer (stirred tank reactor, STR). The tank is normally fitted with fixed baffles that improve the stirring efficiency. A batch reactor is one in which the entire product is removed, as rapidly as is practically possible, after a fixed time. Generally this means that the enzyme and substrate molecules have identical residence times within the reactor, although in some circumstances there may be a need for further additions of enzyme and/or substrate.

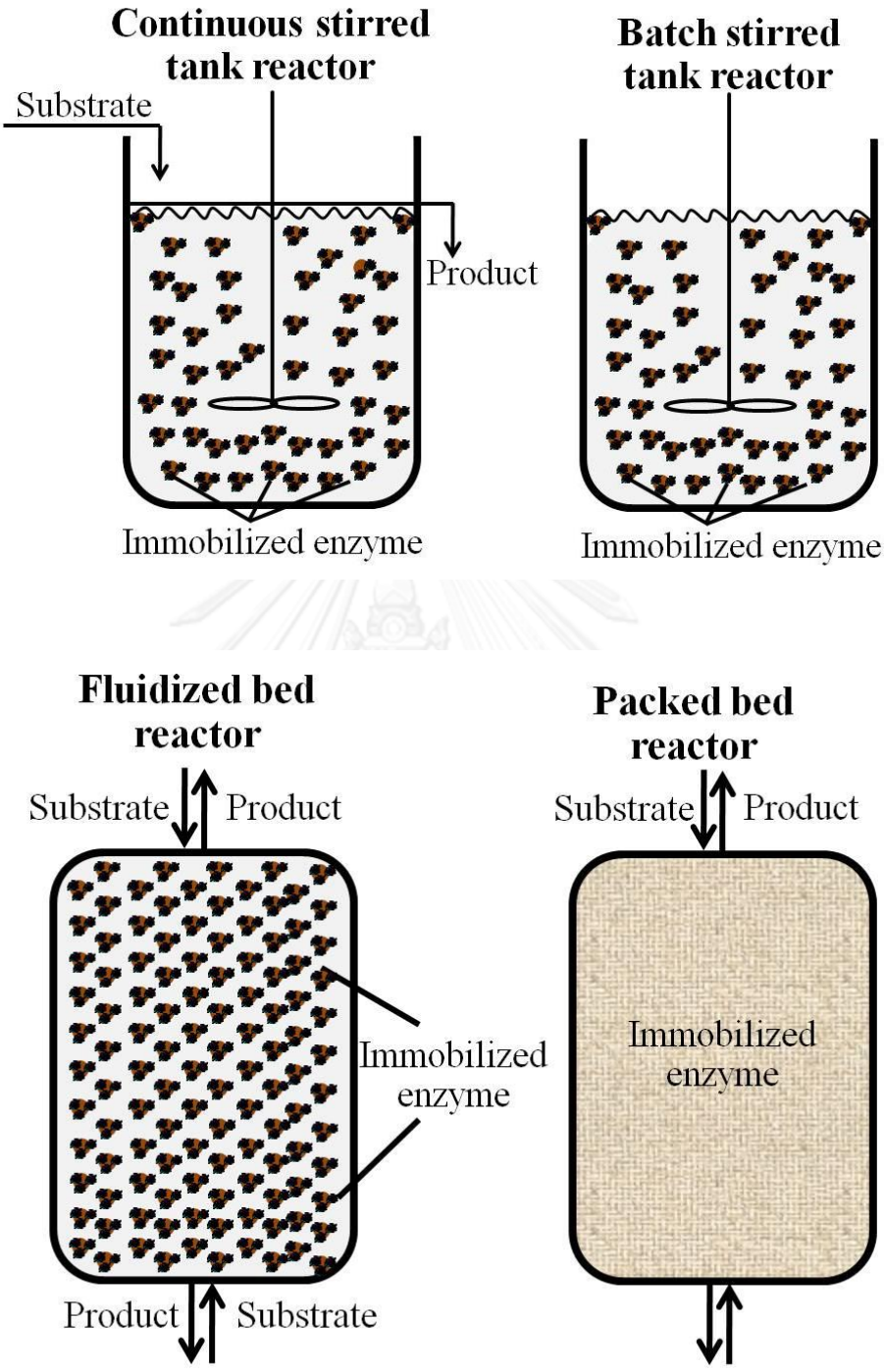


Figure 1.6 Type of reactors

The operating costs of batch reactors are higher than for continuous processes due to the necessity for the reactors to be emptied and refilled both regularly and often. This procedure is not only expensive in itself but means that there are considerable periods when such reactors are not productive; it also makes uneven demands on both labor and services. Batch reactors also suffer from pronounced batch to batch variations, as the reaction conditions change with time, and may be difficult to scale up, due to the changing power requirements for efficient fixing. They do, however, have a number of advantageous features. Primary amongst these is their simplicity both in use and in process development. For this reason they are preferred for small scale production of highly priced products, especially where the same equipment is to be used for a number of different conversions. They offer a closely controllable environment that is useful for slow reactions, where the composition may be accurately monitored, and conditions (e.g. temperature, pH, coenzyme concentrations) varied throughout the reaction. They are also of use when continuous operation of a process proves to be difficult due to the viscous or intractable nature of the reaction mix.

Continuous stirred tank reactor (CSTR)

This reactor consists of a well stirred tank containing the enzyme, which is normally immobilized. The substrate stream is continuously pumped into the reactor at the same time as the product stream is removed. If the reactor is behaving in an ideal manner, there is total back-mixing and the product stream is identical with the liquid phase within the reactor and invariant with respect to time. Some molecules of substrate may be removed rapidly from the reactor, whereas others may remain for substantial periods.

The CSTR is an easily constructed, versatile and cheap reactor, which allows simple catalyst charging and replacement. It’s well mixed nature permits straightforward control over the temperature and pH of the reaction and the supply or removal of gases. CSTRs tend to be rather large as the: need to be efficiently mixed. Their volumes are usually about five to ten time the volume of the contained immobilized enzyme. This, however, has the advantage that there is very little resistance to the flow of the substrate stream, which may contain colloidal or insoluble substrates, so long as the insoluble particles are not able to sweep the immobilized

enzyme from the reactor. The mechanical nature of the stirring limits the supports for the immobilized enzymes to materials which do not easily disintegrate to give fines which may enter the product stream. However, fairly small particle may be used, if they are sufficiently dense to stay within the reactor. This minimizes problems due to diffusional resistance.

Packed bed reactor (PBR)

The most important characteristics of a PBR is that material flows through the reactor as a plug; they are also called plug flow reactors (PFR). Ideally, all of the substrate stream flows at the same velocity, parallel to the reactor axis with no back mixing. All material present at any given reactor cross section has had an identical residence time. The longitudinal position within the PBR is, therefore, proportional to the time spent within the reactor; all product emerging with the same residence time and all substrate molecule having an equal opportunity for reaction. The conversion efficiency of a PBR, with respect to its length, behaves in a manner similar to that of a well stirred batch reactor with respect to its reaction time. Each volume element behaves as a batch reactor as it passes through the PBR. Any required degree of reaction may be achieved by use of an idea PBR of suitable length.

In order to produce ideal plug flow within PBRs, a turbulent flow regime is preferred to laminar flow, as this causes improved mixing and heat transfer normal to the flow and reduced axial back-mixing. Consequent upon the plug flow characteristic of the PBR is that the substrate concentration is maximized, and the product concentration minimized, relative to the final conversion at every point within the reactor; the effectiveness factor being high on entry to the reactor and low close to the exit. This means that PBRs are the preferred reactors, all other factors being equal, for processes involving product inhibition, substrate activation and reaction reversibility. In general, PBRs are used with fairly rigid immobilized enzyme catalysts, because excessive increases in this flow rate may distort compressible or physically weak particles. Particle deformation results in reduced catalytic surface area of particles contacting the substrate-containing solution, poor external mass transfer characteristics and a restriction to the flow, causing increased pressure drop. A vicious circle of

increased back-pressure, particle deformation and restricted flow may eventually result in no flow at all through the PBR.

PBRs behave as deep bed filters with respect to the substrate stream. It is necessary to use a guard bed if plugging of the reactor by small particles is more rapid than the biocatalysts deactivation. They are also easily fouled by colloidal or precipitating material. The design of PBRs does not allow for control of pH, by addition of acids or bases, or for easy temperature control where there is excessive heat output, a problem that may be particularly noticeable in wide reactors (>15 cm diameter).

Fluidized bed reactor (FBR)

These reactors generally behave in a manner intermediate between CSTRs and PBRs. They consist of a bed of immobilized enzyme which is fluidized by the rapid upwards flow of the substrate stream alone or in combination with a gas or secondary liquid stream, either of which may be inert or contain material relevant to the reaction. A gas stream is usually preferred as it does not dilute the product stream. There is a minimum fluidization velocity needed to achieve bed expansion, which depends upon the size, shape, porosity and density of the particles and the density and viscosity of the liquid. This minimum fluidization velocity is generally fairly low as most immobilized enzyme particles have densities close to that of the bulk liquid. In this case the relative bed expansion is proportional to the superficial gas velocity and inversely proportional to the square root of the reactor diameter. Fluidizing the bed requires a large power input but, once fluidized, there is little further energetic input needed to increase the flow rate of the substrate stream through the reactor. At high flow rates and low reactor diameters almost ideal plug flow characteristics may be achieved. However, the kinetic performance of the FBR normally lies between that of the PBR and the CSTR, as the small fluid linear velocities allowed by most biocatalytic particles causes a degree of back mixing that is often substantial, although never total. The actual design of the FBR will determine whether it behaves in a manner that is closer to that of a PBR or CSTR. It can, for example, be made to behave in a manner very similar to that of a PBR, if it is baffled in such a way that substantial back-mixing is avoided. FBRs are chosen when these intermediate characteristics are required, e.g. where a high conversion is needed but the substrate stream is colloidal or the reaction produces a substantial pH change or

heat output. They are particularly useful if the reaction involves the utilization or release of gaseous material.

The FBR is normally used with fairly small immobilized enzyme particles in order to achieve a high catalytic surface area. These particles must be sufficiently dense, relative to the substrate stream that they are not swept out of the reactor. Less-dense particles must be somewhat larger. For efficient operation the particles should be of nearly uniform size otherwise a non-uniform bio-catalytic concentration gradient will be formed up the reactor. FBRs are usually tapered outwards at the exit to allow for a wide range of flow rates. Very high flow rates are avoided as they cause channelings and catalyst loss. The major disadvantage of development of FBR process is the difficulty in scaling-up these reactors. PBRs allow scale-up factors of greater than 50,000 but, because of the markedly different fluidization characteristics of different sized reactors, FBRs can only be scaled-up by a factor of 10 - 100 each time. In addition, changes in the flow rate of the substrate stream causes complex changes in the flow pattern within these reactors that may have consequent unexpected effects upon the conversion rate.

Our goals of immobilizing Ls were to enhance the stability of Ls, to reuse/recycle Ls, to increase production with high percent yield and to produce various molecular weight of L-FOS. Immobilization of Ls derivatives from *Bacillus licheniformis* RN-01 by covalent binding technique was studied. Covalent binding technique is the formation of covalent bonds between the enzyme of interest onto or into the supporting matrices. The LsRN derivatives were covalently bounded to the surface of selected supporting matrices. Covalent binding technique provides a strong binding force between levansucrase and its supporting matrices. By linking LsRN on the surface of the supporting matrices, limitations of substrate/product transfer into the supporting matrices can be eliminated. The immobilized LsRN was incorporated with a suitable system and/or process for L-FOS production.

The aims of this thesis

Our research group had successfully cloned Ls from *Bacillus licheniformis* RN-01 (LsRN), derivatives were generated, expressed and characterized. The LsRN derivatives were used for the production of various molecular weight L-FOS. Short chain L-FOSs (GF_2 – GF_5), medium chain L-FOSs (GF_2 – GF_{11}) and long chain L-FOSs (>GF_{10}) were produced by using LsRN-N251Y, Y246S and Y246W, respectively. In this work; we aim to 1) produce high activity of Ls derivatives from *Bacillus licheniformis* RN-01, 2) immobilize LsRN derivatives on a suitable supporting matrix, and 3) characterize the immobilized LsRN derivatives. Systems and/or processes and optimum reaction conditions for the L-FOS production were established.

CHAPTER II

MATERIALS AND METHODS

Equipments

Autoclave: Model MLS-3020, Sanyo, Japan

Autoclave: Model Hiclave HVP-50, Hirayama, Japan

Autopipette: Model Pipetman, Gilson, France

Autopipette: Model Pipet-Lite, Rainin, USA

Autopipette: Nichipet EX, Japan

Balance, 2 digit: Model PB602-S, Mettler Toledo, USA

Balance, 3 digit: Model PB303-S, Mettler Toledo, USA

Centrifuge, Bench top: Model 5804 R, Eppendorf, USA

Centrifuge, High speed centrifuge: Model Avanti J-30I, Beckman Coulter, USA

Centrifuge, High speed centrifuge: Model Sorvall Legend XTR, Thermo Fisher Scientific, Germany

Cold cabinet: Model SBC-2DA, Sanyo, Thailand

Cold cabinet: Thermo Electron Corporation, USA

Electrophoresis unit: Model Mini protein, Bio-Rad, USA

Fraction collector: Model Frac-920, Amersham Biosciences, United Kingdom

Freezer, -20 ^{o}C: Songserm Intercool, Thailand

Freezer, -80 ^{o}C: Model C660 Premium, New Brunswick Scientific, England

Freezer, -80 ^{o}C: Model ULT Freezer, Thermo Electron Corporation, USA

Gene Pulser: Model Micro Pulser, Bio-Rad, USA

Hot air oven: Memmert, Germany

Hot air oven: Model Designer series, Contherm, Australia

Hot plate and Stirrer: Model C-MAG HS 7, IKA, Germany

Hot plate: Ego, Germany

HPAEC-PAD: Model ICS-5000, Dionex, and CarboPac PA-1 column

HPLC: Model, Shimadazu, Japan and SugarPak column, Water, USA

Incubator: Model Economy Incubator Size 2, Gallenkamp, Germany

Incubator shaker: Model Innova 4000, New Brunswick Scientific, USA

Incubator shaker: Model Innova 4080, New Brunswick Scientific, USA

Incubator shaker: Model KS 4000 I control, IKA, Germany

Incubator shaker: Model Lab-Therm, Kuhner, Switzerland

Laminar Flow: Model ISSCO, International Scientific Supply, Thailand

Magnetic stirrer and heater: Model C-MAG HS 7, IKA, Germany

Overhead Stirrer: Model RW20 digital, IKA, Germany

Peristaltic pump: Model Perista pump AC-2110, ATTO, Japan

pH meter: Model SevanEasy, Mettler Toledo, USA

Power supply: Model EC135-90, E-C Apparatus Corporation, USA

Scanning Electron Microscope: JEOL: Model JSM-5800LV, Japan

Shaker, See-Saw Rocker: Model SSL4, Stuart, United Kingdom

Spectrophotometer: Model 6400, Jenway, England

Spectrophotometer: Model DU 530, Beckman Coulter, USA

Spectrophotometer: Model DU 640, Beckman Coulter, USA

Ultrasonic bath: Model Transsonic T890/H, Elma, Germany

Vacuum pump: Edwards, USA

Vortex mixture: Model Topmix FB15024, Fisher Scientific UK, United Kingdom

Water bath: Heto Birkerod, Denmark

Water bath: Model M20S, Lauda, Germany

Material and Chemical reagents

2-mercaptoethanol, Fluka, Switzerland

3,5-Dinitrosalicylic acid, SRL, India

Acetic acid glacial, Carlo Erba, Italy

Acetone, J.T. Baker, USA

Acrylamide/bis solution 40%, Bio-Rad, USA

Agar, Labchem, Australia

Ammonium persulfate, Loba Chemie, India

Ammonium sulfate, Carlo Erba, Italy

Ampicillin sodium salt, Bio Basic Canada Inc., Canada

Biogel -2, Bio-Rad, USA

Boric acid, Univar, Australia

Bovine serum albumin (BSA), Sigma, USA

Bromophenol blue, Bio-Rad, USA

Butanol, Carlo Erba, Italy

Calcium chloride, Loba Chemie, India

Citric acid, Univar, Australia

Cobalt (II) chloride 6-hydrate, BDH, England

Coomassie brilliant blue G 250, Fluka, Switzerland

Coomassie brilliant blue R 250, Bio-Rad, USA

Copper (II) chloride, Univar, Australia

DEAE cellulose, Sigma, USA

EDTA-4-sodium, Qrec, New Zealand

Ethanol 95%, Carlo Erba, Italy

Ethanol absolute, Carlo Erba, Italy

Fructose, Sigma, USA

Glucose, Carlo Erba, Italy

Glucose oxidase assay kit, Wako, Japan

Glutaraldehyde (25%), Merck, Germany

Glycerol, Univar, Australia

Glycine, Fisher Scientific UK, United Kingdom

Hydrochloric acid 37%, Carlo Erba, Italy

Iron chloride, Unilab, Australia

Magnesium chloride 6-hydrate, Merck, Germany

Magnesium sulfate 7-hydrate, Carlo Erba, Italy

Manganese (II) chloride 4-hydrate, Loba Chemie, India

Methanol, Carlo Erba, Italy

Phenol crystal, Qrec, New Zealand

Phenyl sepharose, Sigma, USA

Phosphoric acid, Carlo Erba, Italy

Potassium chloride, Rankem, India

Potassium sodium tartrate, Univar, Australia

Protein molecular marker, Thermo Scientific, USA

Sepabead EC-EP, Mitsubishi Chemical Corporation, Italy

Sepabead EC-HFA, Mitsubishi Chemical Corporation, Italy

Sephadex G-10, GE Healthcare, Sweden

Sephadex G-25, GE Healthcare, Sweden

Sodium acetate, Carlo Erba, Italy

Sodium azide, Carlo Erba, Italy

Sodium chloride, Univar, Australia

Sodium dodecyl sulfate, Sigma, USA

Sodium hydroxide, Univar, Australia

Sucrose, Merck, Germany

Sucrose commercial, Mitrphol, Thailand

Sulfuric acid, Carlo Erba, Italy

TEMED, Bio-Rad, USA

TLC plate, Merck, Germany

Trichloroacetic acid, Carlo Erba, Italy

Trisodium citrate 2-hydrate, Univar, Australia

Tris, Research Organic, USA

Trisodium citrate 2-hydrate, Carlo Erba Reagenti, Italy

Tryptone, Scharlau, Spain

Yeast extract, Bio Basic Canada Inc., Canada

Zinc sulfate 7-hydrate, Merck, Germany

Host cells

E. coli Top 10 (Invitrogen) with genotype *F-, mcrA, Δ(mrr-hsdRMS-mcrBC), φ80lacZΔM15, ΔlacX74, nupG, recA1, araD139, Δ(ara-leu)7697, galE15, galK16, rpsL(Str^R), endAl, λ$^-$* was used as an expression host cell.

Vector

Plasmid pBluscriptSK(-) (Stratagene) *pls*RN01 containing a levansucrase gene (*ls*RN) of *B. licheniformis* RN-01 (GenBank accession no. FJ171619.1) under the regulation of its putative endogenous promoter was used for levansucrase production.

Media preparation

Luria-Bertani (LB) medium

LB medium consisted of 1% (w/v) tryptone, 0.5% (w/v) yeast extract and 0.5% (w/v) NaCl, pH was adjusted to 7.2. For LB agar medium, 1.5% (w/v) agar was added. Medium was sterilized by autoclaving at temperature of 121 ^oC, pressure 15 lb/in.^{2} for 15 min.

Methodology and Research Plan

Production of recombinant LsRN derivatives in *E.coli*

E. coli Top-10 was used as an expression host and *pls*RN01 containing levansucrase gene (*ls*RN) 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. The derivatives of *ls*RN used in this research were N251Y, Y246S and Y246W.

Competent cells preparation

Competent *E. coli* Top 10 was prepared according to the method demonstrated by Sambrook [86]. An *E. coli* Top 10 single colony was cultured in 3 mL of LB broth medium for used as a starter, incubated at temperature of 37 ^{o}C with shaking at 250 rpm for 16 h. One percent (v/v) of starter was inoculated into 100 mL of LB broth medium, incubated at temperature of 37 ^{o}C with shaking at 250 rpm for 2-3 h until the optical density at 600 nm of culture medium reached 0.4-0.6. The cells were chilled on ice for 15 min and harvested by centrifugation at 3,000 xg at temperature of 4 ^{o}C for 10 min. The cell pellets was washed with a volume of cultured LB broth medium (100 mL) of cold steriled water, resuspended by gently mixing and centrifugated at 3,000 xg at temperature of 4 ^{o}C for 10 min. The supernatant was discarded. The cell pellets was washed again with a 0.5 volume of cultured LB broth medium (50 mL) of cold steriled water, resuspended by gently mixing and centrifugated at 3,000 xg at temperature of 4 ^{o}C for 10 min. The supernatant was discarded. The cell pellets was washed again with a 0.5 volume of cultured LB broth medium (50 mL) of cold steriled 10% (v/v) glycerol, resuspended by gently mixing and centrifugated at 3,000 xg at temperature of 4 ^{o}C for 10 min. The supernatant of glycerol was discarded. The finally, the cell pellets were resuspended in 160 μL of cold steriled 10% (v/v) glycerol. This cell suspension was collected into 40 μL aliquots and stored at -80 ^{o}C until used.

Electrotransformation

The competent *E. coli* Top 10 was mixed with 1 μL of DNA in electroporated cuvette, mixed well and placed on ice. The mixture was electroporated in electroporated cuvette with apparatus setting of the Gene pulser at 25 μF, 200 Ω of the Pulse controller unit and 2.5 kV. After pulsed, the cells were immediately resuspended with 960 μL of LB broth medium and transfer to steriled tube. The suspension cells were incubated at temperature of 37 ^{o}C, 250 rpm of shaking for 45 min. The 100 μL of incubated cell suspension was spread on the LB agar medium containing with 100 μg/mL of Ampicillin, incubated at temperature of 37 ^{o}C for 16 h.

Effect of concentration of LB broth medium on levansucrase activity

E. coli Top-10 harboring plsRN01 was cultured in various culture media, LB broth medium, 2X LB, 3X LB, 4X LB and 5X LB medium at temperature of 37 ^{o}C, 250 rpm. The supernatant was taken at interval time points for 5 d for detect the levansucrase activity. After cultivation, the supernatant was harvested by centrifugation at 8,000 xg, 4 ^{o}C for 10 min and discarded the cell pellets.

Purification of recombinant LsRN derivatives in *E.coli*

Crude recombinant levansucrase derivatives were purified by column chromatography. Crude recombinant levansucrase was dialyzed with 50 mM sodium acetate buffer, pH 6.0 for reduced NaCl concentration.

DEAE-cellulose chromatography

DEAE-cellulose was packed into column 3 x 15 cm. DEAE-cellulose column was equilibrated with 50 mM sodium acetate buffer, pH 6.0 at a flow rate of 45 mL/h. The crude recombinant levansucrase was applied to DEAE-cellulose column in equilibrated buffer at a flow rate of 45 mL/h. The column was eluted with 50 mM sodium acetate buffer, pH 6.0, until A_{280} value was negligible. After, column was eluted using stepwise gradient elution with of 50 mM sodium acetate buffer, pH 6.0 containing 0.1-0.5 M NaCl. The fractions of 3 mL were collected for measurement of levansucrase

activity and protein concentration. The levansucrase containing fractions were pooled and dialyzed to remove NaCl in 50 mM sodium acetate buffer, pH 6.0.

Phenyl-sepharose chromatography

Phenyl-sepharose was packed into column 2 x 10 cm. Phenyl-sepharose column was equilibrated with 25 mM sodium acetate buffer, pH 6.0 containing 1 M of $(NH_4)_2SO_4$ at flow rate 45 mL/h. The partially purified levansucrase was further applied onto Phenyl Sepharose in equilibrated buffer. The column was eluted with equilibrated buffer, until A_{280} value was negligible. Then column was eluted using a reversed salt gradient from 1.0 to 0 M $(NH_4)_2SO_4$. The fractions of 3 mL were collected for measurement of levansucrase activity and protein concentration. Levansucrase containing fractions were pooled and dialyzed to remove NaCl in 50 mM sodium acetate buffer, pH 6.0. The purified levansucrase was stored at 4 °C and used for the following experiments.

Levansucrase assay

Determination of LsRN activity by measuring reducing sugar

The activity of LsRN was measured by quantifying the reducing ability of free glucose liberated from the reaction by the modified DNS method [87]. LsRN assay was carried out in 0.5 mL reaction volume. The reaction mixture contains enzyme (5-10 μL in a typical reaction), 1.6% (w/v) sucrose and 50 mM citrate buffer, pH 6.0. The reaction was incubated at 40 ^oC for 5 min. After incubation, 0.5 mL of DNS reagent was added then heated to 100 ^oC for 15 min. Four milliliters of distilled water was added, the optical density (A_1) of the reaction mixture was measured at absorbance of 540 nm. A blank value (A_0) was obtained by using heat inactivated enzyme. The amount of reducing sugar liberated from the reaction was determined from A_1-A_o using a standard calibration curve generated, using glucose.

One unit of LsRN was defined as the amount of levansucrase producing 1 μmol of reducing sugars equivalent of glucose per minute.

Determination of protein concentration

Protein concentration was determined by dye binding method [88], using a bovine serum albumin (BSA) as a standard protein. A 1.0 mL of reaction volume containing with sample (5-20 μL in a typical reaction) and DI water was mixed with 0.2 mL of Bradford's reagent working solution (5x). The reaction was incubated at RT for 30 min and measured at absorbance of 595 nm. The amount of protein was determined using a standard calibration curve generated by using BSA as the protein.

Concentrating of protein

Proteins were precipitated by 10% (w/v) TCA. Ten percent of TCA were added into protein solution, mixed well and incubated in freezer for 15 min. After that, the protein pellet was collected by centrifugation at 10,000 xg, 4 ^{o}C for 5 min. A supernatant was removed and washed the protein pellet with ice-cold acetone for 3-5 times. The protein pellet was dried with air and resuspended in 50 mM sodium citrate buffer, pH 6.0.

Estimation of molecular weight

The molecular weight of LsRN was estimated by SDS-PAGE [89]. The SDS-PAGE was prepared using a 10% of acrylamide/bis. Samples were mixed with sample loading dye and denatured by heating at temperature of 100 ^{o}C for 5 min. The electrophoresis was performed at constant current of 20 mA per slab gel. Pre-stained standard protein was used as protein marker. After electrophoresis, proteins were stained with 0.25% Coomassie Brilliant Blue R-250 for 1-2 h and then de-staining with a mixture of 10% (v/v) acetic acid and 25% (v/v) of methanol.

Characterization of levansucrase

Optimum pH of LsRN activity

The optimum pH of LsRN was determined using 10 mM Britton-Robinson Universal buffer at pH 3-12. Two hundred milliunits of LsRN were incubated in 1.6% (w/v) sucrose, at 40 ^{o}C for 5 min. The activity of LsRN was measured by modified DNS method.

Optimum temperature of LsRN activity

The optimum temperature of LsRN was determined using temperature range of 20-80 ^{o}C. Two hundred milliunits of LsRN were incubated in 1.6% (w/v) sucrose in 50 mM citrate buffer, pH 6.0 for 5 min. The activity of LsRN was measured by modified DNS method.

Effect of metal ions and chemical reagents on the activity of LsRN

The effect of metal ions and chemical reagents on the activity of LsRN was investigated for some metal ions and chemical reagents**;** Na^{+}, K^{+}, Co^{2+}, Ca^{2+}, Mn^{2+}, Cu^{2+}, Zn^{2+}, Fe^{3+}, EDTA and SDS. Two hundred milliunits of LsRN were incubated in 1.6% (w/v) sucrose in 50 mM citrate buffer, pH 6.0 supplemented with 5 mM of each of the metal ions or chemical reagents, at 40 ^{o}C for 5 min. The activity of LsRN was measured by modified DNS method.

Effect of ionic strength on the activity of LsRN

The effect of ionic strength on the activity of LsRN was investigated using 0-3 M NaCl. Two hundred milliunits of LsRN were incubated with 0-3 M NaCl in 1.6% (w/v) sucrose and 50 mM citrate buffer, pH 6.0, at 40 ^{o}C for 5 min. The activity of LsRN was measured by modified DNS method.

Effect of sucrose concentration on the activity of LsRN

The effect of sucrose concentration on the activity of LsRN was investigated using 10-70% (w/v) of sucrose. Two hundred milliunits of LsRN were incubated with 10-70% (w/v) sucrose in 50 mM citrate buffer, pH 6.0, at 40 ^{o}C for 5 min. The activity of LsRN was measured by modified DNS method.

LsRN stability

The stability of LsRN was investigated by pre-incubating the 200 mU of LsRN in 50 mM citrate buffer, pH 6.0 at 40 ^{o}C for 12 h. The residual activity of LsRN was assayed by modified DNS method.

Storage stability

The storage stability of LsRN was investigated by storing the purified LsRN in 50 mM sodium acetate buffer, pH 6.0 at 4 ^{o}C for 3 mo. The residual activity of LsRN was assayed by once a week with modified DNS method.

Selection of suitable immobilization supporting matrices and characterization of immobilized LsRN

In this research, covalently binding immobilization technique was investigated. Chitosan bead (CTS bead), partially deacetylated chitin/chitosan (PDCC), fine particle chitosan, epoxide sepabeads EC-EP and epoxide sepabeads EC-HFA were used as supporting matrices.

Supporting matrices

Chitosan bead (CTS bead)

Shrimp chitosan polymer (MW= 850,000 Da), 84%DD was prepared as a chitosan bead. The 2% (w/v) chitosan was dissolved in 1% (v/v) acetic acid. The 2% (w/v) CTS beads were prepared by dropping 2% (w/v) chitosan solution into 0.5 N NaOH, with stirring. The CTS beads were washed to remove NaOH with DI H_2O until neutral pH.

Partially deacetylated chitin/chitosan (PDCC)

Squid pen was prepared as a PDCC. The squid pen was reduced particle size by grinding with blender. The flake chitin was partially deacetylated by dissolving in 50% (w/v) NaOH solution for 6 h, with stirring. The PDCC was washed to remove NaOH with DI H_2O until neutral pH.

Fine particle chitosan

Shrimp chitosan polymer (MW= 850,000 Da), 84%DD was prepared as a fibrous chitosan. The 2% (w/v) chitosan was dissolved in 1% (v/v) acetic acid. The 2% (w/v) fibrous chitosan was prepared by mixing 2% (w/v) chitosan solution and 0.5 N NaOH, with stirring. The fibrous chitosan was washed to remove NaOH with DI H_2O until neutral pH.

Epoxide sepabeads EC-EP and sepabeads EC-HFA

Sepabead EC-EP and Sepabead EC-HFA were obtained from Resindion, Mitsubishi Chemical Corporation, Italy. The epoxide sepabeads EC-EP and epoxide sepabeads EC-HFA were incubated with 1 M NaOH at RT for 24 h on orbital rotator shaker to activate the epoxide group. Then wash with DI H_2O until neutral pH.

LsRN immobilization

CTS bead

Effect of glutaraldehyde concentration on cross-linking of LsRN derivative on CTS bead

The 0.5 g (wet weight) of CTS bead was used as supporting matrix in each of immobilization experiments. Glutaraldehyde (GTA) concentration was investigated using 0.1-5% (w/v) as a cross-linking reagent. Forty units, containing 250 μg protein of purified LsRN were incubated with chitosan beads then covalently linked with each of GTA concentration in 50 mM citrate buffer pH 6.0 at 4 ºC under static condition for 2 d. The immobilized LsRN on CTS beads were separated from the unbounded LsRN, then wash with ice cold 50 mM of citrate buffer, pH 6.0 for 3 times. The unbound enzyme and amount of protein remaining in the supernatant was assayed. The immobilized LsRN was stored at 4°C and used for the further experiments.

Partially deacetylated chitin/chitosan (PDCC)

The 0.3 mL of PDCC was used as supporting matrix in each immobilization experiments. GTA was used as a cross-linking reagent. Seven units, containing 50 μg protein of purified LsRN were incubated with PDCC then covalently linked with GTA in 50 mM citrate buffer pH 6.0 at 4 ºC under static condition for 2 d. The immobilized LsRN on PDCC was separated from the unbounded levansucrase, then wash with ice cold 50 mM of citrate buffer, pH 6.0 for 3 times. The unbound enzyme and amount of protein remaining in the supernatant was assayed. The immobilized LsRN was stored at 4 °C and used for the further experiments.

Fine particle chitosan

The 0.3 mL of fine particle chitosan was used as supporting matrix in each immobilization experiments. GTA was used as cross-linking reagent. Seven units, containing 50 μg protein of purified LsRN were incubated with fine particle chitosan then covalently linked with GTA in 50 mM citrate buffer pH 6.0 at 4 ^{o}C under static condition for 2 d. The immobilized LsRN on fine particle chitosan were separated from the unbounded LsRN, then wash with ice cold 50 mM of citrate buffer, pH 6.0 for 3 times. The unbound enzyme and amount of protein remaining in the supernatant was assayed. The immobilized LsRN was stored at 4 °C and used for the further experiments.

Epoxide sepabeads EC-EP and sepabeads EC-HFA

The 0.1 g of Sepabead EC-EP and Sepabead EC-HFA were activated with 1 M NaOH to open the epoxide ring. Thereafter, the activated Sepabead EC-EP and Sepabead EC-HFA were incubated with 7 U of purified LsRN, containing 50 μg proteins of purified levansucrase, in 50 mM citrate buffer pH 6.0 at 4 ^{o}C, with gentle agitation for 2 d. The immobilized LsRN was removed from the unbound LsRN then washed with ice cold 50 mM of citrate buffer, pH 6.0 for 3 times. The unbound enzyme and amount of protein remaining in the supernatant was assayed. The immobilized LsRN on Sepabead EC-EP and Sepabead EC-HFA were stored at 4 °C and used for further experiments.

The immobilized LsRN on each supporting matrices and unbounded LsRN in supernatant were assayed. The immobilization efficiency was calculated from the activity of the immobilized LsRN and unbounded LsRN activity in the supernatant.

Characterization of immobilized LsRN

Optimum pH of LsRN activity

The optimum pH on the activity of free and immobilized LsRN were investigated using 10 mM Britton-Robinson Universal buffer at pH 3-12. Two hundred milliunits of free and immobilized LsRN were incubated in 1.6% (w/v) sucrose, at 40 ^{o}C for 5 min. The activity of LsRN was measured by modified DNS method.

Optimum temperature of LsRN activity

The optimum temperature on the activity of free and immobilized LsRN were investigated using temperature range of 10-80 ^{o}C. Two hundred milliunits of free and immobilized LsRN were incubated in 1.6% (w/v) sucrose in 50 mM citrate buffer, pH 6.0 for 5 min. The activity of LsRN was measured by modified DNS method.

Effect of metal ions and chemical reagents on the activity of LsRN

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

Effect of ionic strength on the activity of LsRN

The effect of ionic strength on the activity of free and immobilized LsRN were investigated using 0-3 M NaCl. Two hundred milliunits of free and immobilized LsRN were incubated with 0-3 M NaCl in 1.6% (w/v) sucrose and 50 mM citrate buffer, pH 6.0, at 40 ^{o}C for 5 min. The activity of LsRN was measured by modified DNS method.

Effect of sucrose concentration on the activity of LsRN

The effect of sucrose concentration on the activity of free and immobilized LsRN were investigated from 10-70% (w/v) sucrose. Two hundred milliunits of free and immobilized LsRN were incubated with 10-70% (w/v) sucrose in 50 mM citrate buffer, pH 6.0, at 40 ^{o}C for 5 min. The activity of LsRN was determined by modified DNS method.

LsRN stability

The stability of free and immobilized LsRN were investigated by pre-incubating the 200 mU of free and immobilized LsRN in 50 mM citrate buffer, pH 6.0 at 40 ^{o}C for 12 h. The residual activity of the free and immobilized LsRN was assayed by modified DNS method.

Storage stability

The storage stability of immobilized LsRN was investigated by stored the immobilized LsRN in 50 mM citrate buffer, pH 6.0 at temperature of 4^{o}C for 3 mo. The residual activity of the immobilized LsRN was assayed by once a week by modified DNS method.

The repeated usability of immobilized LsRN

The repeated usability of the immobilized LsRN was determined. Two hundred milliunits of immobilized LsRN were incubated in 1.6% (w/v) sucrose and 50 mM citrate buffer, pH 6.0, at 40 ^{o}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 LsRN after each reaction cycle was measured by modified DNS method.

Effect of substrate and immobilized LsRN activity ratio on the production of L-FOS

The effect of ratio of substrate: immobilized LsRN activity (1 g sucrose: 1 U of immobilized levansucrase) on the production of L-FOS were investigated at 1:1, 1:10, 1:20, 1:30 1:40 and 1:50. 50 mL of substrate was contained with 50% (w/v) sucrose in 50 mM citrate buffer, pH 6.0. The reaction was incubated with immobilized LsRN-Y246S, at 40 ^{o}C for 12 h. The products of L-FOS and residual sucrose were measured.

Effect of sucrose concentrations on sucrose conversion to L-FOS

The effect of sucrose concentration on sucrose conversion to L-FOS was investigated from 10-70% (w/v) sucrose. Five hundred units of immobilized LsRN was incubated with 50 mL of 10-70% (w/v) sucrose in 50 mM citrate buffer, pH 6.0. The

reaction was incubated at temperature of 40 ºC for 12 h in an orbital shaker, at 150 rpm. The L-FOS concentrations were determined.

Production of L-FOS by immobilized LsRN

Free and immobilized LsRN on each supporting matrices were used for L-FOS production. Substrate was contained of typically 50 mL of 500 U of immobilized LsRN, 50% (w/v) sucrose in 50 mM citrate buffer, pH 6.0. The reaction was incubated at temperature of 40 ºC for 12 h in an orbital shaker, at 150 rpm. Product concentration in effluent was analyzed every hour.

Reusability of immobilized LsRN on the production of L-FOS

Reusability of immobilized LsRN on the production of L-FOS was determined by measuring the efficiency of the immobilized LsRN after repetitive uses. 50 mL of substrate was contained with 500 U of immobilized LsRN, 50% (w/v) sucrose in 50 mM citrate buffer, pH 6.0. The reaction was incubated at 40 ºC under shaking condition at 150 rpm by using an orbital shaker. At the end of each cycle, the supernatant was collected and products were determined. The matrices were washed with cold 50 mM citrate buffer, pH 6.0 and subjected to following cycles.

Process design and optimization of L-FOS production by immobilized LsRN

Immobilized LsRN was packed in to a water jacketed glass column (Figure 2.1). Sucrose concentration, temperature, pH and ratio of substrate : immobilized LsRN activity were maintained based on preliminary results. Substrate was taken in both sides after the elution. Samples were analyzed for L-FOS products. Operational stability of immobilized LsRN was determined. The LsRN activity of immobilized LsRN would be followed during production and residual activity measurement. Feasibility for scale-up (up to 100 g of L-FOS) was investigated.

Effect of substrate feed direction on the production of L-FOS

The effect of substrate feed direction on the production of L-FOS was investigated. The substrates were feed into the column by upward and downward flow directions. Five hundred units of immobilized LsRN were packed in the double jacket column. 50 mL of substrate containing 50% (w/v) sucrose in 50 mM citrate buffer, pH 6.0 was feed into the column with flow rate of 4 mL/min at temperature 40 ^{o}C. The reaction mixture was every hour taken for detection of the L-FOS products.

Effect of flow pattern on the production of L-FOS

The effect of flow pattern on the production of L-FOS was investigated. The samples were feed into the column using single round and multiple rounds. Five hundred units of immobilized LsRN were packed in double jacket column. 50 mL of substrate containing 50% (w/v) sucrose in 50 mM citrate buffer, pH 6.0 was feed into the column with upward flow at temperature 40 ^{o}C. The multiple pass flow, samples were feed into the column with flow rate of 4 mL/min. The samples were collected every hour and assayed the L-FOS products.

The single pass flow, samples were feed into the column with flow rate 0.34 mL/min. At the end of each cycle, the samples were collected and assayed the L-FOS products.

Effect of flow rate of substrate on the production of L-FOS

The effect of flow rate of substrate on the production of L-FOS was investigated by varying the substrate flow rate at 2, 4, 6, 8 and 10 mL/min. Five hundred units of immobilized LsRN were packed in double jacket column. 50 mL of substrate containing 50% (w/v) sucrose in 50 mM citrate buffer, pH 6.0 was feed into the column with upward flow at temperature 40 ^{o}C. The samples were taken at interval time points of 1 h to detect the L-FOS products.

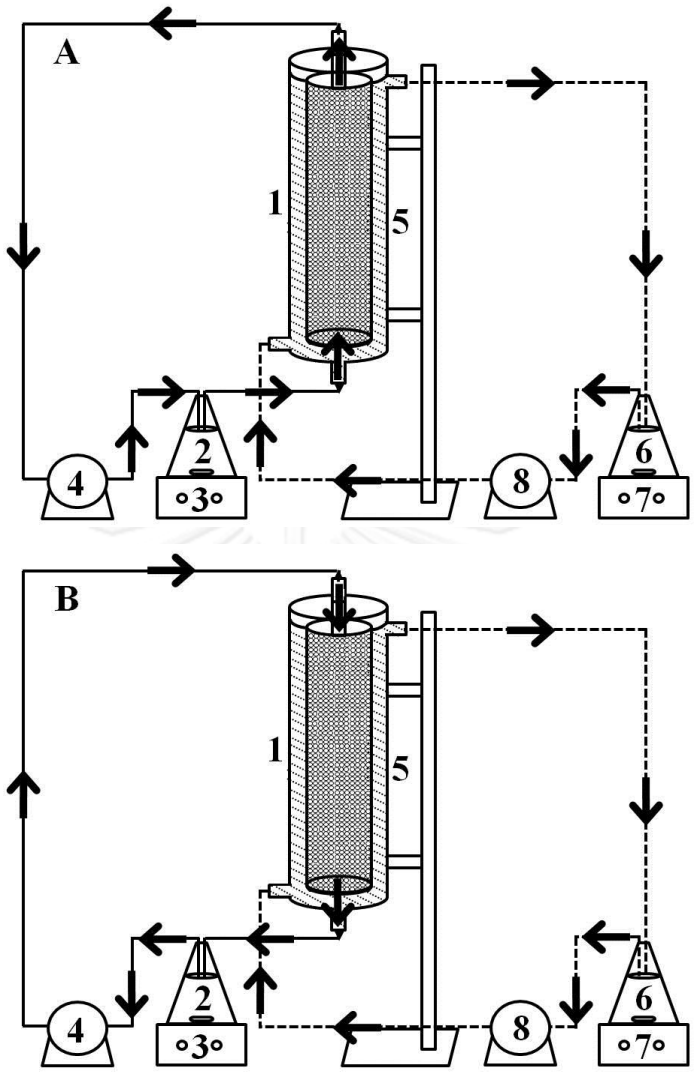


Figure 2.1 Schematic diagram of L-FOS production by packed bed reactor;

A: Upward flow	B: Downward flow
1: packed-bed column	5: heating jacket
2: sample mixer	6: heated water
3: heat and stirrer I	7: heat and stirrer II
4: peristaltic pump I	8: peristaltic pump II

Operational production of L-FOS by immobilized LsRN

The operational production of L-FOS using by packed immobilized LsRN reactor was determined by measuring the efficiency of the immobilized LsRN after repetitive use. Five hundred units of immobilized LsRN were packed in double jacket column. 50 mL of substrate containing 50% (w/v) sucrose in 50 mM citrate buffer, pH 6.0 was feed into the column with upward flow with flow rate of 10 mL/min at temperature 40 ^{o}C. The samples were collected and products were determined every hour. At the end of each cycle, new substrates were taken into the column which same condition.

Scale up production of L-FOS

Scale up production of L-FOS was produced by packed immobilized LsRN reactor. Flow rate, substrate feed direction, flow pattern, and ratio of substrate : immobilized LsRN activity were maintained based on preliminary results. One thousand and five hundred units of immobilized LsRN were packed in double jacket column. 500 mL of substrate containing with 50% (w/v) sucrose in 50 mM citrate buffer, pH 6.0, incubated at temperature of 40 ^{o}C, was feed into the column with upward flow at flow rate of 10 mL/min. The samples were collected and products were determined every hour.

Products analysis

The L-FOS products synthesized by LsRN were examined by thin layer chromatography (TLC), high performance liquid chromatography (HPLC), high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD).

TLC analysis

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 ^{o}C for 5 min to visualize the separated products.

HPLC analysis

Products were determined by HPLC, using SugarPak column, 50 mg/L Na2EDTA used as a mobile phase, flow rate 0.5 mL/min. Detection was achieved by RID.

HPAEC-PAD analysis

The L-FOS products were analyzed by HPAEC-PAD equipped with CarboPac PA-1 column. Elution was performed by gradient of 0-0.6 M NaOAc in 0.1 M NaOH with the flow rate 1 mL/min, detected with a Dionex ED40 electrochemical detector ED40 with an Au working electrode and an Ag/AgCl reference electrode.

Products purification

L-FOS products were purified by Biogel P2 column chromatography using DI water as a mobile phase. The purified fractions were measured by weighting technique. The swelled Biogel P2 was packed in a column (3.2 x 112 cm). The Biogel P2 column was equilibrated with filtrate DI water at a flow rate of 27 mL/hour and temperature of 50 ^{o}C. The crude L-FOS products were applied to Biogel P2 column in equilibrated buffer at flow rate of 27 mL/h and eluted with filtrated DI water. The fractions of 6 mL were collected for measurement of L-FOS concentration. The each size of L-FOS fractions were pooled.

CHAPTER III

RESULTS

Production of recombinant LsRN in *E.coli*

E. coli Top-10 was used as an expression host and *pls*RN01 containing levansucrase gene (*ls*RN) of *B. licheniformis* RN-01 under the regulation of its putative endogenous promoter inserted into pBluscriptSK(-), was used for LsRN production. The derivatives of LsRN used in this research were N251Y, Y246S and Y246W.

Effect of concentration of LB broth medium on LsRN activity

E. coli Top-10 harboring plsRN01 derivatives were cultured in expression LB, 2X LB, 3X LB, 4X LB and 5X LB broth medium containing 100 μg/mL ampicillin at 37 ºC, 250 rpm. The results showed that, the maximum activity of *E. coli* Top-10 and p*ls*RN01 containing Y246S was detected in all LB medium concentrations at day 2 of cultivation and gradually decreased after day 2 to *day 5*, shown in Figure 3.1. There were no significant differences in the activity of LsRN in 3X, 4X and 5X LB of all LsRN derivatives after cultivation at day 2 (Figure 3.2). NaCl concentration had an effect on LsRN activity of all LsRN derivatives when LB medium was used as a control, addition of NaCl from 1X NaCl in LB medium (5 g/L) to 5X NaCl. As shown in Figure 3.3, 3X NaCl in LB medium gave highest LsRN activity about 2 fold in all LsRN derivatives when compared with LB medium containing 1X NaCl. Decreasing in LsRN activity was observed when the concentration of NaCl was higher than 3X NaCl (15 g/L). This result revealed that 3X LB medium could increase LsRN activity about 4, 12 and 7 folds for LsRN-N251Y, Y246S and Y246W, respectively. Thus, 3X LB medium containing 3X NaCl was selected for further cultivation of all LsRN derivatives and harvested at day 2.

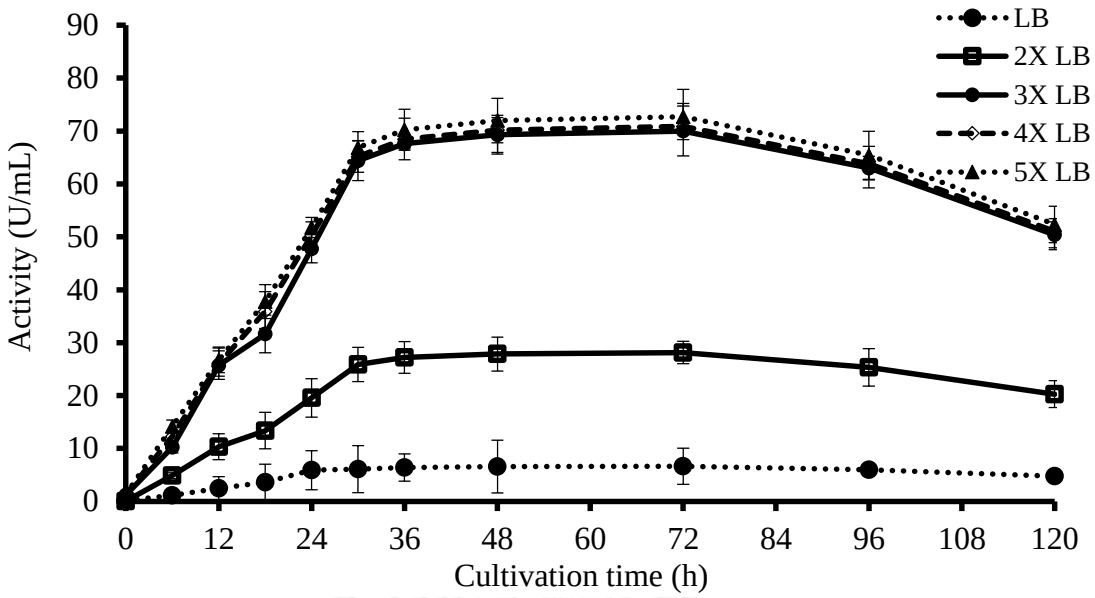


Figure 3.1 LsRN production profile of *E. coli* Top-10 harboring plsRN01 containing *ls*RN Y246S in various LB medium concentrations

E. coli Top-10 recombinant was cultured in LB, 2X LB, 3X LB, 4X LB and 5X LB broth medium at temperature of 37 ºC, 250 rpm for 5 d.

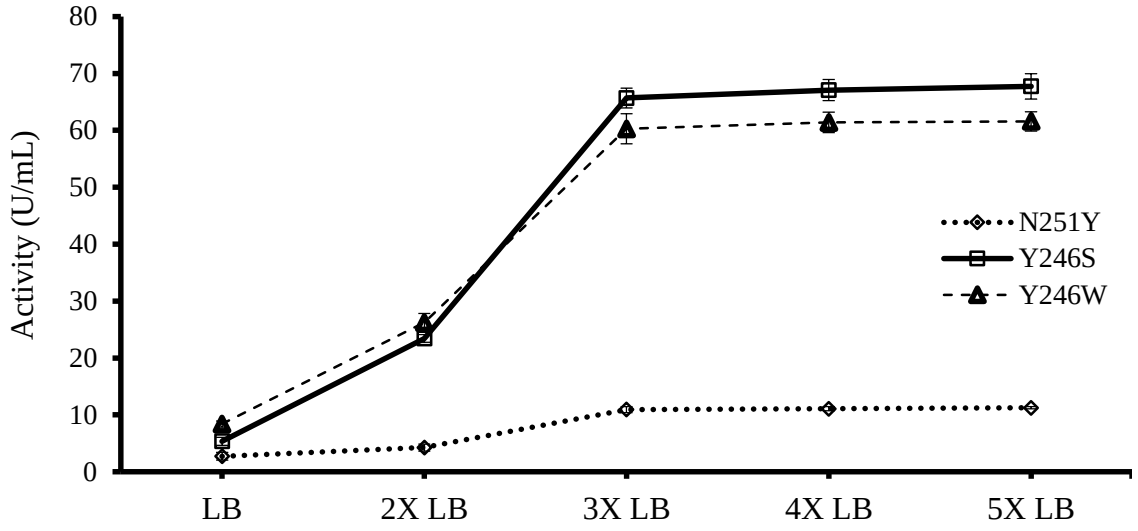


Figure 3.2 Effect of LB medium concentration on the LsRN production

E. coli Top-10 recombinant containing N251Y, Y246S and Y246W – *ls*RN was cultured in LB, 2X LB, 3X LB, 4X LB and 5X LB broth medium at temperature of 37 ºC, 250 rpm for 30 h.

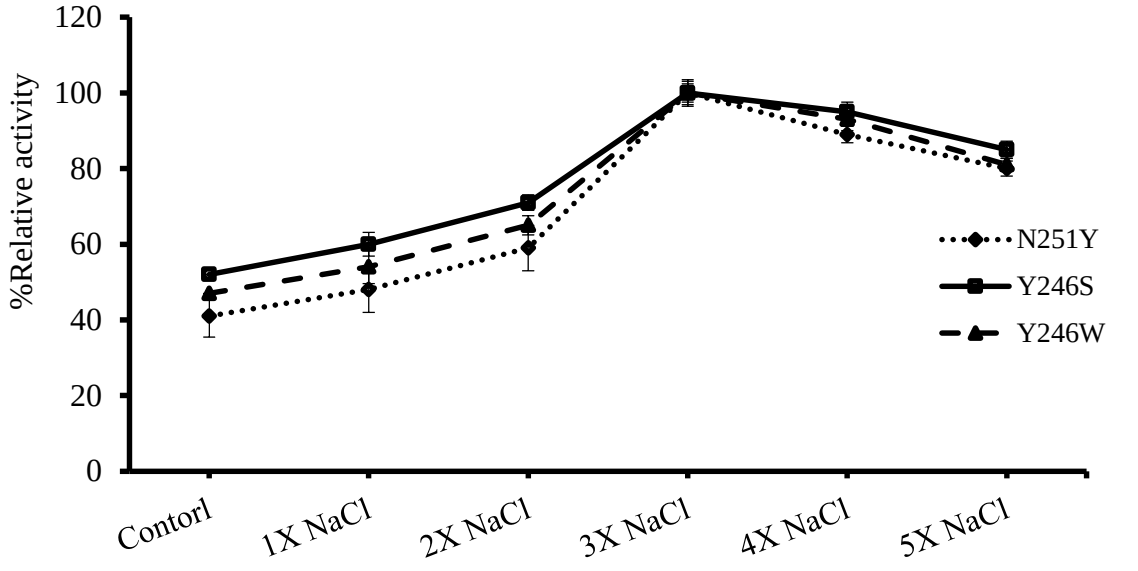


Figure 3.3 Effect of NaCl concentration on the LsRN production

E. coli Top-10 recombinant was cultured in 3X LB broth medium and various NaCl concentration, at temperature of 37 ºC, 250 rpm for 30 h.

Purification of recombinant LsRN derivatives in *E.coli*

Crude recombinants LsRN-N251Y, Y246S and Y246W were purified by column chromatography. The DEAE-cellulose chromatogram of LsRN-N251Y and Y246S purification, in Figure 3.4 and 3.5, showed that there were at least two protein peaks containing LsRN activity. In contrast, The LsRN-Y246W demonstrated that only single peak of LsRN activity, shown in Figure 3.6. The fractions containing LsRN activity were applied to a phenyl-sepharose column. The result indicated that only a single peak contained LsRN activity in LsRN-N251Y, Y246S and Y246W, as shown in Figure 3.7 - 3.9, respectively. The LsRN purification table of all LsRN derivatives were shown in Table 3.1. The specific activity of purified LsRN-N251Y, Y246S and Y246W fraction increased to 375, 168 and 425 U/mg protein, respectively, with 9.6, 6.5 and 12.1 purification fold, and 56, 62 and 69% yield of LsRN-N251Y, Y246S and Y246W, respectively. Analysis of the purified LsRN by SDS-PAGE showed the purified LsRN had a MW of approximately 52 kDa and revealed a high degree of apparent homogeneity, as shown in Figure 3.10 – 3.12.

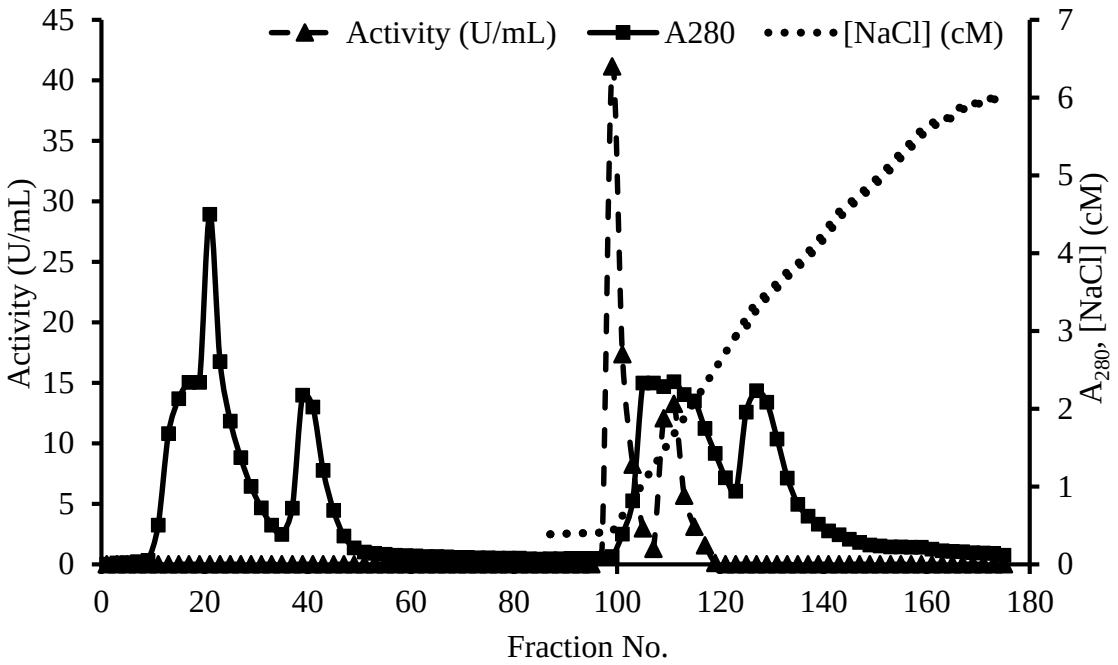


Figure 3.4 LsRN-N251Y purification profile by DEAE-cellulose chromatography

Crude LsRN-N251Y was applied to DEAE-cellulose column with 50 mM sodium acetate buffer, pH 6.0 at flow rate 45 mL/h and eluted using by stepwise gradient elution with of 50 mM sodium acetate buffer, pH 6.0 containing 0.1-0.5 M NaCl.

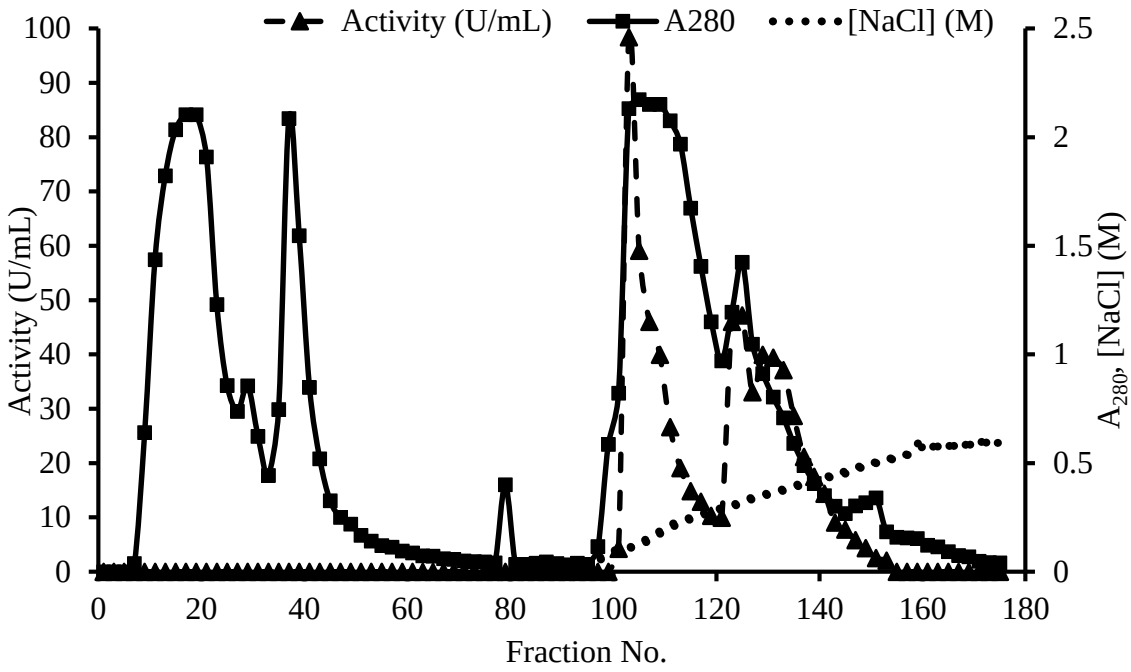


Figure 3.5 LsRN-Y246S purification profile by DEAE-cellulose chromatography

Crude LsRN-Y246S was applied to DEAE-cellulose column with 50 mM sodium acetate buffer, pH 6.0 at flow rate 45 mL/h and eluted using by stepwise gradient elution with of 50 mM sodium acetate buffer, pH 6.0 containing 0.1-0.5 M NaCl.

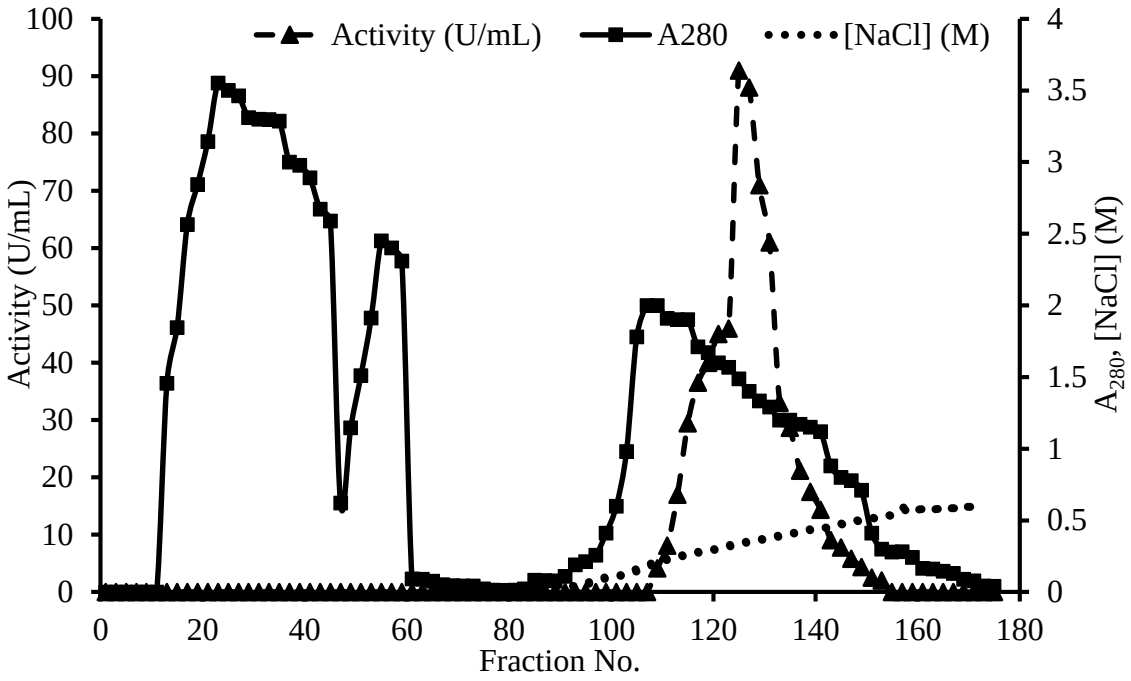


Figure 3.6 LsRN-Y246W purification profile by DEAE-cellulose chromatography

Crude LsRN-Y246W was applied to DEAE-cellulose column with 50 mM sodium acetate buffer, pH 6.0 at flow rate 45 mL/h and eluted using by stepwise gradient elution with of 50 mM sodium acetate buffer, pH 6.0 containing 0.1-0.5 M NaCl.

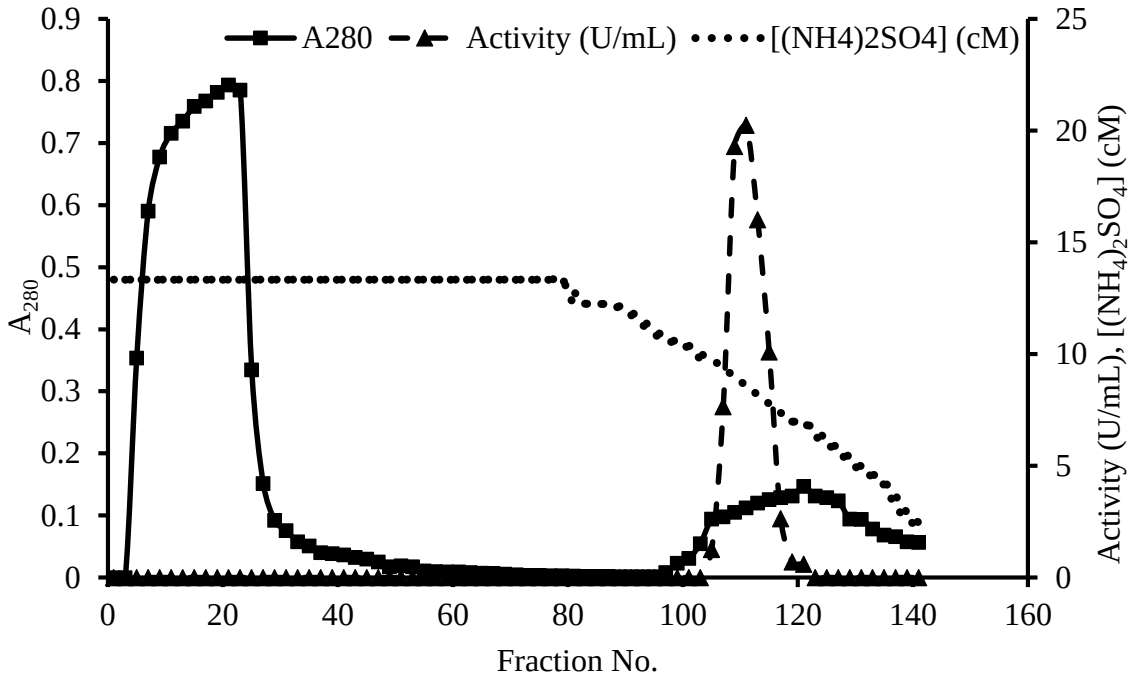


Figure 3.7 LsRN-N251Y purification profile by Phenyl-sepharose chromatography

Partially purified LsRN-N251Y was applied to Phenyl-sepharose column with 25 mM sodium acetate buffer, pH 6.0 containing 1 M $(NH_4)_2SO_4$ at flow rate 45 mL/h and eluted using by a reversed salt gradient from 1.0 to 0 M $(NH_4)_2SO_4$.

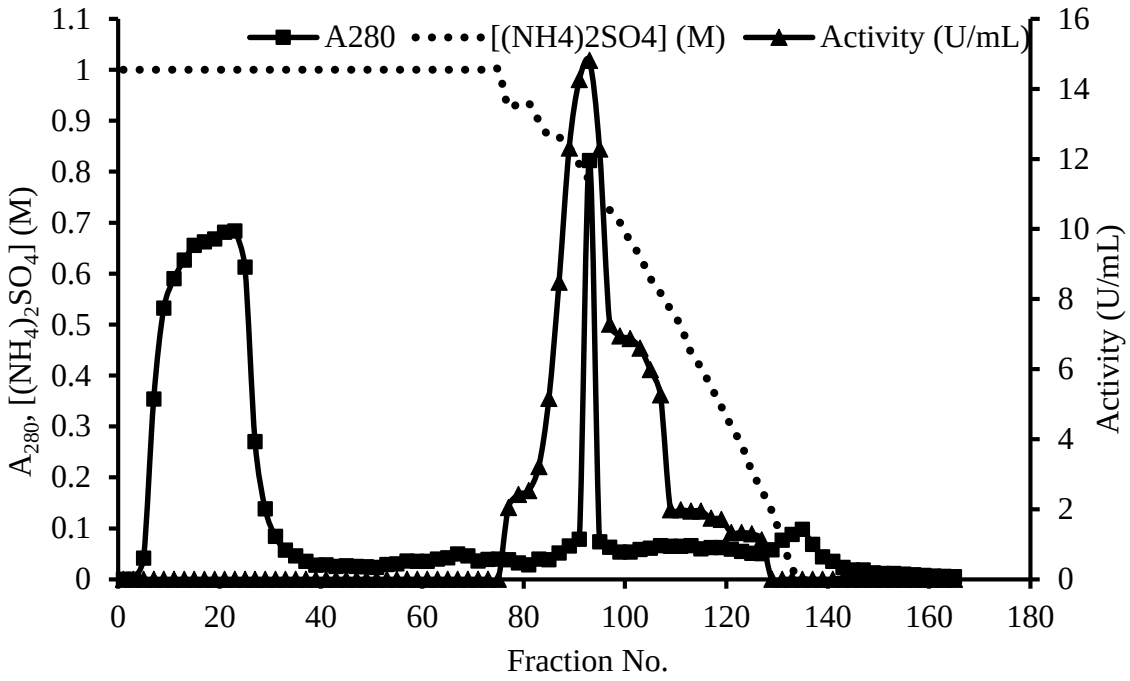


Figure 3.8 LsRN-Y246S purification profile by Phenyl-sepharose chromatography

Partially purified LsRN-Y246S was applied to Phenyl-sepharose column with 25 mM sodium acetate buffer, pH 6.0 containing 1 M $(NH_4)_2SO_4$ at flow rate 45 mL/h and eluted using by a reversed salt gradient from 1.0 to 0 M $(NH_4)_2SO_4$.

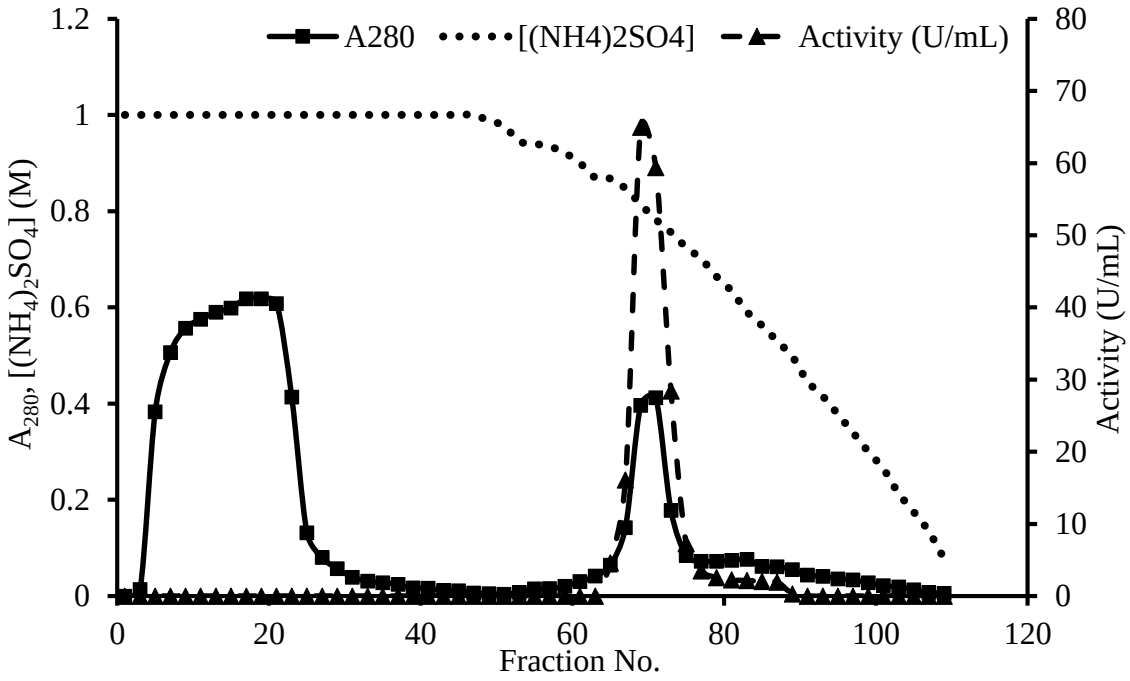


Figure 3.9 LsRN-Y246W purification profile by Phenyl-sepharose chromatography

Partially purified LsRN-Y246W was applied to Phenyl-sepharose column with 25 mM sodium acetate buffer, pH 6.0 containing 1 M $(NH_4)_2SO_4$ at flow rate 45 mL/h and eluted using by a reversed salt gradient from 1.0 to 0 M $(NH_4)_2SO_4$.

Table 3.1 Table of LsRN purification

Fraction	Volume (mL)	Activity (U/mL)	Total Activity (U)	Protein content (μg/mL)	Total protein (mg)	Specific activity (U/mg)	%Yield	Fold
	LsRN-N251Y							
Crude	50	93	4,650	2,391	120	39	100	1
DEAE	81	45	3,645	501	41	89	78	2.3
DEAE and Phenyl	64	41	2,624	115	7	375	56	9.6
	LsRN-Y246S							
Crude	50	49	2,450	1,890	95	26	100	1
DEAE	82	24	1,968	334	27	73	80	2.8
DEAE and Phenyl	50	30	1,514	178	9	168	62	6.5
	LsRN-Y246W							
Crude	50	111	5,550	3,183	159	35	100	1
DEAE	85	54	4,590	671	57	81	83	2.3
DEAE and Phenyl	75	51	3,825	124	9	425	69	12.1

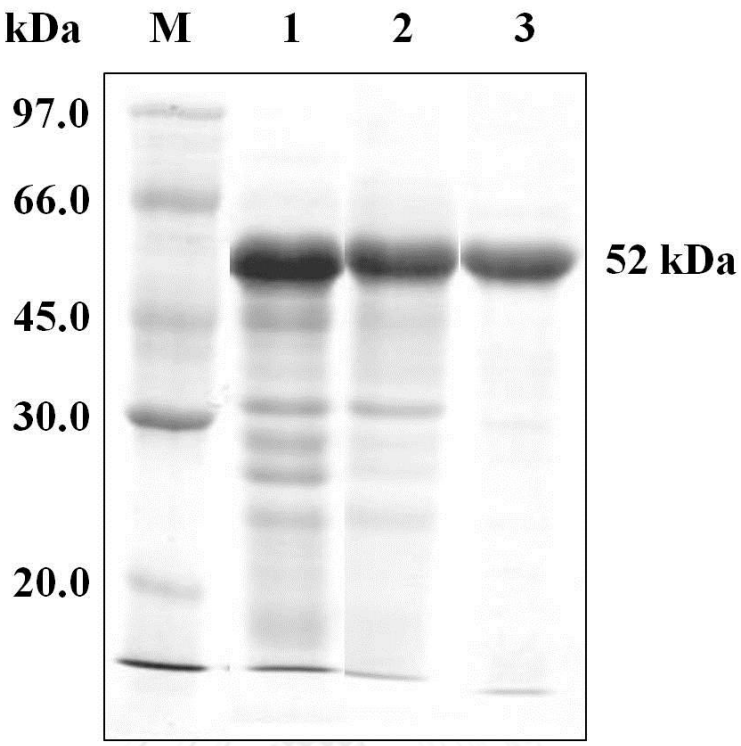


Figure 3.10 Analysis of purified LsRN-N251Y by 10% SDS-PAGE

Lane M: Low MW protein marker

Lane 1: Crude LsRN-N251Y

Lane 2: Purified LsRN-N251Y by DEAE-cellulose column

Lane 3: Purified LsRN-N251Y by DEAE-cellulose and Phenyl-sepharose fraction column

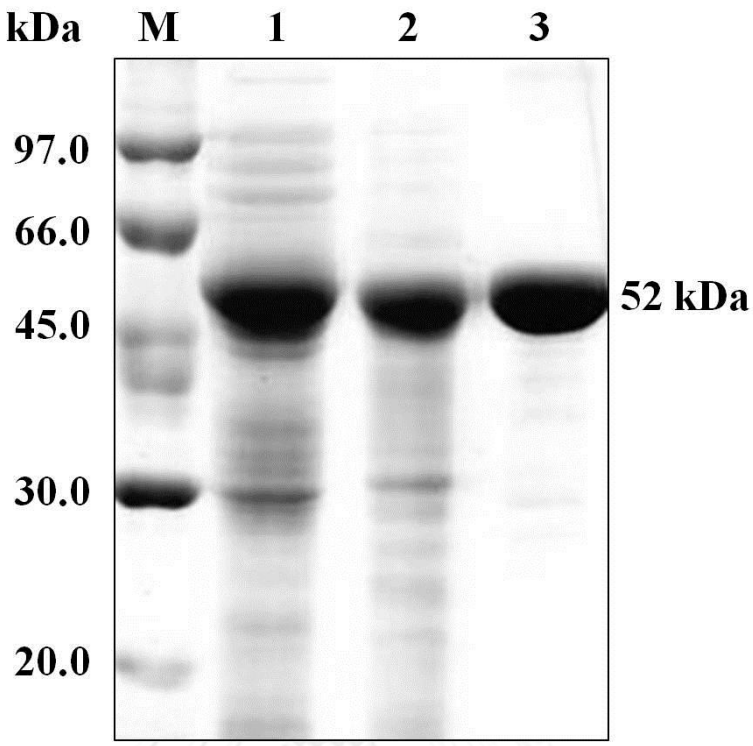


Figure 3.11 Analysis of purified LsRN-Y246S by 10% SDS-PAGE

Lane M: Low MW protein marker

Lane 1: Crude LsRN-Y246S

Lane 2: Purified LsRN-Y246S by DEAE-cellulose column

Lane 3: Purified LsRN-Y246S by DEAE-cellulose and Phenyl-sepharose column

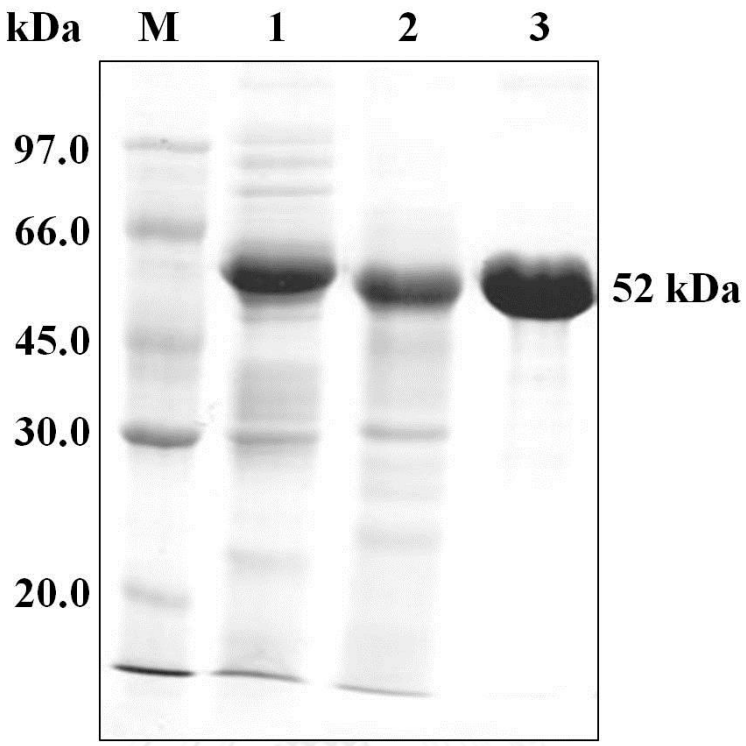


Figure 3.12 Analysis of purified LsRN-Y246W by 10% SDS-PAGE

Lane M: Low MW protein marker

Lane 1: Crude LsRN-Y246W

Lane 2: Purified LsRN-Y246W by DEAE-cellulose column

Lane 3: Purified LsRN-Y246W by DEAE-cellulose and Phenyl-sepharose column

Characterization of levansucrase

Optimum pH of LsRN activity

The optimum pH of LsRN was determined using 10 mM Britton-Robinson Universal buffer at pH 3-12. The optimum pH of LsRN-N251Y, Y246S and Y246W were similar at pH 6.0 (Figure 3.13). However, the LsRN-N251Y had a broader activity range, at pH 5-8 than that LsRN-Y246S and Y246W.

Optimum temperature of LsRN activity

The optimum temperature of LsRN was determined using temperature range of 10-80 ^{o}C. The optimum temperatures of all LsRN derivatives were similar, at 40 ^{o}C as shown in Figure 3.14. All LsRN derivatives had a board temperature range for enzymatic activity, 30 ^{o}C – 50 ^{o}C.

Effect of metal ions and chemical reagents on the activity of LsRN

The effect of metal ions and chemical reagents on the activity of LsRN was investigated with 5 mM of each the metal ions or chemical reagents. The effects of various metal ions and chemical reagents on the activity of LsRN were shown in Figure 3.15. Most metal ions tested slightly affect the activity of LsRN. Metal ions that had inhibitory effect on the activity of levansucrase of LsRN were Cu^{2+}, Zn^{2+} and Fe^{3+}. Ionic surfactant, SDS and chelating agents such as EDTA can also drastically inhibit the activity of LsRN. Interestingly, Ca^{2+} and Mn^{2+} significantly enhanced the LsRN activity.

Effect of ionic strength on the activity of levansucrase

The effect of ionic strength on the activity of levansucrase was investigated using 0-3 M NaCl. Two hundred milliunits of levansucrase were incubated with 0-3 M NaCl in 1.6% (w/v) sucrose and 50 mM citrate buffer, pH 6.0, at 40 ^{o}C for 5 min. As shown in Figure 3.16, the result showed that the levansucrase activity gradually increased from without NaCl to 2 M NaCl in all LsRN and decreased when NaCl concentration was increased to more than 2 M.

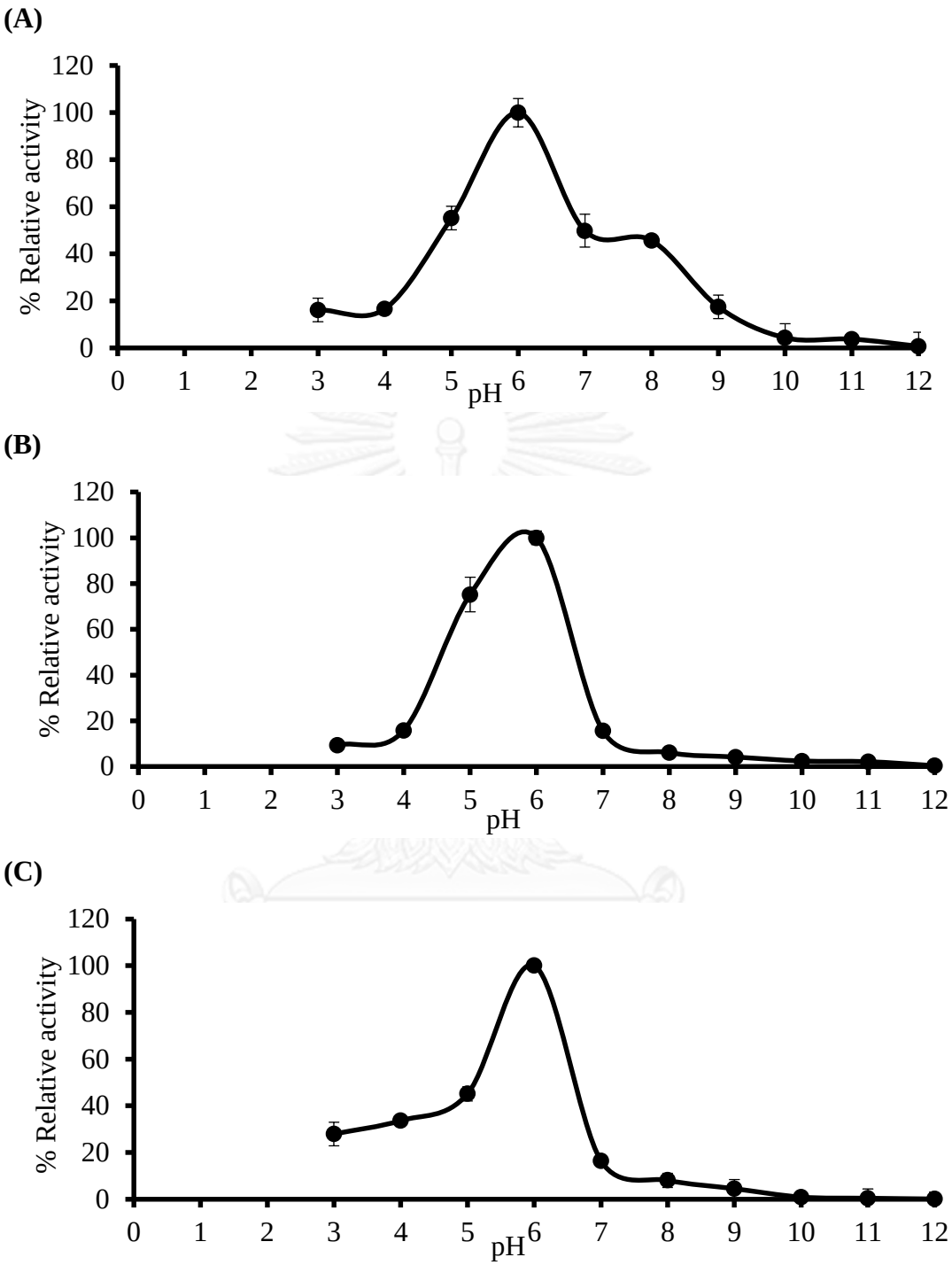


Figure 3.13 Effects of pH on the activity of LsRN

Two hundred milliunits of LsRN: (A) N251Y, (B) Y246S and (C) Y246W were incubated in 10 mM Britton-Robinson Universal buffer in 1.6% (w/v) sucrose, at 40 ^{o}C for 5 min.

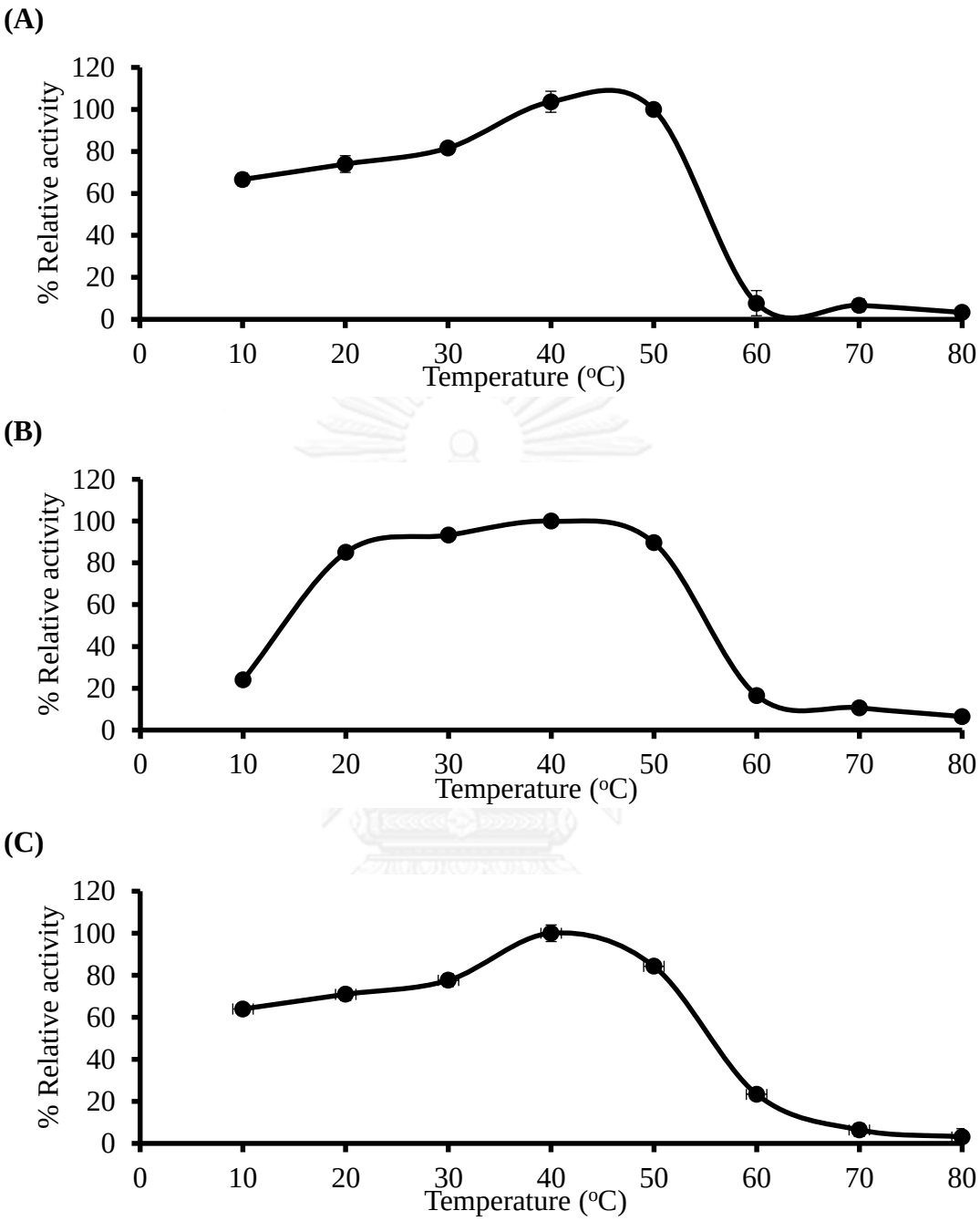


Figure 3.14 Effect of temperature on the activity of LsRN

Two hundred milliunits of LsRN: (A) N251Y, (B) Y246S and (C) Y246W were incubated in 1.6% (w/v) sucrose in 50 mM citrate buffer, pH 6.0 for 5 min.

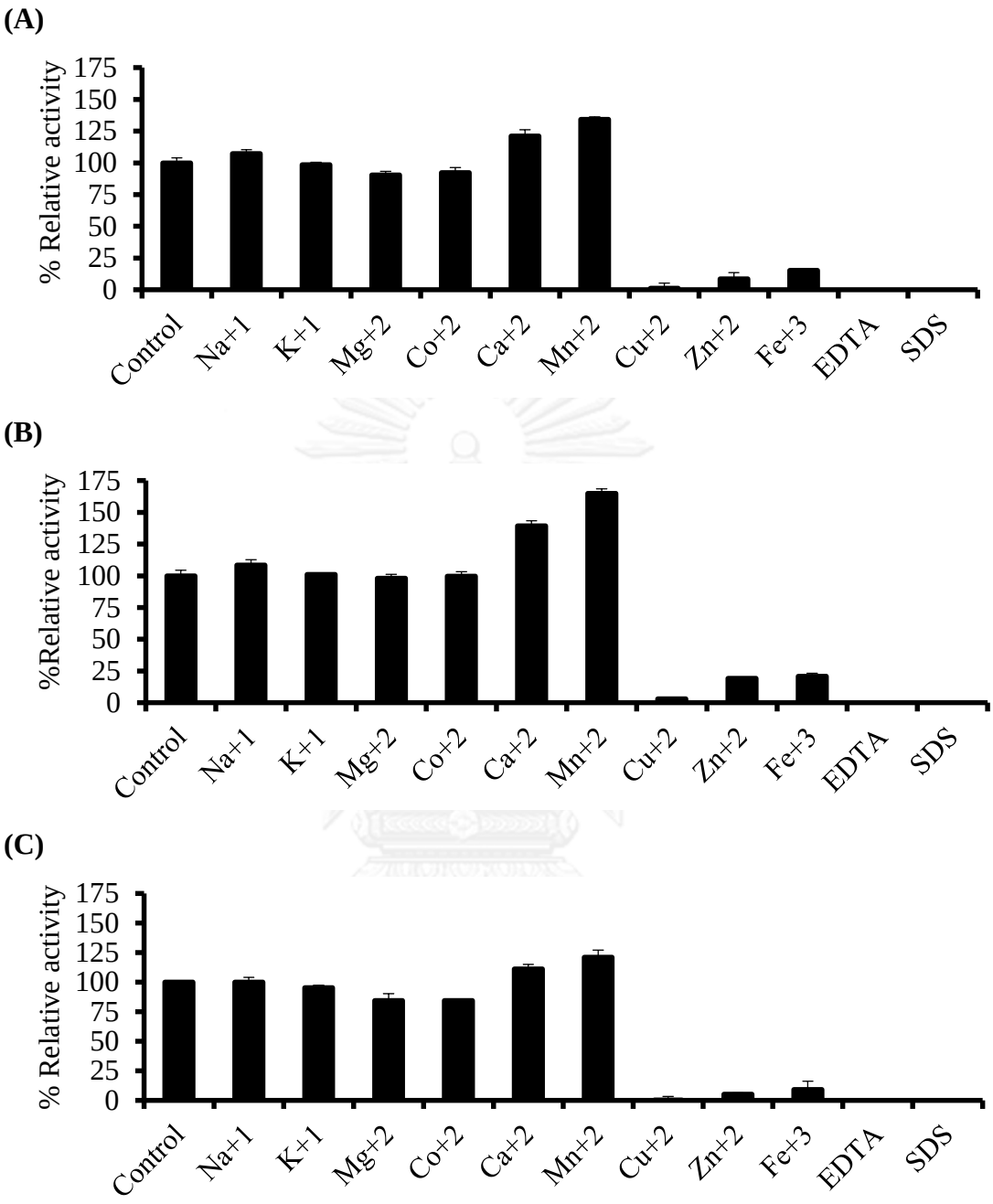


Figure 3.15 Effect of metal ions and chemical reagents on the activity of LsRN

Two hundred milliunits of LsRN: (A) N251Y, (B) Y246S and (C) Y246W were incubated in 1.6% (w/v) sucrose, 50 mM citrate buffer, pH 6.0 supplemented with 5 mM of each of the metal ions or chemical reagents, at 40 ^{o}C for 5 min.

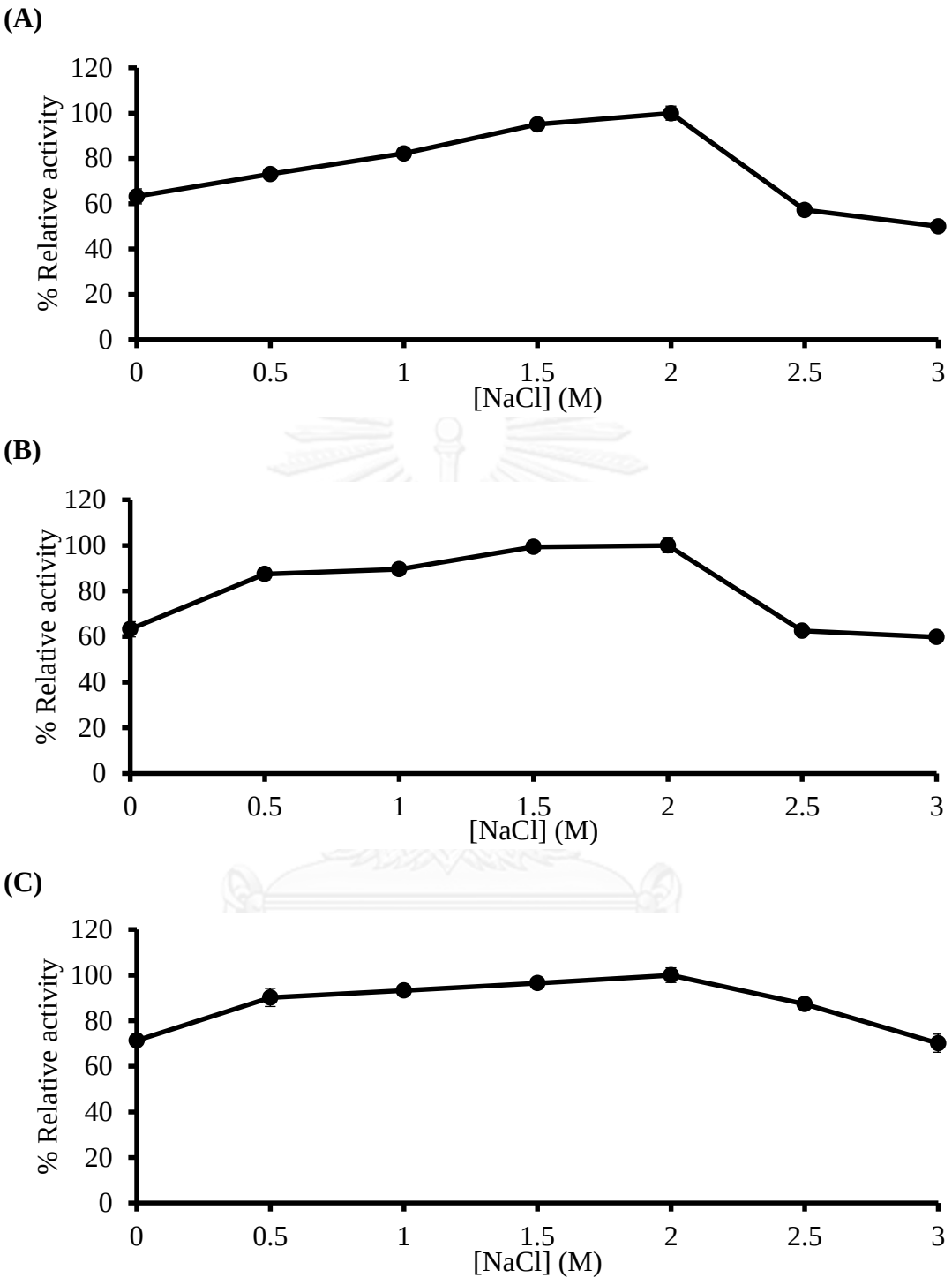


Figure 3.16 Effect of ionic strength on the activity of LsRN

Two hundred milliunits of LsRN: N251Y, (B) Y246S and (C) Y246W were incubated with 0-3 M NaCl in 1.6% (w/v) sucrose and 50 mM citrate buffer, pH 6.0, at 40 ^{o}C for 5 min.

Effect of sucrose concentration on the activity of LsRN

The effect of sucrose concentration on the activity of LsRN was investigated using 10-70% (w/v) of sucrose. The result of the effect of sucrose concentration on LsRN of all LsRN derivatives was shown in Figure 3.17. Forty percent of sucrose concentration gave highest activity of LsRN in all LsRN derivatives. At sucrose concentration ranging from 20%-40% (w/v) there were no significant differences in the activity of LsRN-Y246S.

LsRN stability

The stability of LsRN was investigated by pre-incubating the 200 mU of levansucrase in 50 mM citrate buffer, pH 6.0 at 40 ^{o}C for 12 h. After pre-incubation of LsRN-N251Y, Y246S and Y246W at 40 ^{o}C, they lost approximately 50% activity after 2, 3 and 2 h, respectively, shown in Figure 3.18. When prolonged the incubation period to 12 h, LsRN-N251Y and Y246W completely lost their activity.

Storage stability

Storage stability of LsRN was investigated by storing the LsRN in 50 mM sodium acetate buffer, pH 6.0 at 4 ^{o}C for 3 mo. Storing of LsRN for 1 mo, N251Y, Y246S and Y246W retained 77%, 81% and 80% of their activity, respectively, as shown in Figure 3.19. Activity of all LsRN derivatives decreased to 50% when storage the LsRN for 9 wk, 11 wk and 10 wk of N251Y, Y246S and Y246W, respectively.

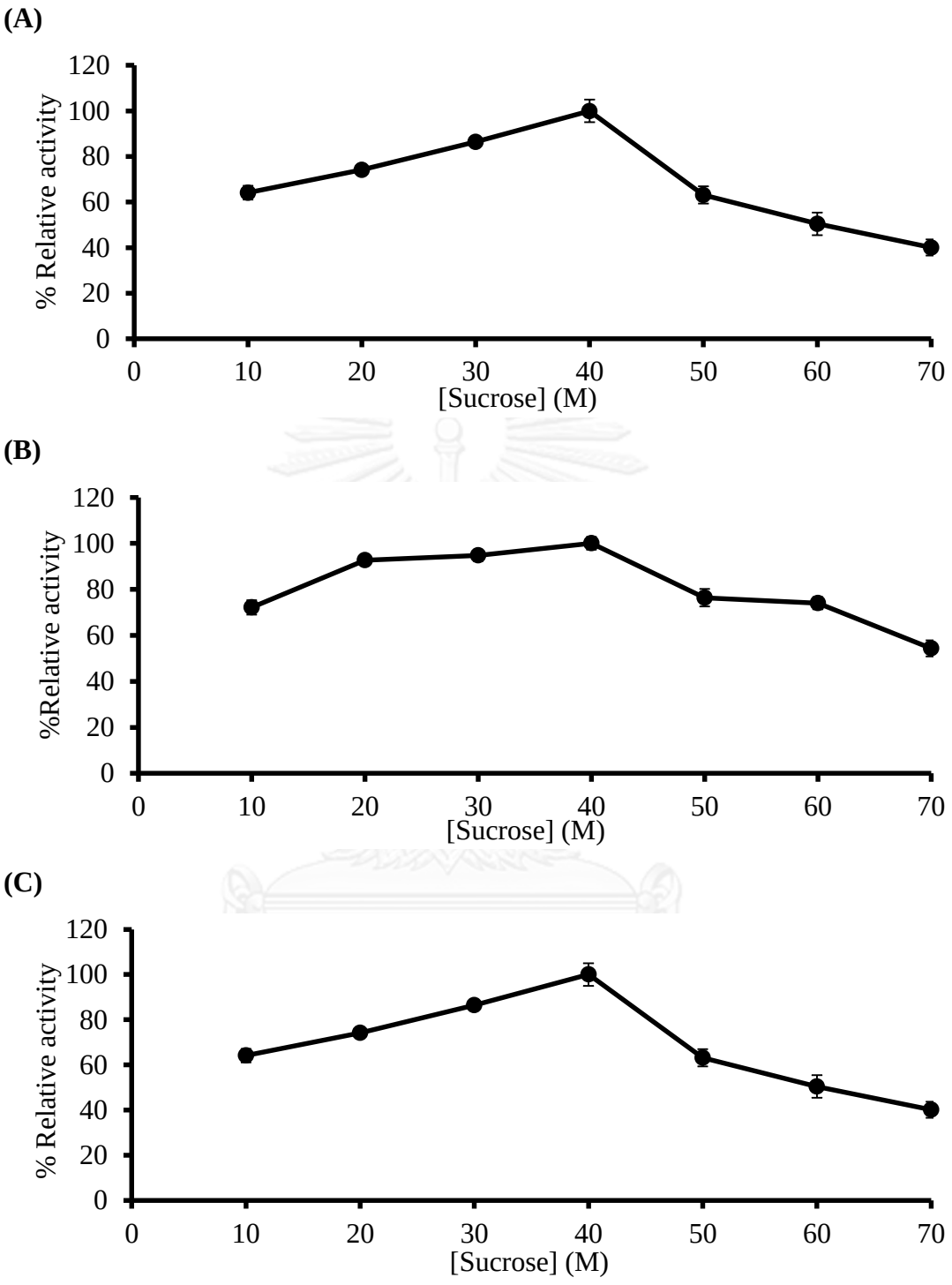


Figure 3.17 Effect of sucrose concentration on the activity of LsRN

Two hundred milliunits of LsRN: (A) N251Y, (B) Y246S and (C) Y246W were incubated with 10-70% (w/v) sucrose in 50 mM citrate buffer, pH 6.0, at 40 ^{o}C for 5 min.

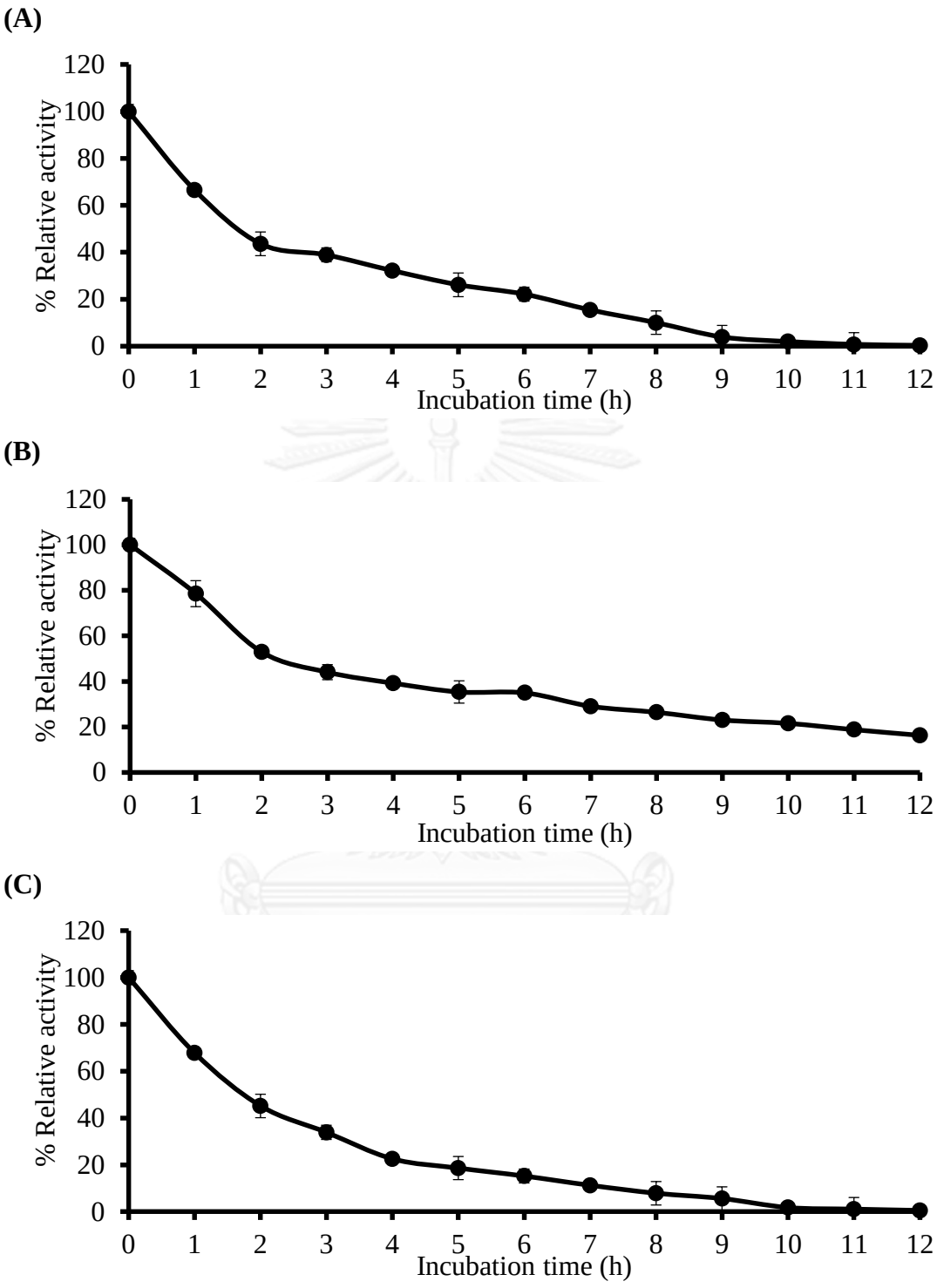


Figure 3.18 Stability of LsRN

Stability of LsRN: (A) N251Y, (B) Y246S and (C) Y246W was investigated by pre-incubating the 200 mU of levansucrase in 50 mM citrate buffer, pH 6.0 at 40 ^{o}C for 12 h.

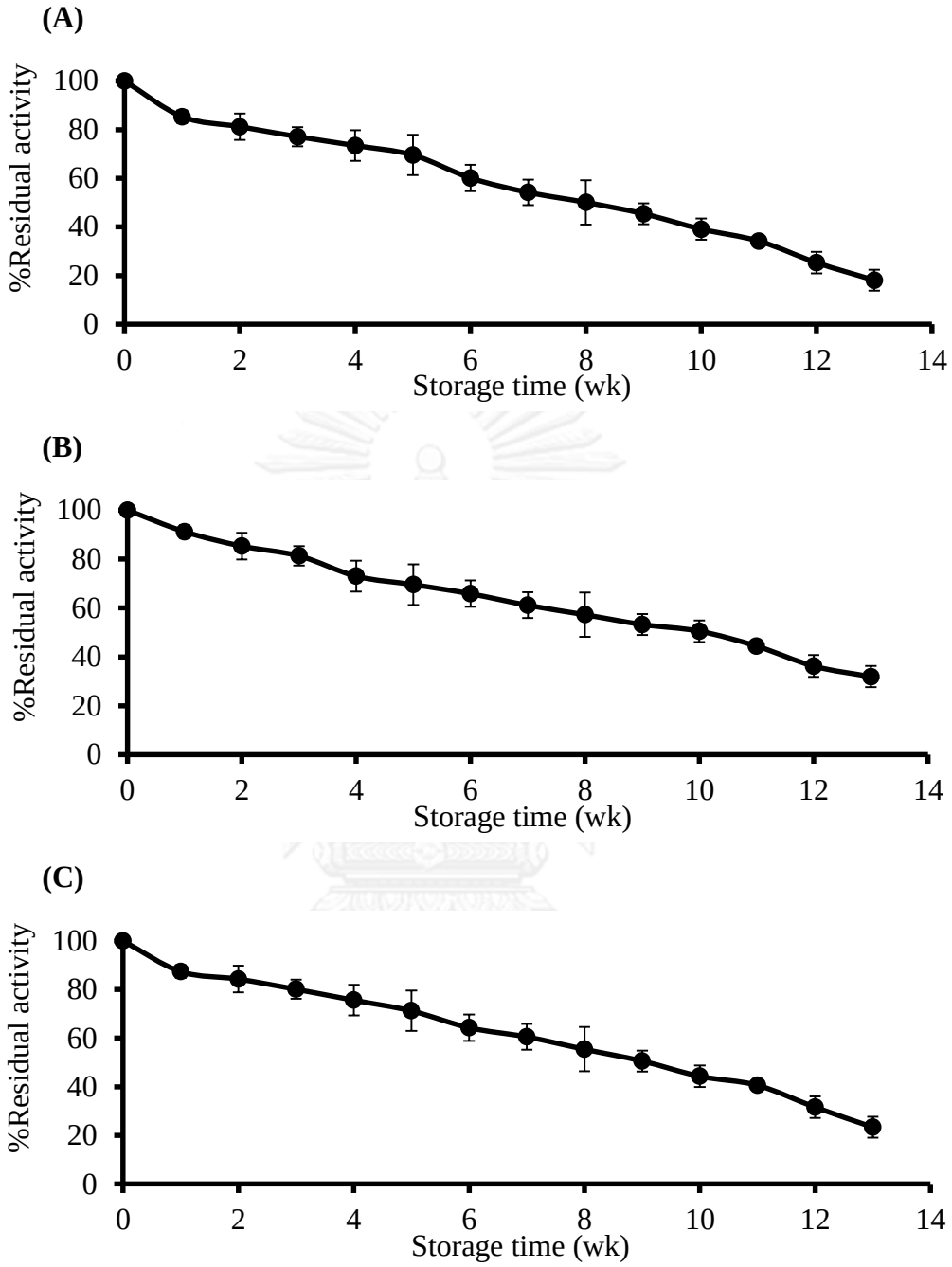


Figure 3.19 Storage stability LsRN

The storage stability of LsRN: (A) N251Y, (B) Y246S and (C) Y246W was investigated by storing the purified LsRN in 50 mM sodium acetate buffer, pH 6.0 at 4 ^{o}C for 3 mo.

Selection of suitable immobilization supporting matrices and characterization of immobilized LsRN

Effect of GTA concentration on cross-linking of LsRN-Y246S on CTS bead

0.5 g (wet weight) of CTS bead was incubated with 250 μg LsRN-Y246S containing 40 U of LsRN activity and GTA. As shown in Figure 3.20, the result indicated the immobilization of LsRN-Y246S by covalently cross-linking on CTS bead, the immobilization yield gradual increased when the GTA concentration was increased ranging from 0.25% - 4% and gave the highest immobilization yield, approximately 97%, when 4% GTA was used as a cross-linking agent. Although the immobilization yield increased when the GTA concentration was increased to 4%, the physical property of CTS bead was not appropriate for using as a supporting matrices. It was found that the CTS bead changed from elastic bead to hard bead and easily cracked. Thus, in further work, 2% GTA concentration was used as a cross-linking agent.

Immobilization efficiency of LsRN-Y246S on various matrices

The immobilization efficiency was calculated from the activity of immobilized LsRN-Y246S and unbounded LsRN-Y246S activity in the supernatant. LsRN-Y246S was immobilized on different supporting matrices using covalently linking method. The percentages of immobilized LsRN-Y246S on fine particle chitosan, Sepabead EC-HFA, Sepabead EC-EP, PDCC and CTS beads after immobilization were 11%, 30%, 38%, 94 and 96%, respectively, as shown in Table 3.2. Sepabead EC-HFA and Sepabead EC-EP had approximately 30-40% of LsRN-Y246S activity immobilized on the matrices, with an approximately 50% lost in the enzymatic activity. Although PDCC had a good immobilization efficiency but the immobilized LsRN-Y246S on PDCC could be used only 3 times after which the immobilized LsRN-Y246S completely lost activity (data not shown). Surprisingly, 96% of LsRN-Y246S activity remained when immobilized on the CTS beads. Two percent of the total activity was found left over in the supernatant and only a 2% lost in activity was observed. When observed structure of CTS bead with SEM the result showed that the CTS bead had a solid core and the surface of the CTS bead has nano-scale fibrous like texture as shown in Figure 3.21.

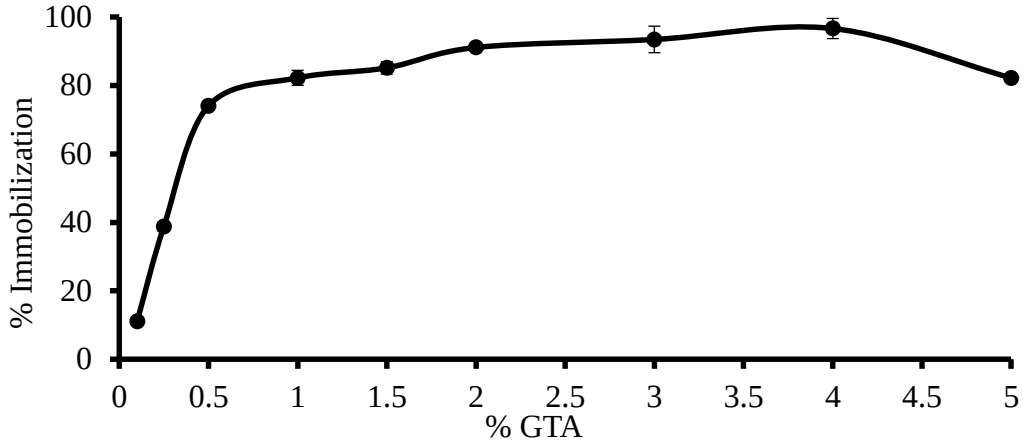


Figure 3.20 Effect of GTA concentration on cross-linking of LsRN-Y246S on the CTS bead

0.5 g (wet weight) of CTS bead was incubated with 250 μg LsRN-Y246S containing 40 U of activity and GTA in 50 mM citrate buffer pH 6.0 at 4 ºC under static condition for 2 d.

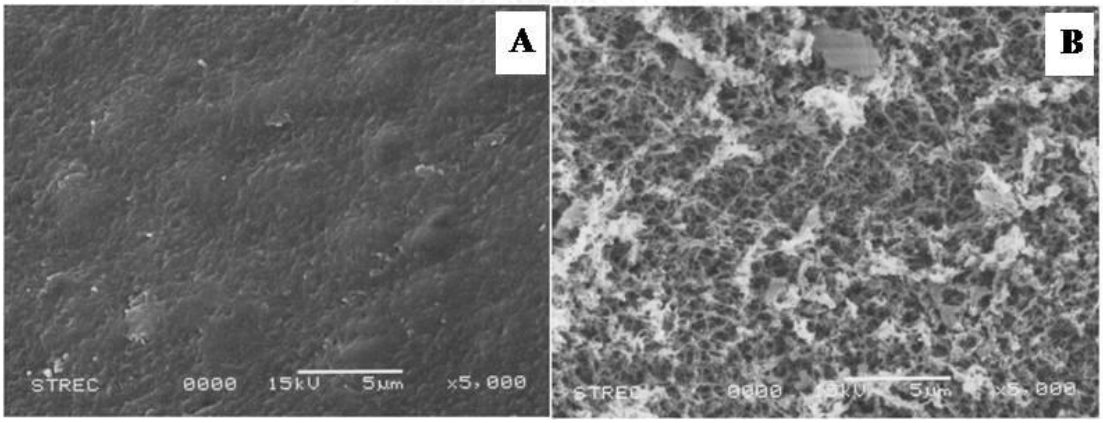


Figure 3.21 Scanning electron micrographs (SEM) of shrimp chitosan bead

A: cross-section of the CTS bead, at 5,000X magnification

B: outer surface of the CTS bead at 5,000X magnification

Table 3.2 Immobilization efficiency of LsRN-Y246S on various matrices

Matrix	Total activity 7 U and 50 μg Protein		
	% Activity on matrix	% Activity in supernatant	% Activity lost
CTS bead	96 (6.72 U)	2 (0.14 U), (1.15 μg Protein)	2
PDCC	93.57 (6.55 U)	4.57 (0.32 U), (2.19 μg Protein)	1
Fine particle chitosan	11 (0.77 U)	86 (6.02 U). (45 μg Protein)	2
Sepabead EC-EP	38 (2.66U)	15 (1.05 U), (7.65 μg Protein)	47
Sepabead EC-HFA	30 (2.10U)	18 (1.26 U), (9.17 μg Protein)	52

Characterization of immobilized LsRN

Optimum pH of LsRN activity

The optimum pH on the activity of free and immobilized LsRN were investigated using 10 mM Britton-Robinson Universal buffer at pH 3-12. The optimum pH of all LsRN derivatives were shown in Figure 3.22. The optimum pH of all free and immobilized LsRN were similar, at pH 6.0. However, the immobilized LsRN had a broader activity range in all LsRN derivatives. Moreover, it was observed that in all immobilized LsRN protected its activity in the acidic range (pH 3-5). All LsRN derivatives immobilized on CTS bead could retain more than 58% of their activity at pH range 3.0-7.0. An increased pH stability was also observed when Sepabead EC-EP and Sepabead EC-HFA beads were used as the matrix for LsRN immobilization, though quite inferior when compared to CTS beads.

Optimum temperature of LsRN activity

The optimum temperature on the activity of free and immobilized LsRN were investigated using temperature range of 10-80 ^{o}C. The optimum temperature for all LsRN derivatives when immobilized on supporting matrices were similar at 50 ^{o}C. All free LsRN derivatives had optimal activity at 40 ^{o}C, and exhibited higher activity than the immobilized LsRN at low temperatures, below 35 ^{o}C. However, at temperatures above 35 ^{o}C the immobilized LsRN was found to be more stable and showed a significant increase in enzymatic activity comparing to the free LsRN. The LsRN immobilized by covalently linking on the matrix may result in an increase of the enzyme stability by multiple point attachment while retaining the enzymatic activity.

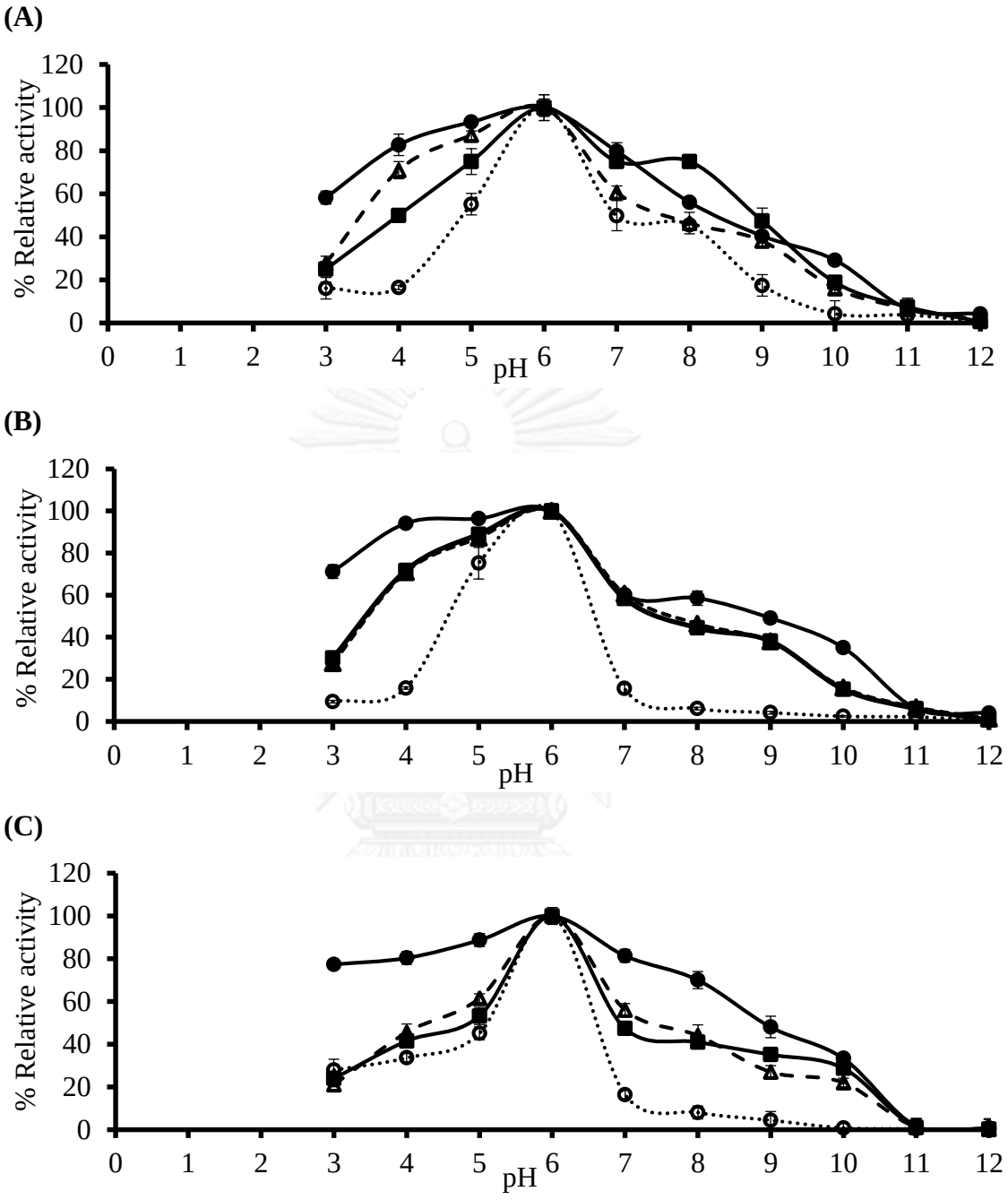


Figure 3.22 Effects of pH on the activity of free and immobilized LsRN

Two hundred milliunits of LsRN: (A) N251Y, (B) Y246S and (C) Y246W of (···o···) free levansucrase, (—●—) immobilized on CTS, (—■—) immobilized on EC-EP and (-▲-) immobilized on EC-HFA were incubated in 10 mM Britton-Robinson Universal buffer, 1.6% (w/v) sucrose, at 40 ^{o}C for 5 min.

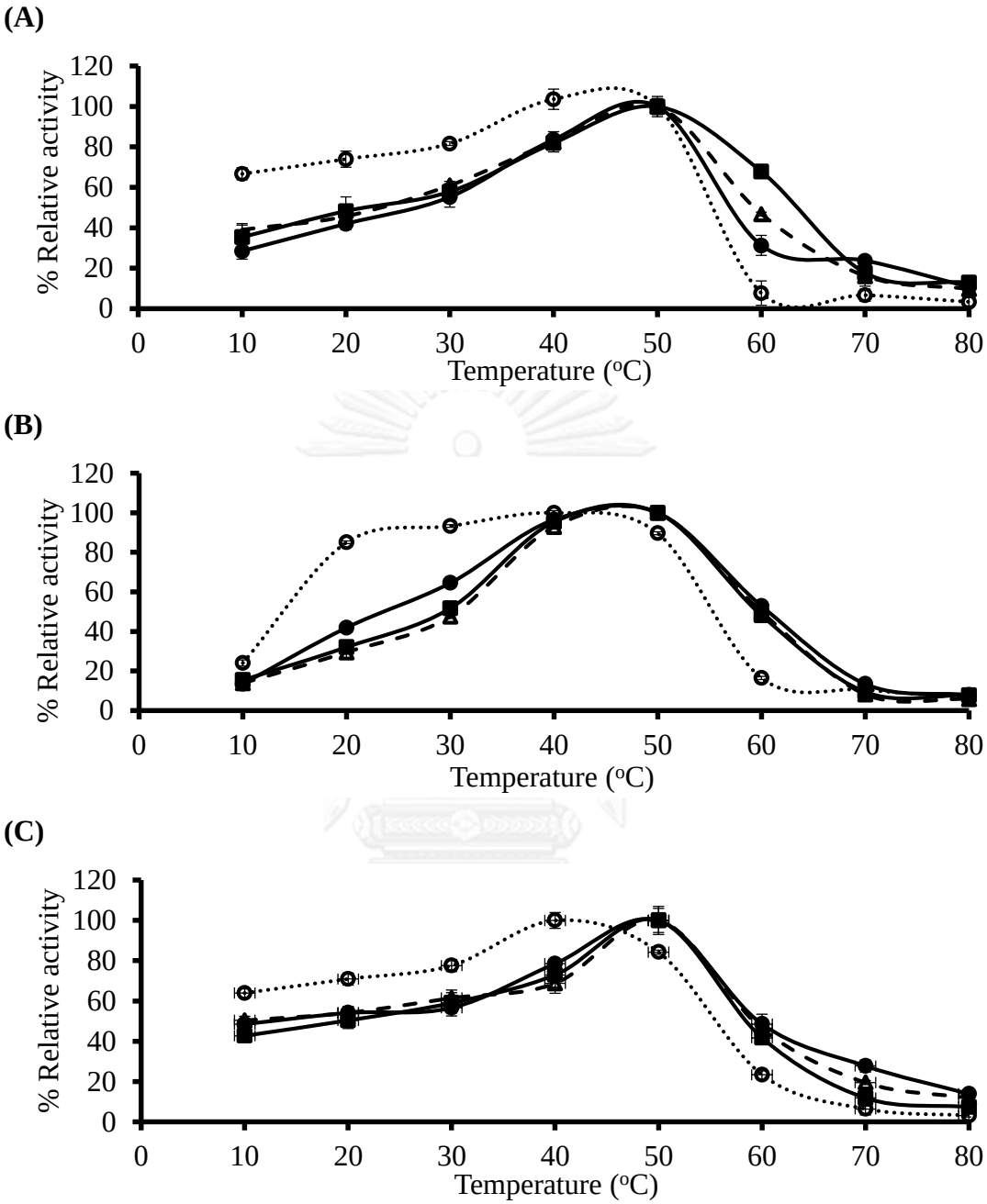


Figure 3.23 Effect of temperature on the activity of free and immobilized LsRN

Two hundred milliunits of LsRN: (A) N251Y, (B) Y246S and (C) Y246W of free levansucrase, immobilized on CTS, immobilized on EC-EP and immobilized on EC-HFA were incubated in 1.6% (w/v) sucrose in 50 mM citrate buffer, pH 6.0 for 5 min.

Effect of metal ions and chemical reagents on the activity of LsRN

The effect of metal ions and chemical reagents on the activity of free and immobilized LsRN were investigated from 5 mM of each of the metal ions or chemical reagents. The effects of various metal ions and chemical reagents on the activity of free and immobilized LsRN were shown in Figure 3.24. The results indicated that most metal ions tested slightly affect the activity of the all free and immobilized LsRN. Metal ions that had inhibitory effect on the activity of LsRN in either the free or immobilized form were Cu^{2+}, Zn^{2+} and Fe^{3+}. Ionic surfactant, SDS and chelating agents such as EDTA could also drastically inhibit the activity of both the free or immobilized LsRN. Immobilization of LsRN on CTS bead could slightly protect LsRN from the inactivation by Cu^{2+}, Zn^{2+} and Fe^{3+}, as well as SDS and EDTA, as trace activity was detected.

Interestingly, immobilizing LsRN on the CTS beads further enhanced the LsRN activity by Mn^{2+} more than 2 folds when compared with the effect observed on free enzyme. This phenomenon was not observed for LsRN immobilized on Sepabead EC-EP and Sepabead EC-HFA beads.

Effect of ionic strength on the activity of LsRN

The effect of ionic strength on the activity of free and immobilized LsRN was investigated by using 0-3 M NaCl. All immobilized LsRN derivative on all supporting matrices had an activity less than the free LsRN when increased the ionic strength over than 1 M of NaCl, shown in Figure 3.25.

Effect of sucrose concentration on the activity of LsRN

The effect of sucrose concentration on the activity of free and immobilized LsRN was investigated from 10-70% (w/v) sucrose. The result showed in Figure 3.26, at sucrose concentration ranging from 20%-40% (w/v) there were no significant differences in the activity of the free or immobilized LsRN. However at sucrose concentration above 40% (w/v) we observed that immobilized LsRN on CTS, Sepabead EC-EP and Sepabead EC-HFA beads could retain more of its activity than free LsRN. The concentration of sucrose up to 60% (w/v) could be used with the immobilized LsRN on the CTS beads, retaining more than 90% of activity in all LsRN derivatives.

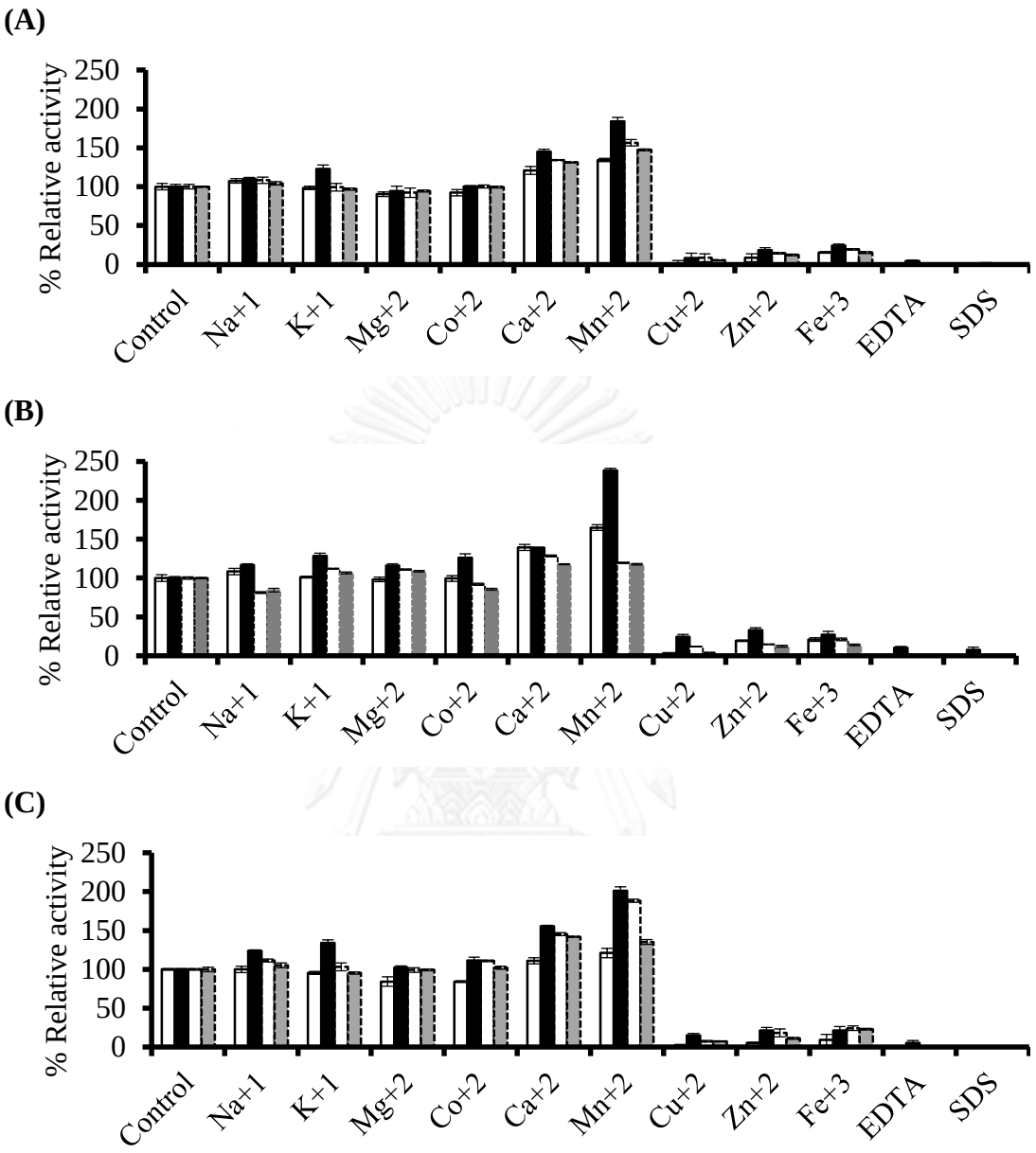


Figure 3.24 Effect of metal ions and chemical reagents on the activity of free and immobilized LsRN

Two hundred milliunits of LsRN: (A) N251Y, (B) Y246S and (C) Y246W of (□) free levansucrase, (■) immobilized on CTS, (⬚) immobilized on EC-EP and (⬚) immobilized on EC-HFA were incubated with 1.6% (w/v) sucrose in 50 mM citrate buffer, pH 6.0 supplemented with 5 mM of each of the metal ions or chemical reagents, and incubated at 40 ^{o}C for 5 min.

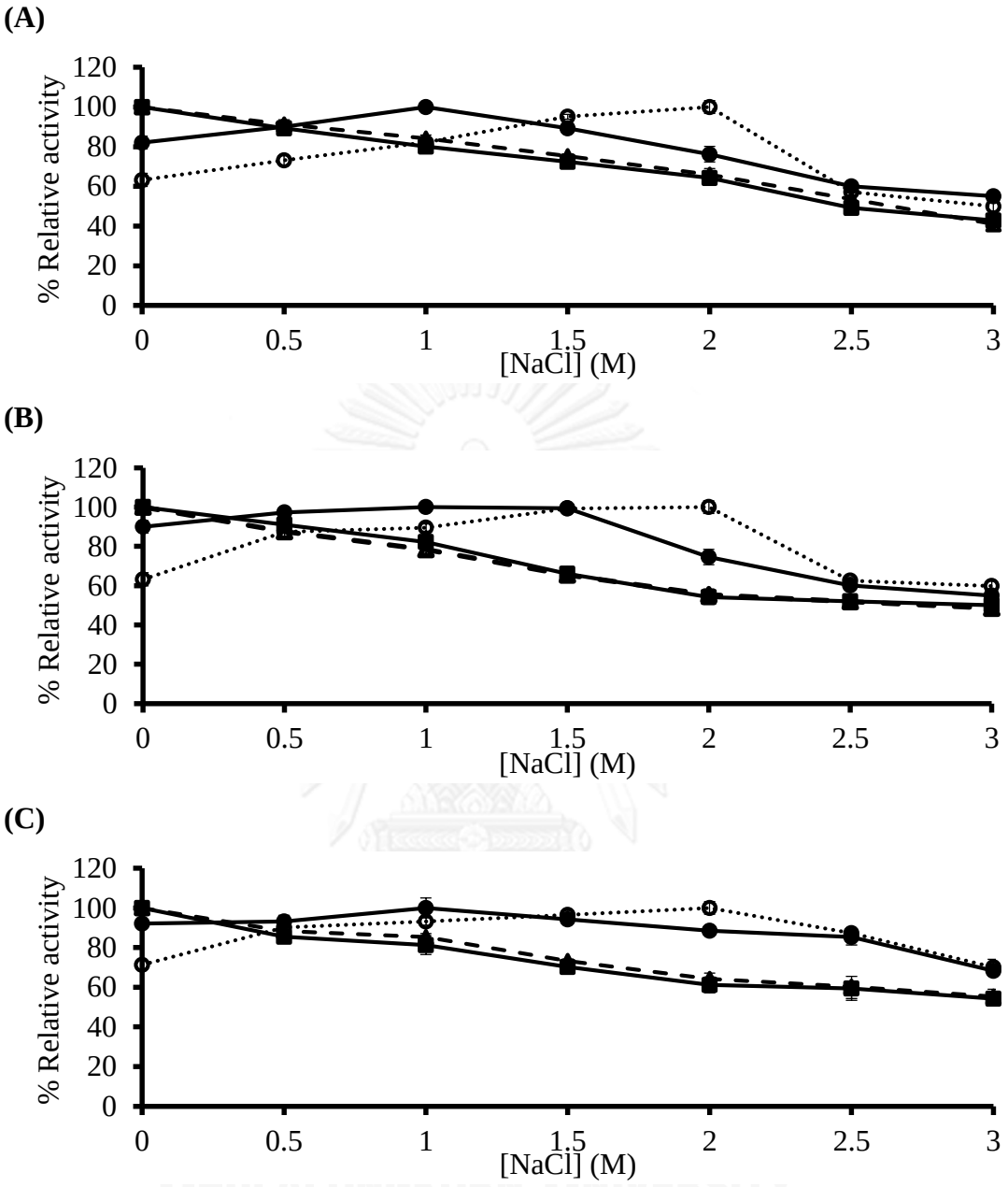


Figure 3.25 Effect of ionic strength on the activity of free and immobilized LsRN

Two hundred milliunits of LsRN: (A) N251Y, (B) Y246S and (C) Y246W of free levansucrase, immobilized on CTS, immobilized on EC-EP and immobilized on EC-HFA were incubated with NaCl in 1.6% (w/v) sucrose and 50 mM citrate buffer, pH 6.0, at 40 ºC for 5 min.

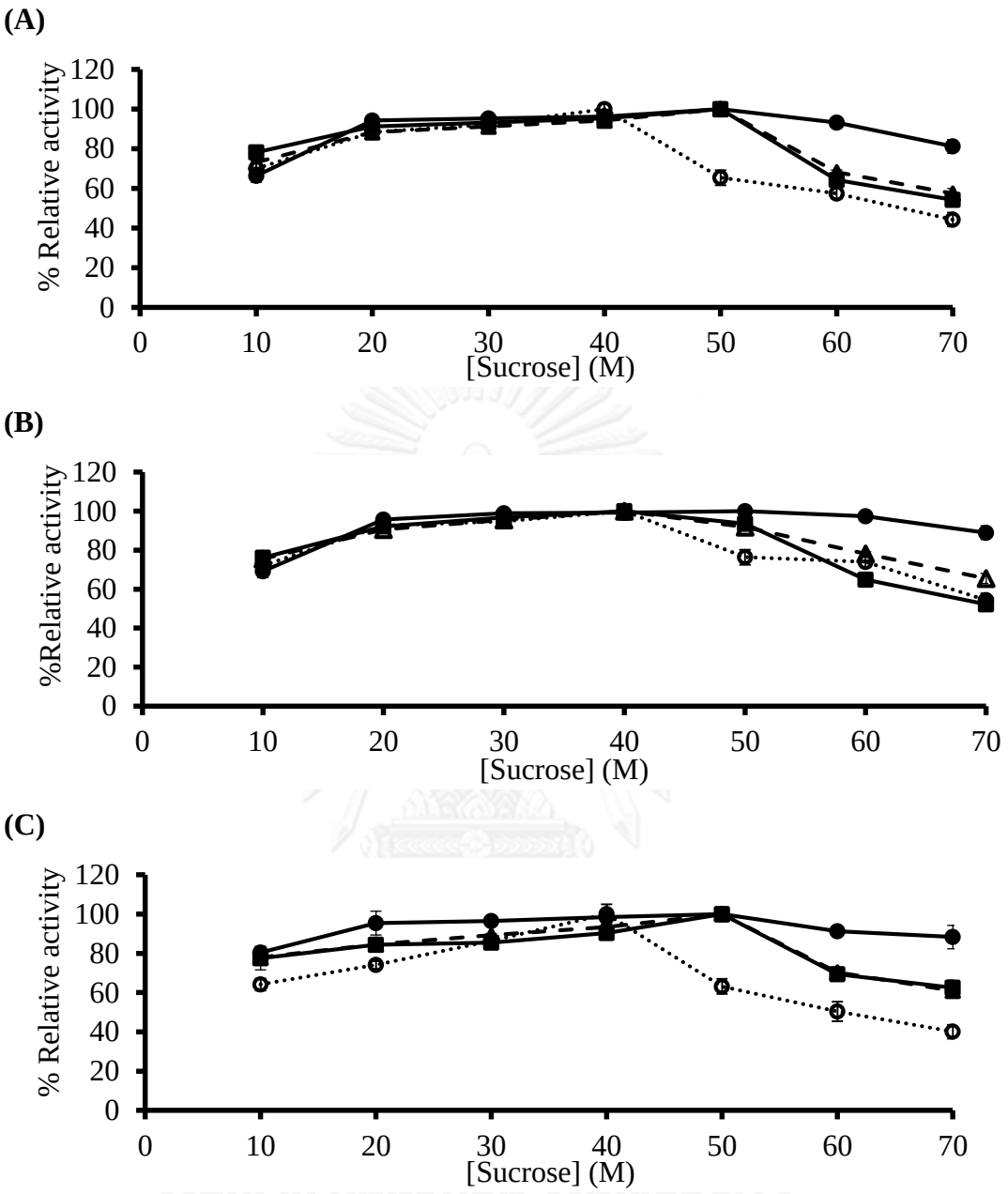


Figure 3.26 Effect of sucrose concentration on the activity of free and immobilized LsRN

Two hundred milliunits of LsRN: (A) N251Y, (B) Y246S and (C) Y246W of (···o···) free levansucrase, (—●—) immobilized on CTS, (—■—) immobilized on EC-EP and (- -▲- -) immobilized on EC-HFA were incubated with 10-70% (w/v) sucrose in 50 mM citrate buffer, pH 6.0, at 40 ^{o}C for 5 min**.**

LsRN stability

The stability of free and immobilized LsRN were investigated by pre-incubating 200 mU of free and immobilized LsRN in 50 mM citrate buffer, pH 6.0 at 40 ^{o}C for 12 h. When the free and immobilized LsRN were subjected to prolonged incubation at its optimum temperature of 40 ^{o}C, the immobilized LsRN retained higher activity than the free LsRN. The free LsRN rapidly lost its activity more than 50% within the first 2 h (Figure 3.27). The LsRN immobilized on the CTS beads showed a better stability than the LsRN immobilized on Sepabead EC-EP and Sepabead EC-HFA, which showed similar stability profile.

Storage stability

The storage stability of the immobilized LsRN was investigated by storing the immobilized LsRN in 50 mM citrate buffer, pH 6.0 at temperature of 4 ^{o}C for 3 mo. The immobilized LsRN derivatives on supporting matrices showed good stability in all supporting matrices. When storage immobilized LsRN derivatives on all supporting matrices, activity of LsRN-N251Y, Y246S and Y246Ws were retained about 50% of its activity for 10, 14 and 11 wk, respectively (Figure 3.28).

The reusability of immobilized LsRN

The LsRN-Y246S immobilized on CTS beads retained over 75% of its activity after 10 cycles of repetitive use and over 60% activity after 17 cycles of repeated use. In contrast, Sepabead EC-EP and Sepabead EC-HFA lost 67% and 70% of its activity after only 5 cycles of repeated use, shown in Figure 3.29. Moreover, The CTS bead retained the activity more than 50% after used for 8 and 10 cycles of LsRN-N251Y and Y246W, respectively. However, Sepabead EC-EP and Sepabead EC-HFA lost its activity of LsRN-N251Y and Y246W over 50% only 4 and 8 cycles of repetitive use, respectively.

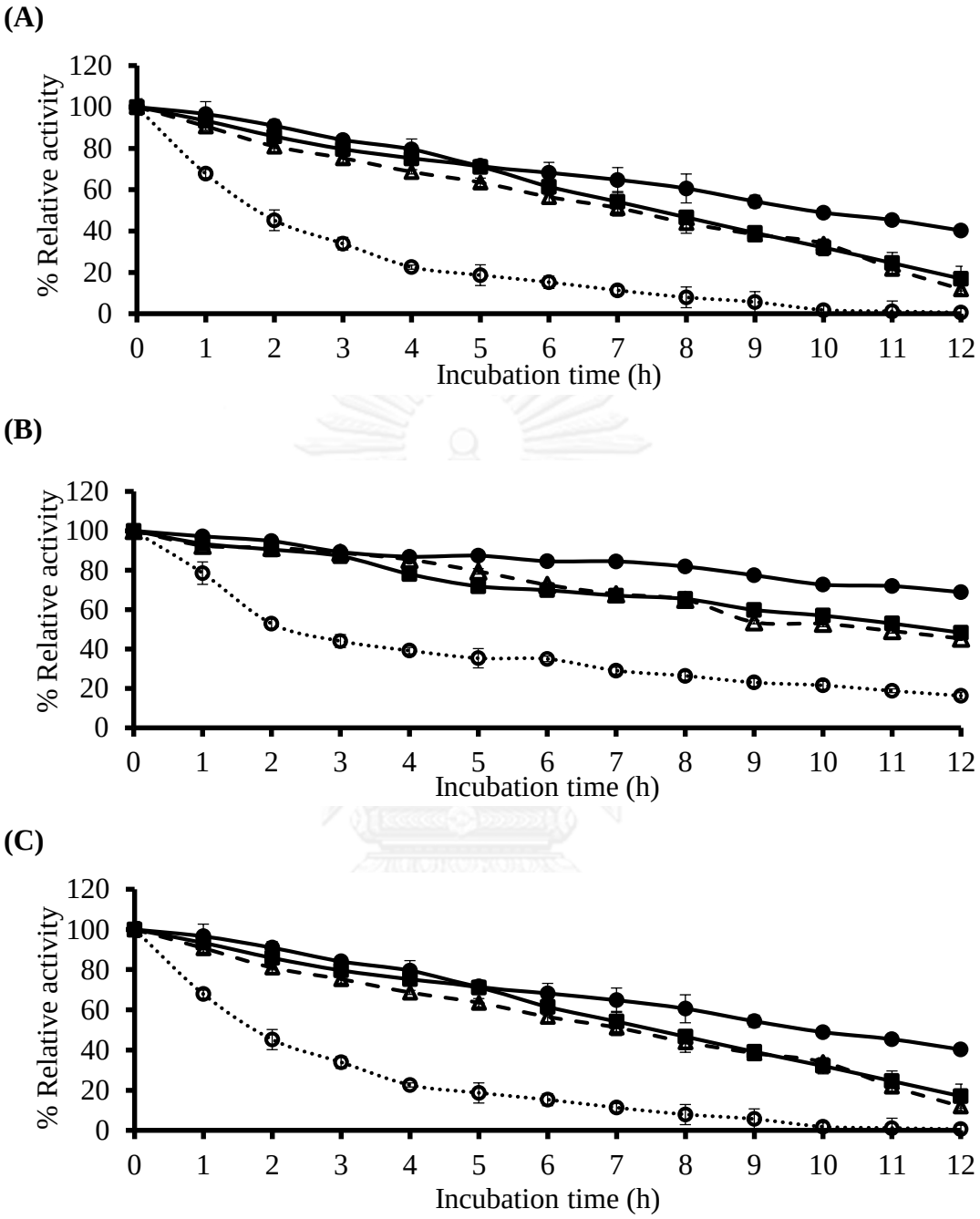


Figure 3.27 Stability of free and immobilized LsRN

The stability of LsRN: (A) N251Y, (B) Y246S and (C) Y246W of (···o···) free levansucrase, (—●—) immobilized on CTS, (—■—) immobilized on EC-EP and (–▲–) immobilized on EC-HFA was investigated by pre-incubating the 200 mU of free and immobilized LsRN in 50 mM citrate buffer, pH 6.0 at 40 ^{o}C for 12 h.

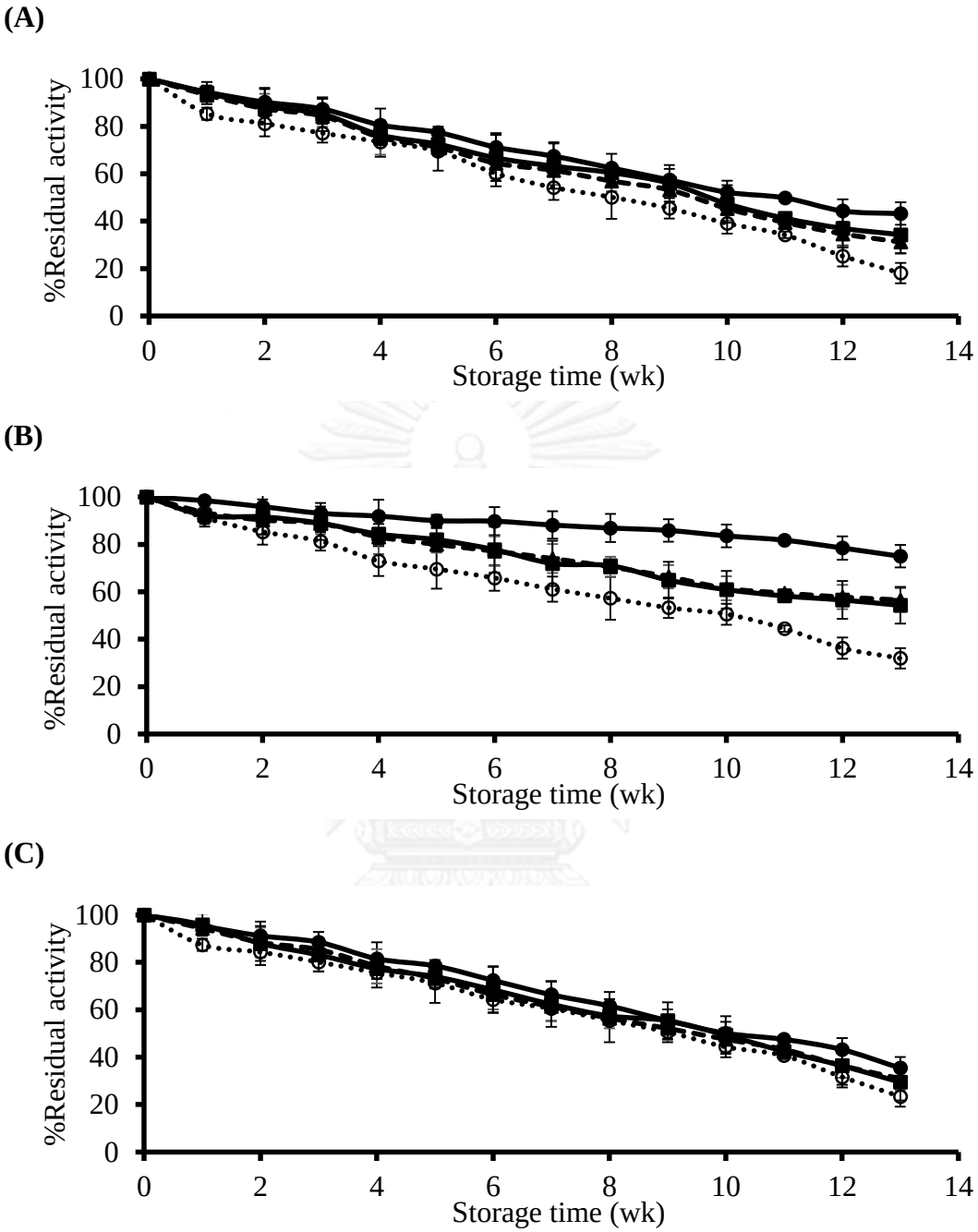


Figure 3.28 Storage stability of immobilized LsRN

The storage stability of LsRN: (A) N251Y, (B) Y246S and (C) Y246W of (···o···) free, (—●—) immobilized on CTS, (—■—) immobilized on EC-EP and (-▲-) immobilized on EC-HFA was investigated by stored the immobilized LsRN in 50 mM citrate buffer, pH 6.0 at temperature of 4 ºC for 3 mo.

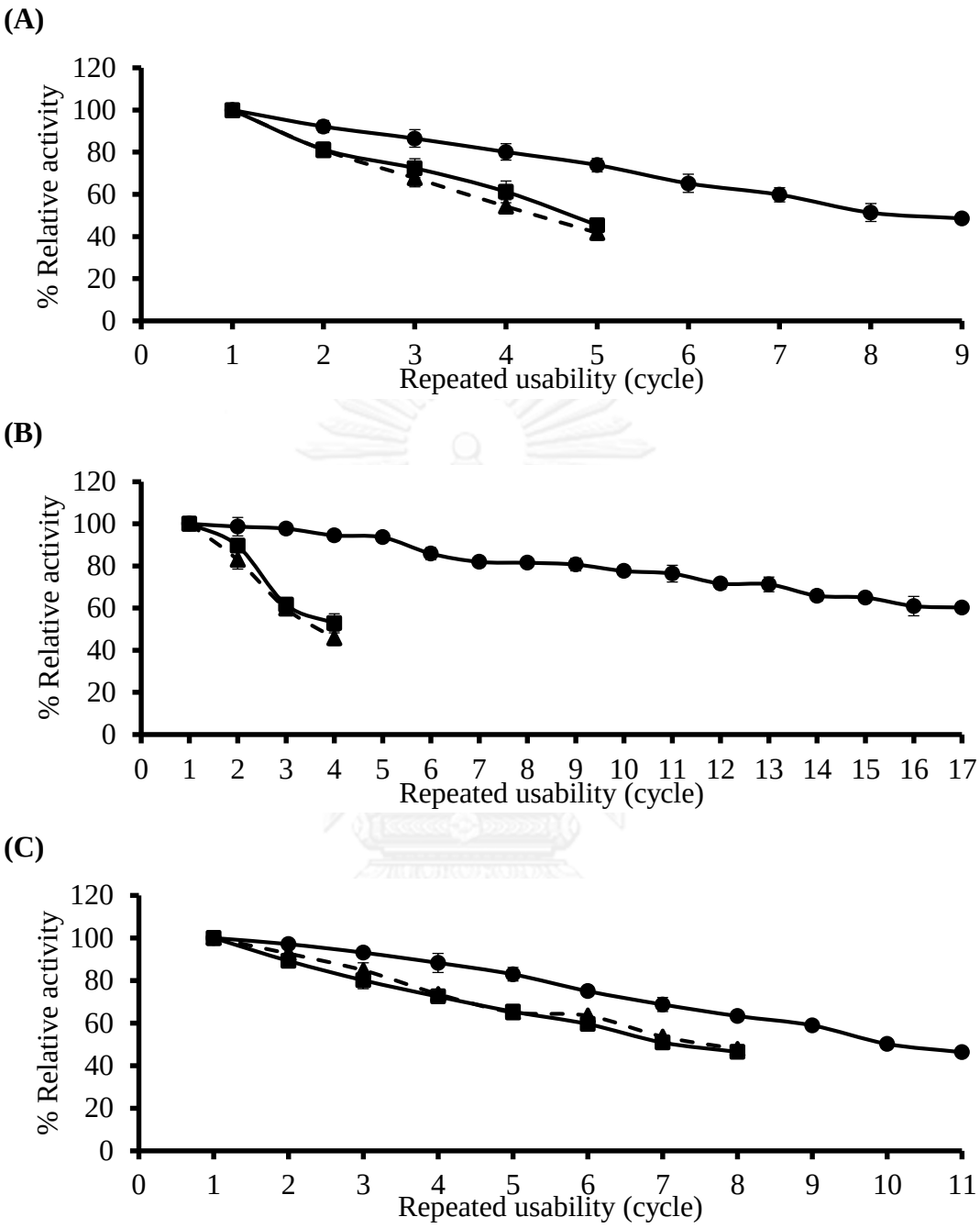


Figure 3.29 Reusability of immobilized LsRN

Two hundred milliunits of LsRN: (A) N251Y, (B) Y246S and (C) Y246W of (—●—) immobilized on CTS, (—■—) immobilized on EC-EP and (- -▲- -) immobilized on EC-HFA were incubated in 1.6% (w/v) sucrose and 50 mM citrate buffer, pH 6.0, at 40 ^{o}C for 5 min.

Product analysis by TLC

The products analyzed by TLC were shown in Figure 3.30. The result demonstrated that immobilized LsRN could completely hydrolyze sucrose and synthesize products within 12 h. However, free LsRN did not completely utilize sucrose substrate, as large amount of sucrose was still presented and only small amount of high molecular weight L-FOS was produced.

Effect of substrate and immobilized LsRN activity ratio on the production of L-FOS

The effect of ratio of substrate: immobilized LsRN activity (1 g sucrose: 1 U of immobilized LsRN) on the production of L-FOS were investigated at 1:1, 1:10, 1:20, 1:30 1:40 and 1:50. As shown in Figure 3.31, L-FOS was produced around 7.1 g, using the ratio of substrate and LsRN activity of 1:20. The ratio of 1:20 was used for further experiments.

Effect of sucrose concentrations on sucrose conversion to L-FOS

The effect of sucrose concentration on the sucrose conversion to L-FOS was investigated by using 10-70% (w/v) sucrose. The result was shown in Figure 3.32. This result revealed that the trend of sucrose conversion of all LsRN derivatives were similar. When increasing the sucrose concentration from 10% to 50%, the products of L-FOS were immediately increased. However, when the sucrose concentration was increased more than 50% and up to 70%, the products of L-FOS were gradual increased.

Production of L-FOS by immobilized LsRN

All LsRN derivatives immobilized on chitosan beads were used for the L-FOS production. As shown in Figure 3.33, trends of reaction of all LsRN derivatives were similar. At the first 3 h the conversion yield for the production of L-FOS was slightly increased. According by the reaction rate was gradually progressed. From 3 to 5 h, the reaction rate was enhanced, resulting in a significant increase in the amount of L-FOS products. The reaction reached equilibrium after the reaction was prolonged over 5 h.

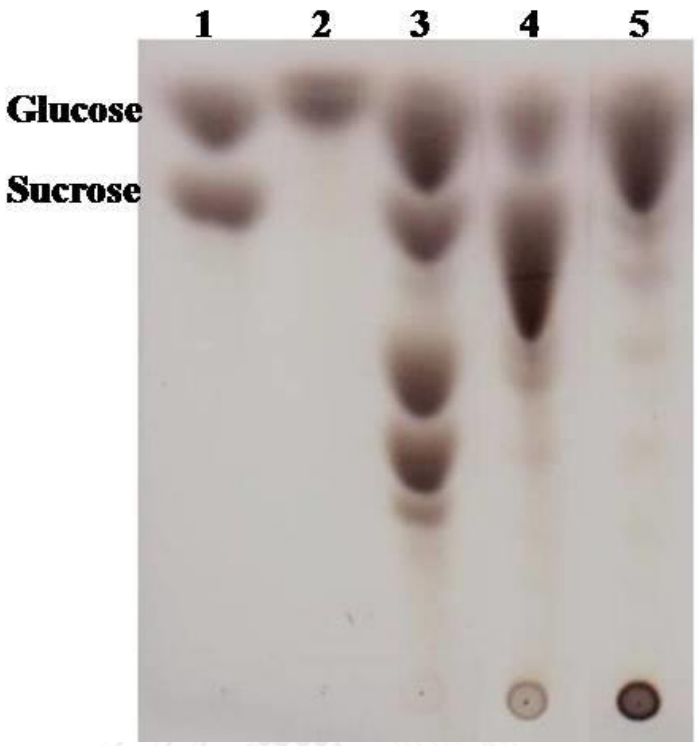


Figure 3.30 TLC chromatogram of the reaction products

The reaction condition consist of 1 U of either free or immobilized LsRN, 20% (w/v) Sucrose, 50 mM citrate buffer pH 6.0 at 50 ^{o}C for 12 h with agitation at 200 rpm. The TLC plate was ran in a mobile phase containing acetic acid: butanol: distilled water at the ratio of 3 : 3 : 2. The TLC plate was stained with ethanol: sulfuric acid ratio 9 : 1. Lane 1: standards glucose (G) and sucrose (S), lane 2: standards fructose (F), lanes 3: standards oligosaccharide (F_2, F_3, F_4 and F_5), lane 4: products from free levansucrase, lane 5: products from immobilized levansucrase on CTS.

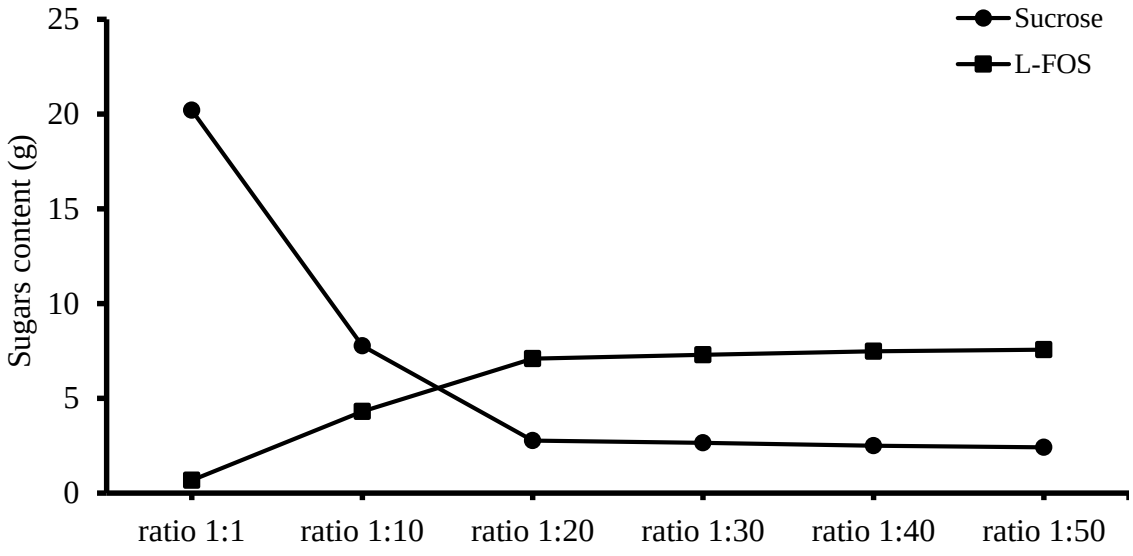


Figure 3.31 Effect of substrate and immobilized LsRN-Y246S activity ratio on the production of L-FOS

The effect of ratio of substrate: immobilized LsRN-Y246S activity (1 g sucrose: 1 U of immobilized LsRN-Y246S) on the production of L-FOS were investigated at 1:1, 1:10, 1:20, 1:30 1:40 and 1:50. 50 mL of substrate was contained with 50% (w/v) sucrose in 50 mM citrate buffer, pH 6.0. The reaction was incubated with immobilized LsRN-Y246S, at 40 ºC for 12 h.

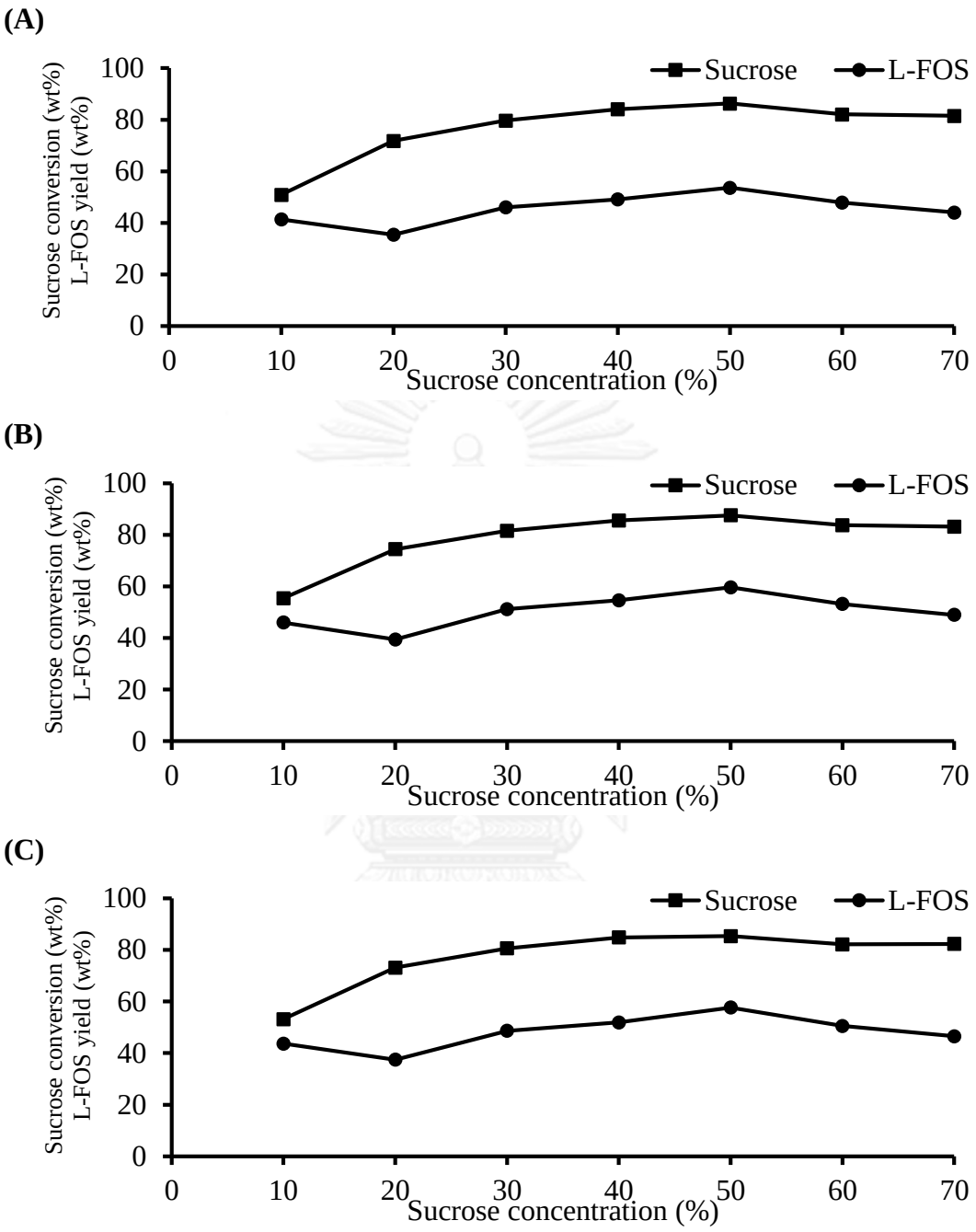


Figure 3.32 Effect of sucrose concentrations on sucrose conversion and L-FOS yield

Five hundred units of immobilized LsRN: (A) N251Y, (B) Y246S and (C) Y246W were incubated with 50 mL of 10-70% (w/v) sucrose in 50 mM citrate buffer, pH 6.0, 40 ^{o}C for 12 h in an orbital shaker, at 150 rpm**.**

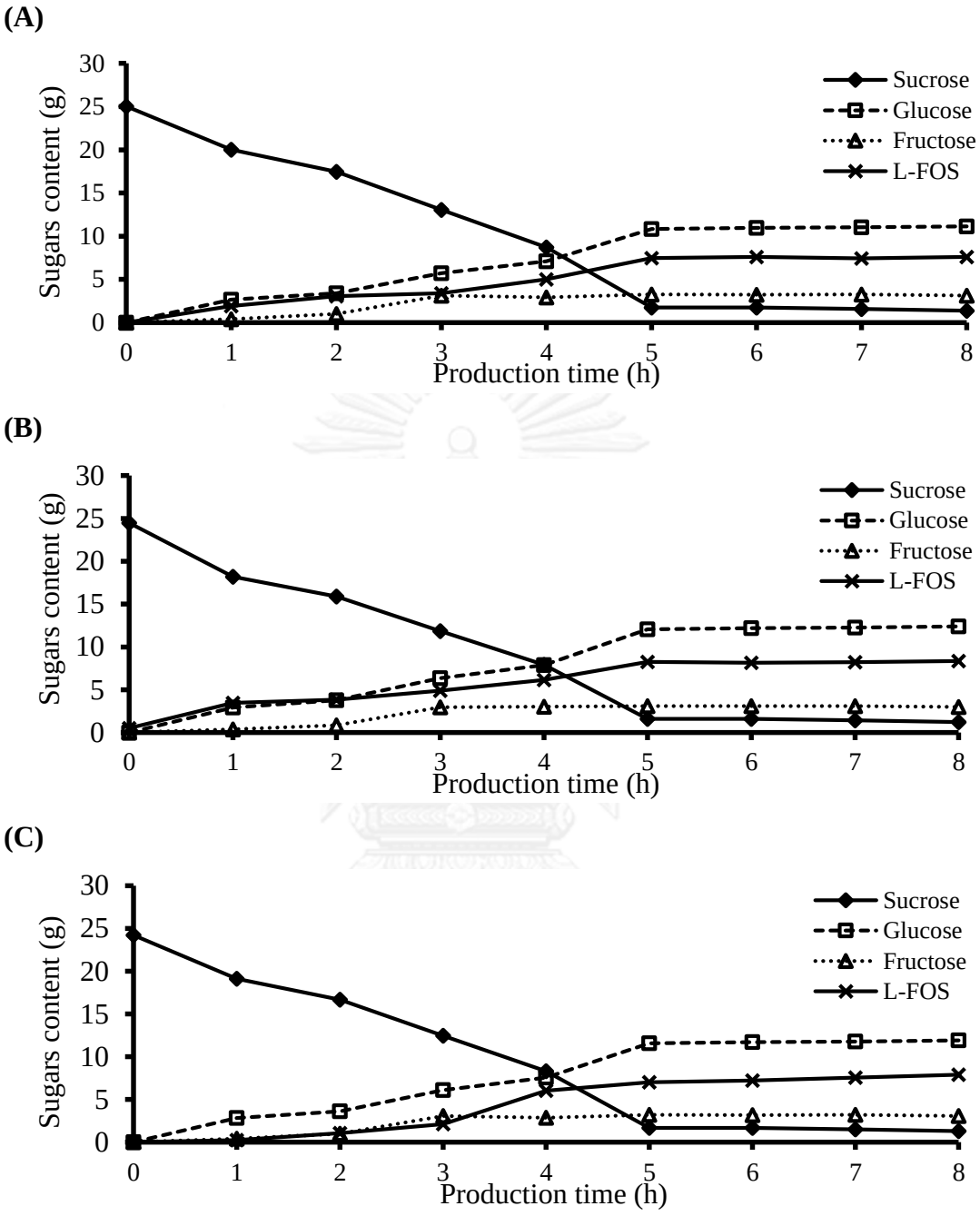


Figure 3.33 Batch production of L-FOS by immobilized LsRN

Five hundred Units of immobilized LsRN on CTS bead: (A) N251Y, (B) Y246S and (C) Y246W were used for the L-FOS production. Substrate was contained of typically 50 mL, 50% (w/v) sucrose in 50 mM citrate buffer, pH 6.0. The reaction was incubated at temperature of 40 ^{o}C for 12 h in an orbital shaker (150 rpm).

Reusability of immobilized LsRN on the production of L-FOS

The reusability of immobilized LsRN for the production of L-FOS was determined by measuring the efficiency of the immobilized LsRN after repeated use. The repeated use of immobilized LsRN on CTS bead was shown in Figure 3.34. LsRN-N251Y immobilized on CTS bead could be reused for 5 cycles for production of L-FOS was 66% (5.43 g) from the first cycle (8.23 g). The efficiency for L-FOS production of immobilized LsRN-Y246S was retained about 75% (6.26 g) after repeated use for 5 cycles (8.36 g from first cycle). And 71% (5.43 g) efficiency of repeated use for 5 cycles (7.62 g from first cycle) of LsRN-Y246W was observed.

Process design and optimization of L-FOS production by immobilized LsRN

Effect of substrate feed direction on the production of L-FOS

The effect of substrate feed direction on the production of L-FOS was investigated from LsRN-Y246S. The substrates were feed into the column by upward or downward flow directions with flow rate of 4 mL/min. The result indicated that upward flow of substrate had higher efficiency than downward flow. Approximately 3.57 g of L-FOS was observed after 3 h proceeded and slightly increased to 7.93 g at 8 h when feed substrate with upward flow, shown in Figure 3.35.

Effect of flow pattern on the production of L-FOS

The effect of flow pattern on the production of L-FOS was investigated. The samples were feed into the column using single and multiple pass flow. For the multiple pass flow, samples were feed into the column with flow rate of 4 mL/min. The single pass flow, samples were feed into the column with flow rate 0.34 mL/min. The result showed that L-FOS products were produced 5.47 g at 12 h when feed sample with single pass flow. In contrast, the multiple pass flow produced 7.93 g of L-FOS products in 8 h, shown in Figure 3.36. Therefore, multiple pass flow was more favorable for the production of L-FOS.

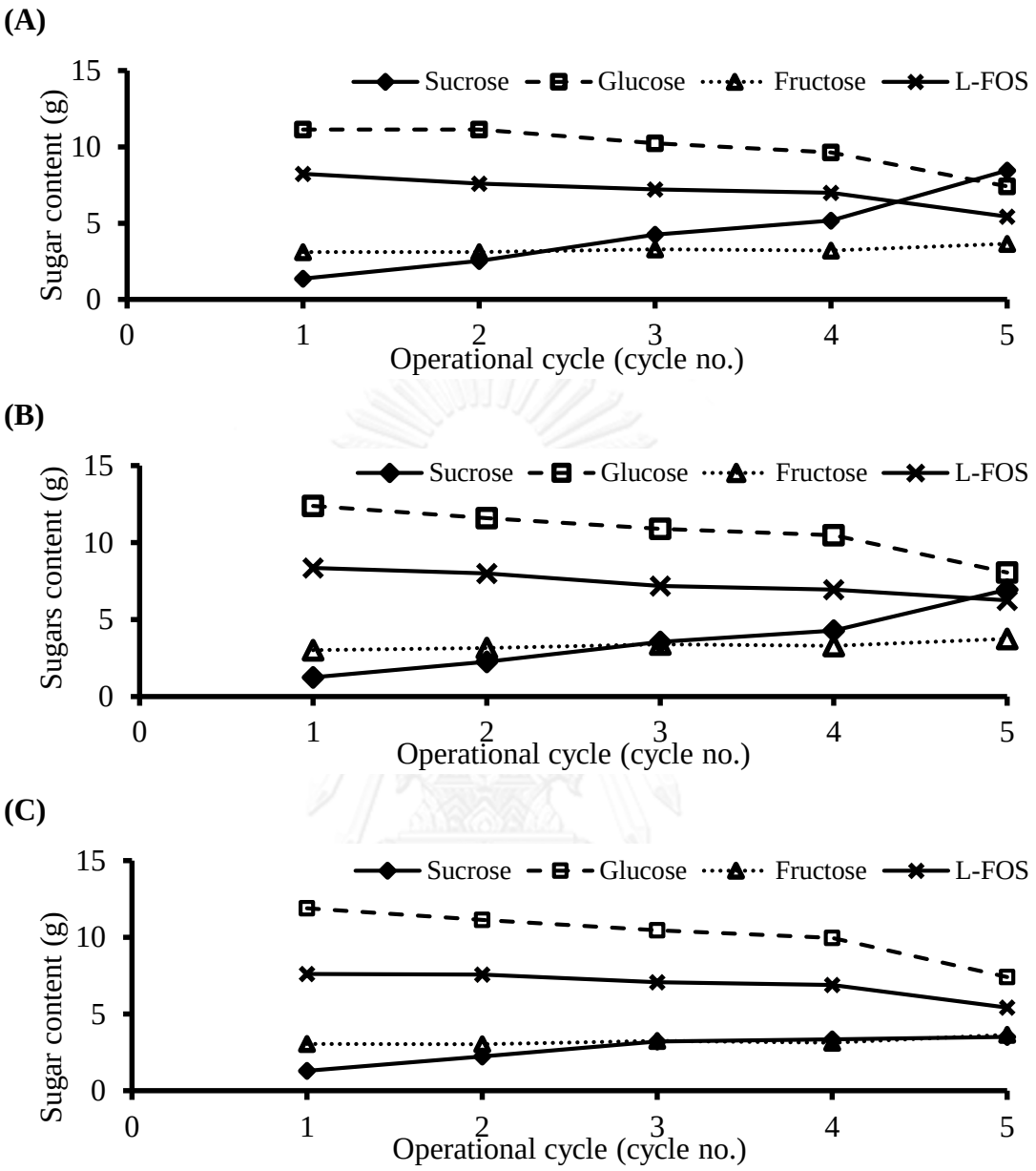


Figure 3.34 Reusability of immobilized LsRN for L-FOS production

The reusability of LsRN: (A) N251Y, (B) Y246S and (C) Y246W immobilized on CTS bead for the production L-FOS were determined by measuring the efficiency of the immobilized LsRN after repeated use. 50 mL of substrate was contained with 500 U of immobilized LsRN, 50% (w/v) sucrose in 50 mM citrate buffer, pH 6.0. The reaction was incubated at 40 ^{o}C under shaking at 150 rpm on orbital shaker.

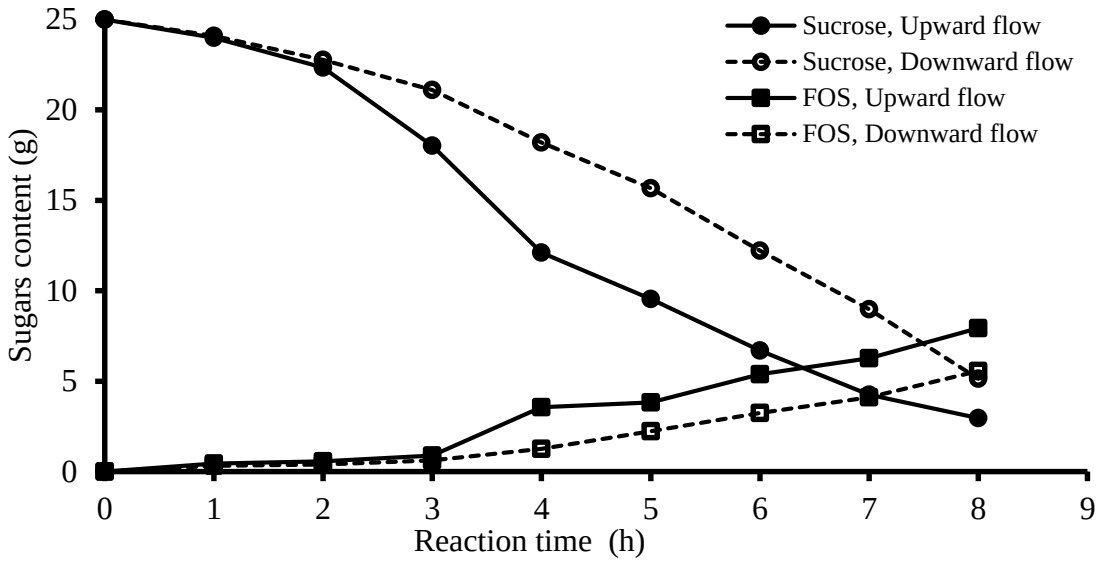


Figure 3.35 Effect of substrate feed direction on the production of L-FOS by immobilized LsRN-Y246S

The substrates were feed into the column by upward and downward flow directions. Five hundred units of immobilized LsRN-Y246S were packed in double jacket column. 50 mL of substrate containing 50% (w/v) sucrose in 50 mM citrate buffer, pH 6.0, was feed into the column with flow rate of 4 mL/min at temperature 40 ^{o}C.

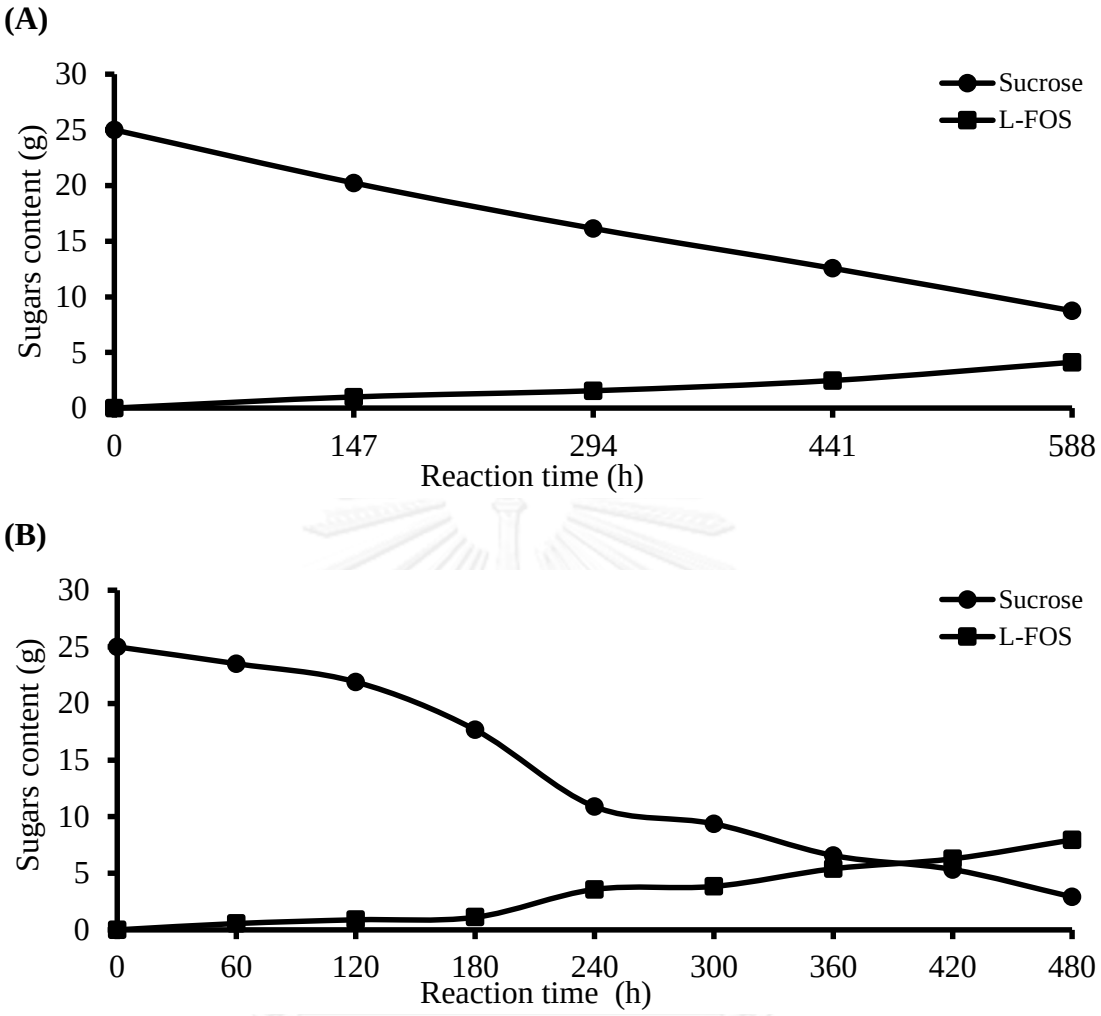


Figure 3.36 Effect of flow pattern on the production of L-FOS by immobilized LsRN-Y246S

(A) single pass flow, samples were feed into the column with flow rate 0.34 mL/min. (B) multiple pass flow, samples were feed into the column with flow rate of 4 mL/min. Five hundred units of immobilized LsRN-Y246S were packed in double jacket column. 50 mL of substrate containing 50% (w/v) sucrose in 50 mM citrate buffer, pH 6.0, at temperature 40 ^{o}C, was feed into the column with upward flow.

Effect of flow rate of substrate on the production of L-FOS

The effect of flow rate of substrate on the production of L-FOS was investigated by 2, 4, 6, 8 and 10 mL/min. The result showed that at flow rate range from 2 – 8 mL/min there were no significantly difference in the rate of the reaction, shown in Figure 3.37. Interestingly, when the flow rate was increased to 10 mL/min the reaction rate significantly increased when compared to low substrate flow rate.

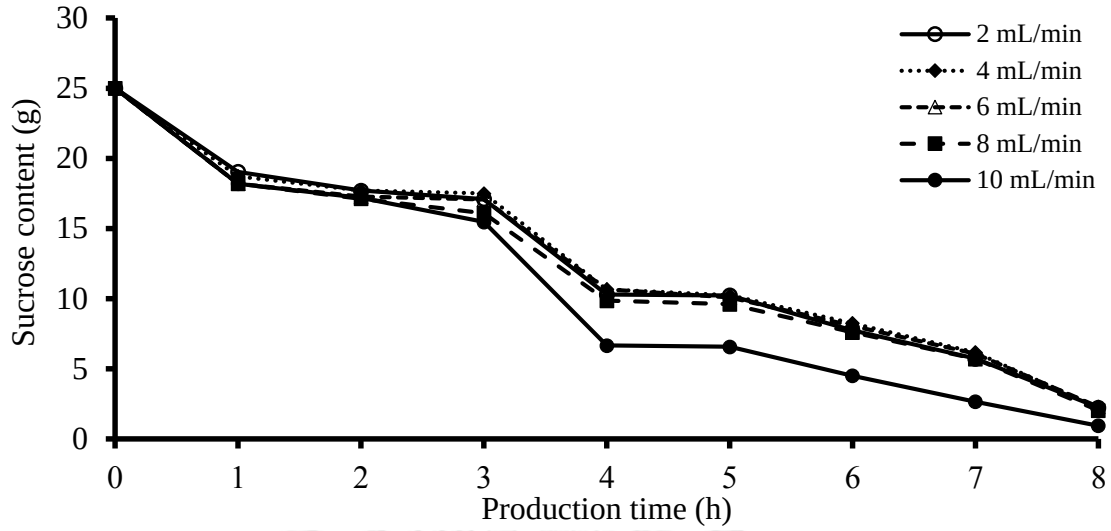
Operational production of L-FOS by immobilized levansucrase

The operational production of L-FOS by using packed immobilized levansucrase reactor was determined by measuring the efficiency of the immobilized levansucrase after repeated use. The L-FOS products were produced by LsRN-N251Y for 7.83 g at the first cycle and decreased to 5.22 g at fifth cycle. LsRN-Y246S was used to produce L-FOS about 8.36 g and 6.26 g for the first and fifth cycle, respectively. The 8.14 g and 4.56 g of L-FOS at the first and fifth cycle, respectively were produced from LsRN-Y246W (Figure 3.38).

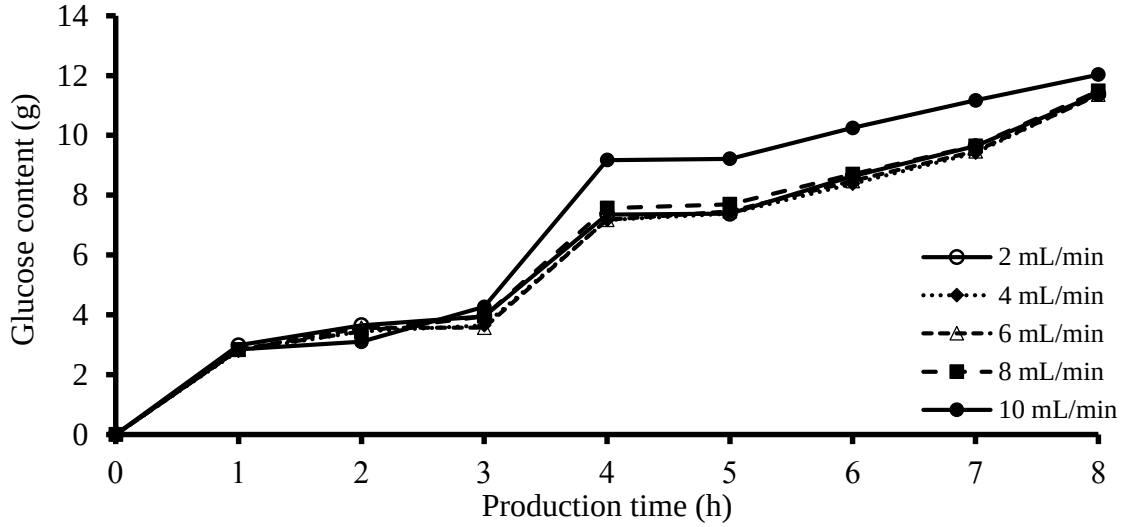
Scale up production of L-FOS

Scale up production of L-FOS was performed by using packed-bed immobilized levansucrase reactor. As shown in Figure 3.39, the result demonstrated that L-FOS products yield at 12 h of production time was 118.39 g and 130.45 g from LsRN-Y246S and Y246W, respectively. However, when the production time was extended to 24 h, the L-FOS yields were slightly increased 7.09 g and 4.84 g from LsRN-Y246S and Y246W, respectively. LsRN-N251Y could produce the L-FOS products of 102.93 g at 24 h.

(A)



(B)



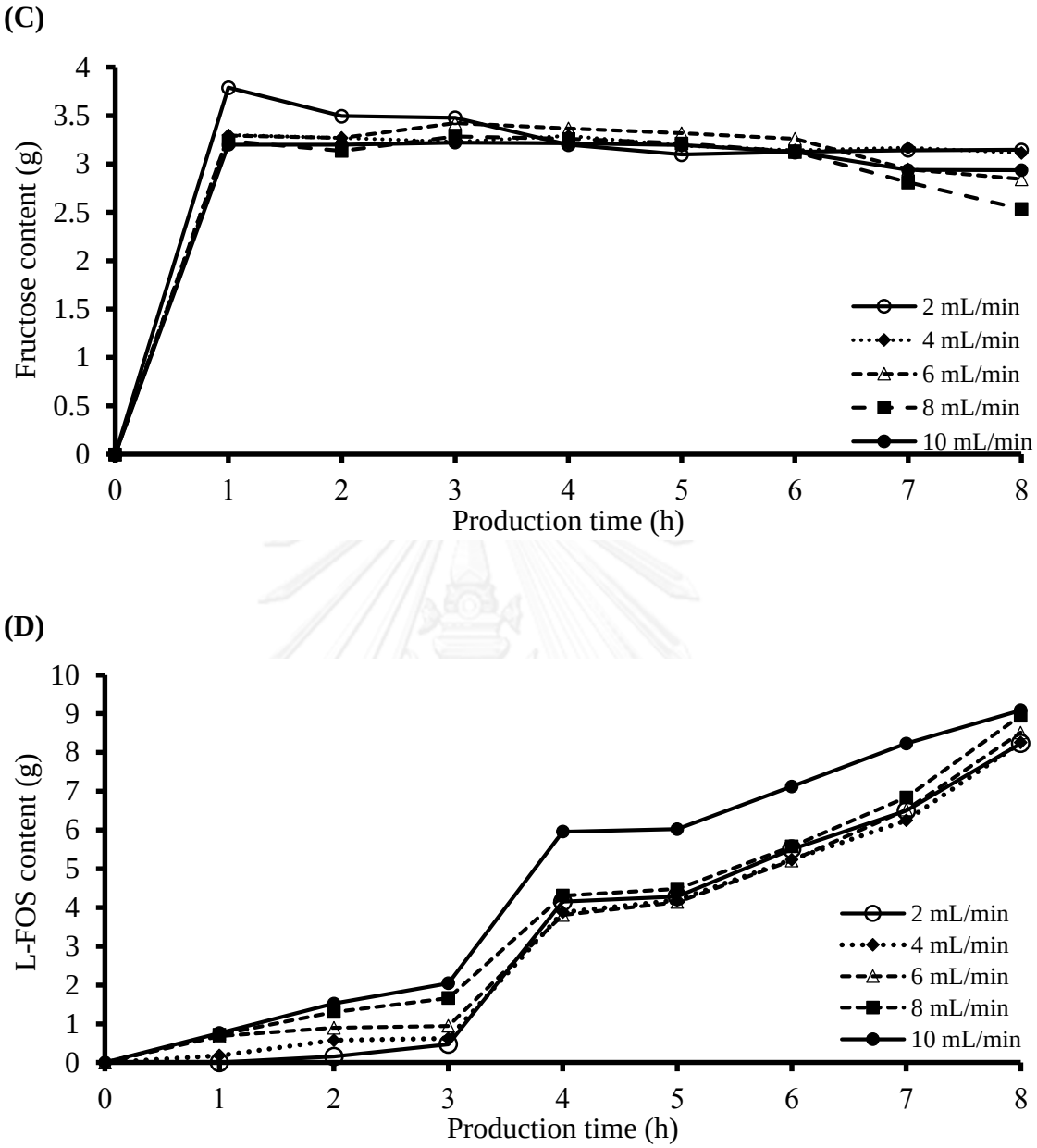


Figure 3.37 Effect of flow rate of substrate on the production of L-FOS by immobilized LsRN-Y246S

The effect of flow rate of substrate on the production of L-FOS was investigated by 2, 4, 6, 8 and 10 mL/min. Five hundred units of immobilized LsRN-Y246S was packed in double jacket column. 50 mL of substrate containing 50% (w/v) sucrose in 50 mM citrate buffer, pH 6.0, incubated at temperature of 40 ºC, was feed into the column with upward flow.

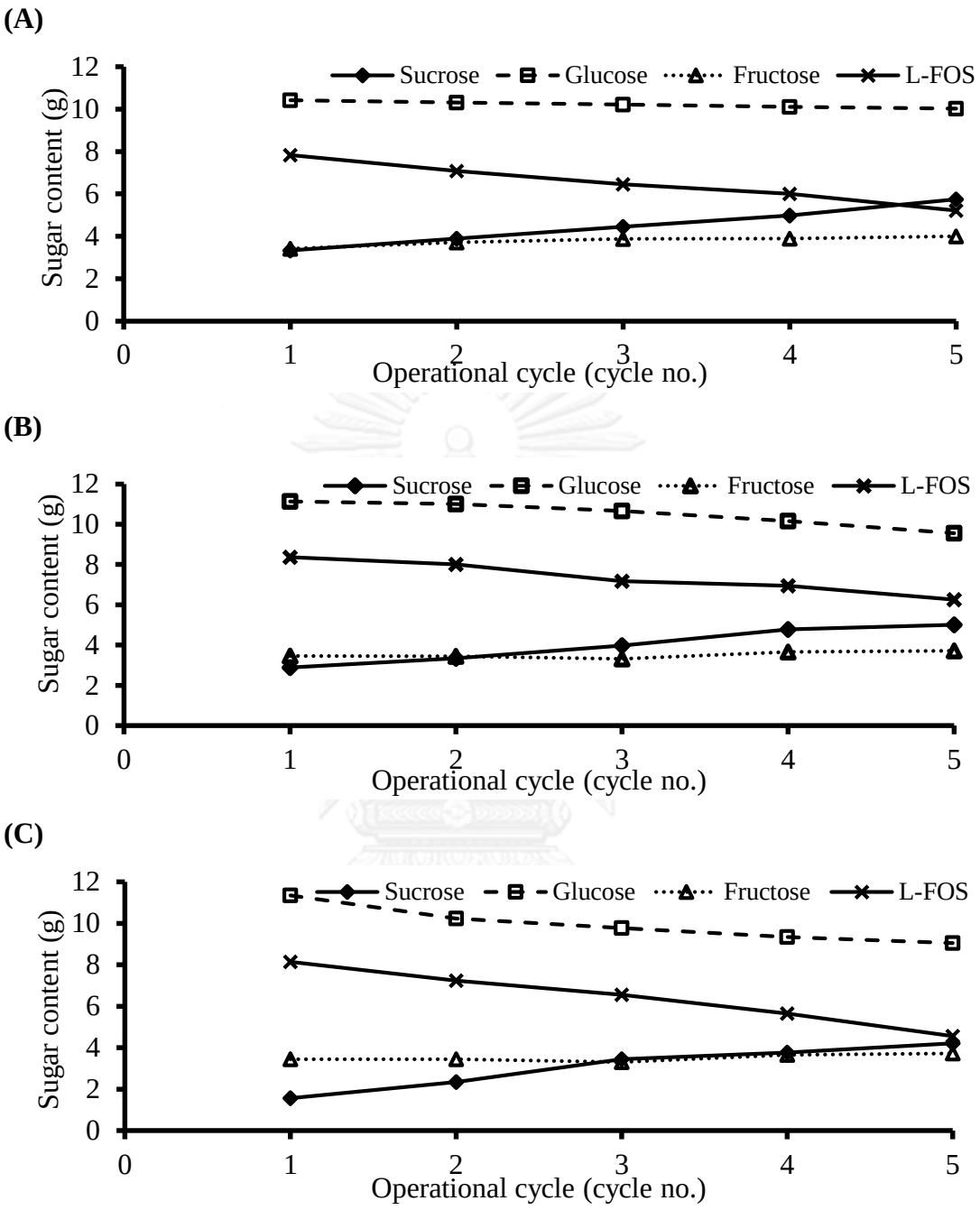


Figure 3.38 Operational production of L-FOS by packed immobilized LsRN

The repeated use of LsRN: (A) N251Y, (B) Y246S and (C) Y246W was investigated. Five hundred units of immobilized levansucrase in double jacket column. 50 mL of substrate containing 50% (w/v) sucrose in 50 mM citrate buffer, pH 6.0, 40 ^{o}C, was feed into the column with upward flow at flow rate of 10 mL/min.

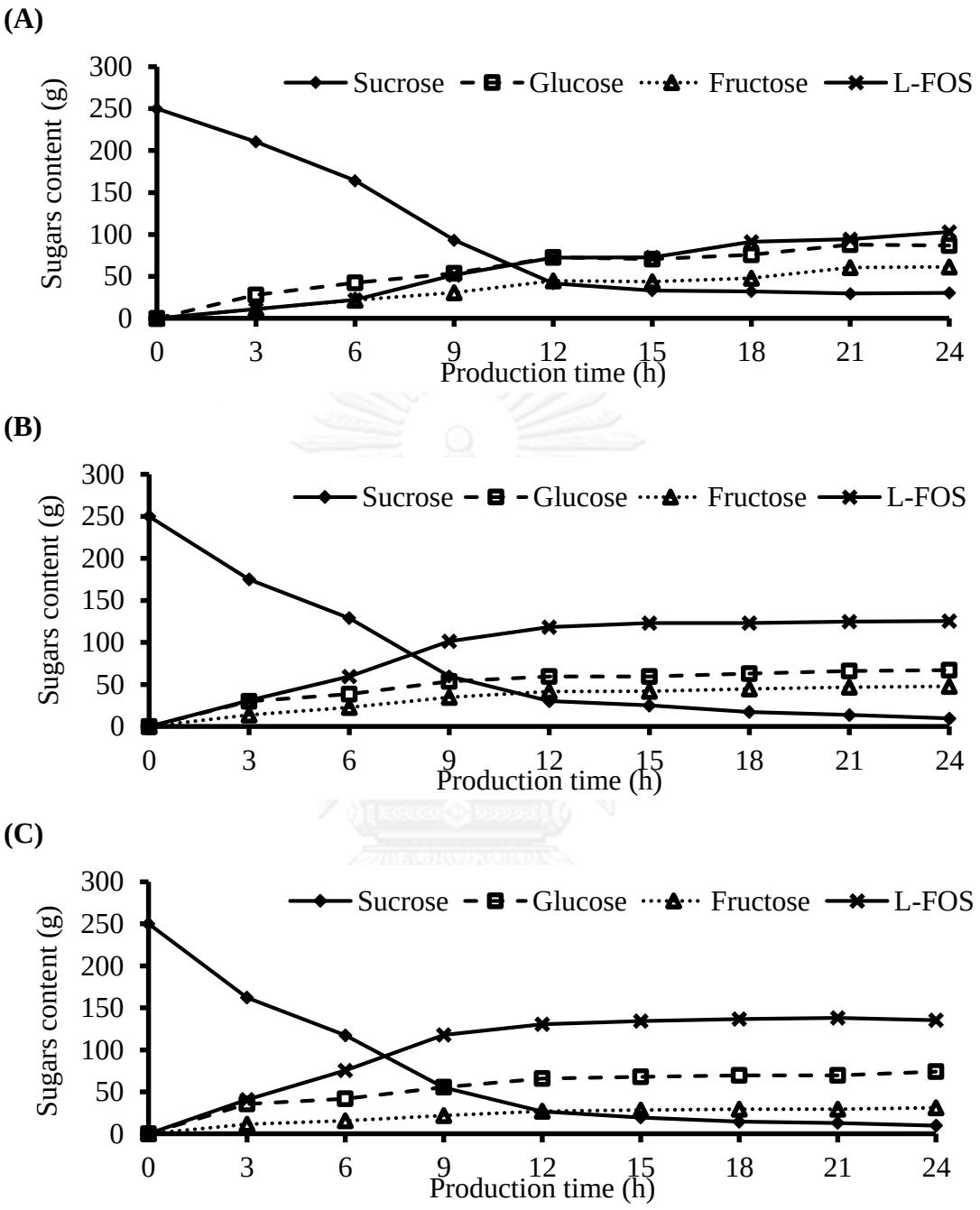


Figure 3.39 Scale up production of L-FOS by immobilized LsRN

The 1,890 U of immobilized LsRN: (A) N251Y, (B) Y246S and (C) Y246W were packed in double jacket column. 500 mL of substrate containing 50% (w/v) sucrose in 50 mM citrate buffer, pH 6.0, at temperature 40 ^{o}C, was feed into the column with upward flow at flow rate 10 mL/min.

Products analysis

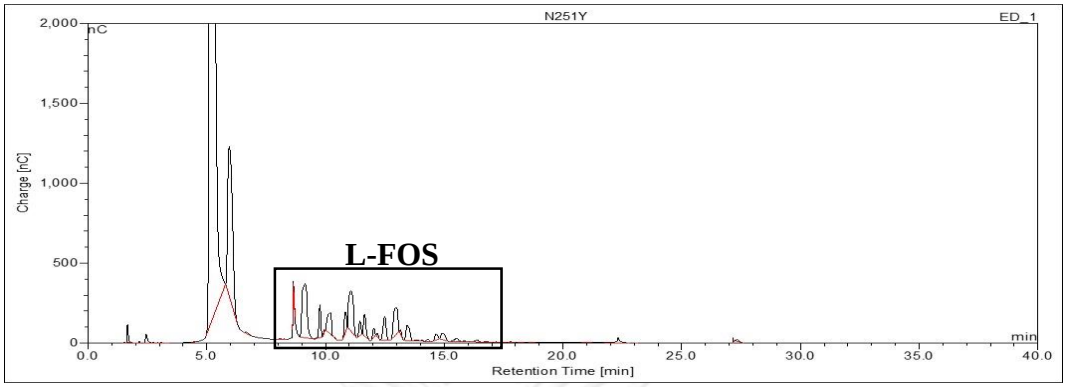
HPAEC-PAD analysis

The L-FOS products were analyzed by HPAEC-PAD equipped with CarboPac PA-1 column. Elution was performed by gradient of 0-0.6 M NaOAc in 0.1 M NaOH with the 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 result demonstrated that LsRN-N251Y could produce short chain (up to DP5) L-FOS. Medium chain (up to DP11) L-FOS was produced by LsRN-Y246S and long chain (up to DP20) L-FOS was produced by LsRN-Y246W (Figure 3.40).

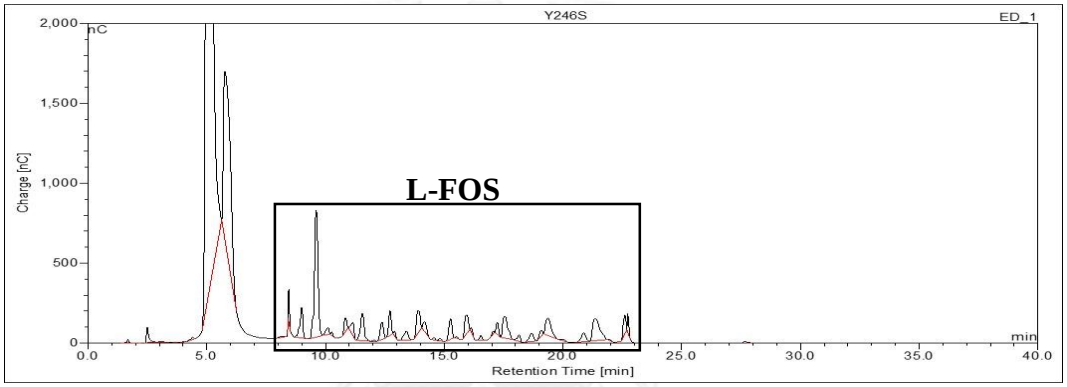
Products purification

L-FOS products were purified by Biogel P2 column chromatography using DI water as a mobile phase. The result demonstrated that size exclusion chromatography with Biogel P2 could purify L-FOS by DP1 – DP9 of products (Figure 3.41).

(A)



(B)



(C)

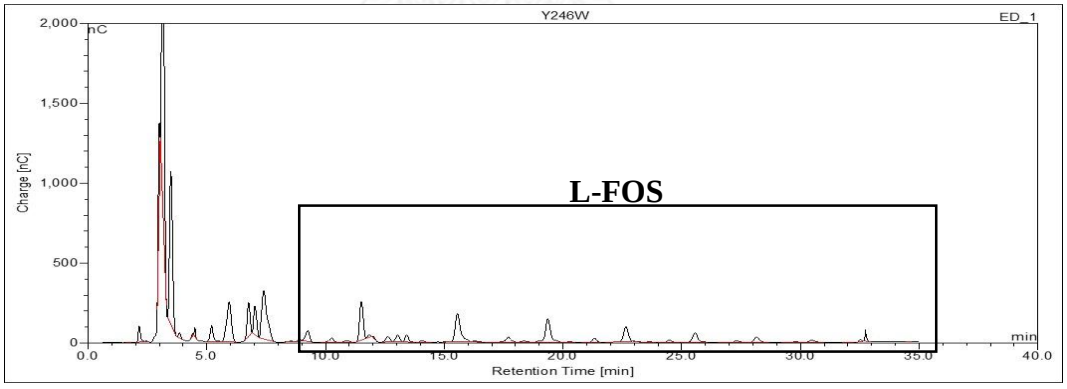


Figure 3.40 HPAEC-PAD chromatogram of L-FOS products

The L-FOS products from LsRN: (A) N251Y, (B) Y246S and (C) Y246W were analyzed by HPAEC-PAD equipped with CarboPac PA-1 column. Elution was performed 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.

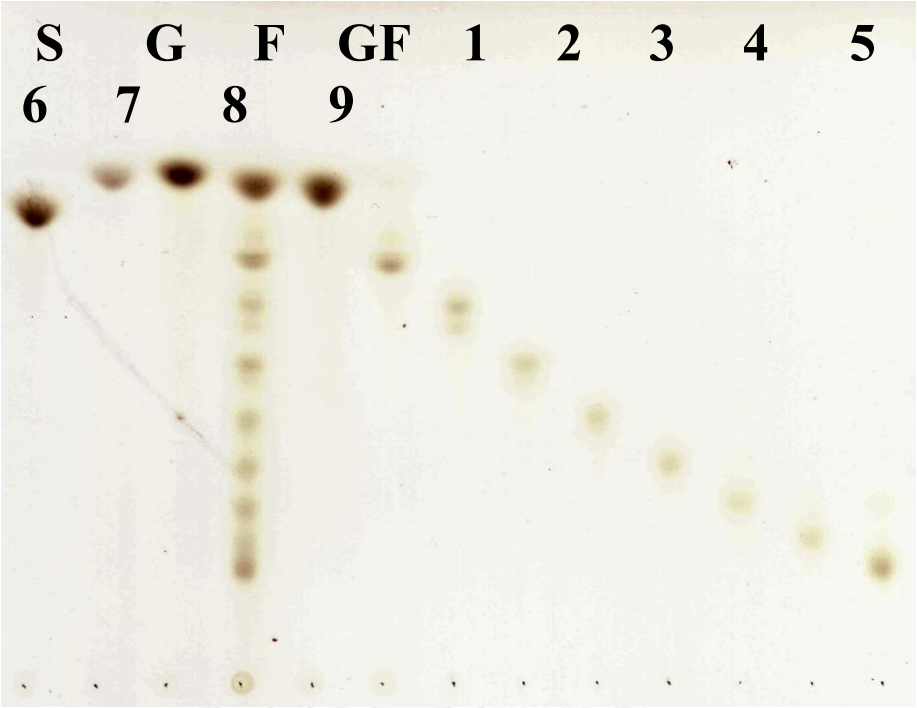


Figure 3.41 TLC chromatogram of the products purification by Biogel P2 column chromatography

L-FOS products were purified by Biogel P2 column chromatography. The crude L-FOS products were applied to Biogel P2 column at flow rate 27 mL/h and eluted with filtrated DI water. The TLC plate was ran in a mobile phase containing acetic acid : butanol : distilled water at the ratio of 3 : 3 : 2. The TLC plate was stained with ethanol : sulfuric acid ratio 9 : 1. Lane S: standards sucrose, lane G: standards glucose, lanes F: standards fructose, lane GF: standards GF, lane 1 - 9: purified product DP1 – DP9, respectively.

CHAPTER IV

DISCUSSION

Production of recombinant LsRN derivatives in *E.coli*

The results demonstrated that the maximum activity of *E. coli* Top-10 and p*ls*RN01 containing LsRN-Y246S was detected in all LB medium concentration at day 2 of cultivation and gradually decreased after day 3 to *day 5*. There were no significant differences in the activity of LsRN in 3X, 4X and 5X LB of all LsRN derivatives after the second day of cultivation. NaCl concentration had effect on levansucrase activity of all LsRN derivatives when LB medium was used as a control and addition of NaCl, increasing from 1X NaCl in LB medium to 5X NaCl. 3X NaCl in LB medium, gave the highest levansucrase activity in all LsRN derivatives, approximately 2 fold, when compared with LB medium containing 1X NaCl. The LsRN activity decreased when NaCl concentration was higher than 3X NaCl. This result revealed that culturing *E. coli* Top-10 in 3X LB medium could increase the LsRN activity approximately 4, 12 and 7 folds for N251Y, Y246S and LsRN Y246W, respectively, compared to LB medium. These results suggested that higher levels of nutrients in the medium could provide more nutrients for the cells or induce the cells to produce more enzymes. The elevated salt concentration may also play a role in promoting cells to secret more enzymes in to the culture medium. Furthermore, when the cultivation time was extended to 5 days, the activity did not further increase when compared to day 2, at every concentration of LB medium, suggesting that the cells cease enzyme production after cell saturation. Thus, 3X LB medium, containing 3X NaCl and 100 μg/mL ampicillin, was selected for the further cultivation of all LsRN derivatives for 2 days, at 37 ^{o}C, 250 rpm.

Purification of recombinant LsRN derivatives in *E.coli*

The DEAE-cellulose chromatogram of LsRN-N251Y and Y246S were shown least two protein peaks containing LsRN. In contrast, the LsRN-Y246W gave only a single peak of LsRN. The fractions containing LsRN were pooled and applied to a phenyl-sepharose column. The result showed a single peak containing LsRN for all

LsRN derivatives. The specific activity of LsRN-N251Y, Y246S and Y246W increased to 375, 168 and 425 U/mg protein, respectively. LsRN-N251Y, Y246S and Y246W were purified 9.6, 6.5 and 12.1 fold, with 56, 62 and 69% yield, respectively. The results indicate that the crude LsRN produced by recombinant *B. licheniformis* RN-01 in *E.coli* were successfully purified to homogeneity. In previous studies, recombinant Ls was purified by generating His-tag fusion protein then purified by Ni-NTA column chromatography [51], [90] and [91], however, the addition of His-Tag might have modified or affect the native activity of the enzyme. In our studies no His-tags were added, thus the purified enzyme would exhibit its native properties. Hettwer *et a*l. [92] also reported the purification of *P. syringa*eusing levansucrase by two step of column chromatography, TMAE-Fraktogel and Butyl-Fraktogel column chromatography, with the yield of approximately 23% purification fold, and 44% recovery. The Ls from *Streptoccocussalivarius* was purified by Resource-Q and Sephacryl S-300 column chromatography [93], only 5 purification fold and a specific activity of 53 U/mg.protein was acheived. Our purification protocol have proven to be better than most of the work previously reported.

Analysis of the purified LsRN by SDS-PAGE showed that the purified LsRN had a MW of about 52 kDa and revealed a high degree of apparent homogeneity. Previously, there were reports of the molecular weight of Ls from *B. circulans* (52 kDa) [52], *B. megaterium* (52 kDa) [94], *B. subtilis*(50 kDa) [95] and *B. amyloliquefaciens*(52 kDa) [96], which demonstrated that our enzyme has similar molecular weight with levansucrase of other *Bacillus spp*. as expected.

Characterization of LsRN derivatives

The optimum pH of LsRN-N251Y, Y246S and Y246W were similar, at pH 6.0. However, LsRN-N251Y exhibit a broader pH range, pH 5-8, than Y246S and Y246W. Previously reported optimum pH for other *Bacillus* species were also in the pH range of 5.0 to 7.0 [43].

The optimum temperature of LsRN-N251Y, Y246S and Y246W were similar, at 40 ^{o}C. All LsRN derivatives had a board temperature range for enzymatic activity, 30 to 50 ^{o}C. In previous studies the optimum temperature of recombinant LsRN was 50 ^{o}C [97]. Similarly, the optimum temperature of levansucrase from other *Bacillus spp*.,

such as, *B. circulans* [94], *B. subtilis* [98], *B. Megaterium* [52], *Z. Mobilis* [99] and *L. Reuteri* [100] were also reported, approximately range from 45 to 50 ºC.

Most metal ions tested slightly affect the activity of all LsRN derivatives. Metal ions that had inhibitory effect on the activity of LsRN derivatives were Cu^{2+}, Zn^{2+} and Fe^{3+}. Ionic surfactant, SDS, and chelating agents, EDTA, could also drastically inhibit the activity of LsRN. Interestingly, Ca^{2+} and Mn^{2+} significantly enhanced LsRN derivatives activity. Our result also revealed that the LsRN was significantly activated by calcium and manganese ion. Ca^{2+} has been shown to bind and subsequently activating of fructosyltranferase [100] and [101]. Mn^{2+} had also reported to activate levansucrase from *B. subtilis* [84]. This suggest that Ca^{2+} and Mn^{2+} might be a cofactor for the enzyme or could modulate the activity of the enzyme via interaction with the enzyme or enzyme substrate complex. We also found that Mn^{2+} can increase the hydrolytic activity of the enzyme (data not shown). Cu^{2+}, Zn^{2+} and Fe^{3+} have also been shown to inhibited the activity of levansucrase from *B. subtilis* and *B. circulans* [84].

There was a slight increase in LsRN activity when the ionic strength was increased from 0 to 2 M NaCl, in all LsRN derivatives. However, LsRN activity decreased when NaCl concentration was elevated higher than 2 M. This result indicated that moderate ionic strength is required for optimal activity, but high ionic strength over 2 M may disrupt the enzyme-substrate binding or affect the overall structure of the enzyme reducing its activity.

Sucrose concentration has an effect on the activity of LsRN derivatives we found that at 40% (w/v) sucrose, all LsRN derivatives had the highest activity. At sucrose concentration ranging from 20%-40% (w/v), there were no significant differences in the activity of LsRN-Y246S. Previous reports have shown that at low sucrose concentration, the hydrolytic activity of Ls was predominant, which would yield twice the amount of reducing moiety when assayed with DNS method. As the sucrose concentration increases transglycosylation rate increases. Thus the ratio between transglycosylation versus hydrolysis increases this ratio. Furthermore, hydrolysis will undergo a minor inhibition and effect from the released free glucose [102].

The stability of LsRN derivatives in the reaction conditions, were determined by pre-incubating of LsRN-N251Y, Y246S and Y246W at 40 ºC, LsRN activity

remained at about 50% after 2, 3 and 2 h, respectively. When the incubation period was prolonged to 12 h, the LsRN-N251Y and Y246W lost almost all of its activity. Previously, Ls from *R. aquatilis* was shown to lose 50% of initial activity after pre-incubation at temperature of 60 ^{o}C for 20 min [103]. When increased the temperature more over to 60 ^{o}C, Ls undergoes an irreversible denaturation, this may be due to the permanent temperature-dependent unfolding [101].

After storage the all purified LsRN derivatives for a month, LsRN activity of N251Y, Y246S and Y246W were retained at 77%, 81% and 80%, respectively. The activity of all LsRN derivatives decreased to 50% after it have been stored for 9 wk, 11 wk and 10 wk for N251Y, Y246S and Y246W, respectively. Finally, when all LsRN derivatives were stored for 3 mo the activity of 18%, 32% and 23% was retained for N251Y, Y246S and Y246W, respectively. Fortunately, we could store all purified LsRN derivatives more than a month.

Selection of suitable immobilization supporting matrices and characterization of immobilized LsRN

The immobilization yield of LsRN immobilized by covalently cross-linking on CTS bead, using GTA as a cross-linking agent, increased as the GTA concentration increases from 0.25% - 4%. The highest immobilization yield of 97% was achieved when 4% GTA was used. Although, the immobilization yield increased when the GTA concentration was increased from 2 - 4%, the physical property of CTS bead was not suitable for using, as a supporting matrix. It was found that the CTS bead lost its elasticity and became hard and brittle that easily cracks. Thus, in this work 2% GTA concentration was used to cross-link the enzyme onto CTS beads. Because at 2% GTA there was a good compromise between efficiency and physical properties of the bead. Earlier reports have used either higher concentration of 3% GTA as a cross-linking reagent, for immobilized *Bacillus subtilis* NRC33a levansucrase onto chitosan [84] or low percent of GTA, about 0.05%, for immobilization of catalase onto chitosan bead [104]. The concentration of the cross-linker seems to be dependent of the nature of the system, each enzyme and supporting matrix. However, our immobilization yield exceeded all previously reported experiments of Ls being crossed-linked on supporting matrices.

The percentage of immobilized LsRN on fine particle chitosan, Sepabead EC-HFA, Sepabead EC-EP, PDCC and CTS beads after immobilization were 11%, 30%, 38%, 94 and 96%, respectively. Sepabead EC-HFA and Sepabead EC-EP had approximately 30-40% of LsRN activity immobilized on the matrices, with an approximately 50% lost in the enzymatic activity. This loss of activity was probably due to the improper orientation of the enzyme on the supporting matrix when it was covalently linked. The active site of the enzyme may become inaccessible. Some intra-molecular or inter-molecular cross-linking of the enzyme molecules might also render the enzyme inactive, which normally occurs in the immobilization process. We also observed a 15-18% of the enzyme that was not immobilized.

Although, PDCC had good immobilization efficiency, the immobilized LsRN on PDCC was unstable, as the immobilized levansucrase completely lost activity after 3 repetitive use (data not shown). Surprisingly, 96% of LsRN activity could be immobilized on the CTS beads. Two percent of the total activity was found left over in the supernatant and only a 2% lost in activity was observed. This result made CTS bead a very efficient matrix for LsRN immobilization. This result had, so far, the highest immobilization yield found for the immobilization of LsRN, comparing to the 81.5% and 75% immobilization yield reported earlier [84] and [105]. This extraordinary high immobilization yield suggests that CTS beads may play a role in orientating the enzyme molecule as well as serving as a supporting matrix.

Our study showed that CTS bead has a solid core, however, the surface of the CTS bead has nano-scale fibrous like texture. This nano-scale fibrous like structures provides vast surface area increasing the loading capacity of CTS beads. It might have also helped orientating LsRN, by interacting with the proper surface on enzyme molecule. This will allow LsRN active site to be exposed, and remain functionally active.

Characterization of immobilized LsRN derivatives

The optimum pH of all LsRN derivatives of free and immobilized levansucrase were similar, at pH 6.0. However, the immobilized LsRN had a broader activity range in all LsRN derivatives. Moreover, it was observed that all immobilized LsRN derivatives extended its activity into the acidic range (pH 3-5). The LsRN immobilized

on CTS bead could retain more than 70% activity for Y246S and Y246W and about 58% for N251Y, 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 LsRN immobilization, though quite inferior when compared to CTS beads. Similar results were reported for immobilized FTFase [106] and *B. subtillis* LS [84] where its activity was also extended into the acidic range.

The optimum temperature of all LsRN derivatives when immobilized on supporting matrices were similar, at 50 ^{o}C. Free form levansucrase of all LsRN derivatives had optimal activity at 40 ^{o}C, but at lower temperatures, below 35 ^{o}C, free LsRN exhibit higher activity than all immobilized LsRN derivatives. This phenomena could be contributed to the lack of mobility of the immobilized enzyme, thus reducing its activity at lower temperature where the mass exchange rate drops. However, at temperatures above 35 ^{o}C immobilized LsRN was found to be more stable and showed a significant increase in enzymatic activity comparing to free LsRN. The immobilization of LsRN by covalently linking it to the matrix may result in an increase of the enzyme stability by multiple point attachment. This would lead to the increase in stability at higher temperatures, 40 to 50 ^{o}C. [107].

Our results results indicated that most metal ions tested slightly affect the activity of all free and immobilized LsRN derivatives. Metal ions that had inhibitory effect on the activity of LsRN in both free and immobilized form were Cu^{2+}, Zn^{2+} and Fe^{3+}. Ionic surfactant, SDS and chelating agents such as EDTA could also drastically inhibit the activity of both the free or immobilized LsRN. Immobilization of LsRN on CTS bead could slightly protect LsRN from the inactivation by Cu^{2+}, Zn^{2+} and Fe^{3+}, as well as SDS and EDTA, as trace activity was detected. Metal ions and ionic detergents are well known for their protein denaturation properties. The ability of immobilized enzyme to withstand the presence of Cu^{2+}, Zn^{2+} and Fe^{3+}, as well as SDS, implies the structural stabilization of LsRN by CTS bead. The EDTA ability to abolish the enzyme activity suggests that some divalent metal ions might be needed to retain the enzymatic activity.

Interestingly, immobilizing LsRN on CTS beads further elevate the enhancement of LsRN activity by Mn^{2+}, more than 2 folds. This phenomenon was not observed with LsRN immobilized on Sepabead EC-EP and Sepabead EC-HFA beads,

suggesting that there were indeed differences in the way the enzyme was bound to these different supporters.

Effect of ionic strength was demonstrated that all immobilized LsRN derivatives. LsRN on supporting matrices had higher activity than free LsRN derivatives, at ionic strength over 1 M of NaCl. This result may possibly be due to a better interaction between the substrate and/or the nascent product, and immobilized enzyme, which may play an important role in the processivity of the enzyme.

At sucrose concentration ranging from 20%-40% (w/v), there were no significant differences in the activity of all free nor immobilized LsRN derivatives. However at sucrose concentration above 40% (w/v) we observed that immobilized LsRN on CTS, Sepabead EC-EP and Sepabead EC-HFA beads retained more of its activity than free LsRN. The concentration of sucrose up to 60% (w/v) could be used with immobilized LsRN on CTS beads, which retains more than 90% of activity for all LsRN derivatives. The ability of the immobilized LsRN to tolerate higher concentration of sucrose is preferable for L-FOS production, since higher amount of substrate could be applied and further condensation steps could be kept to a minimum.

When free and immobilized LsRN were subjected to prolonged incubation at its optimum temperature 40 ^{o}C, the immobilized LsRN retained higher activity than the free LsRN. The free LsRN rapidly lost its activity, more than 50% within the first 2 h. The LsRN immobilized on the CTS beads showed better thermo-stability than LsRN immobilized on Sepabead EC-EP and Sepabead EC-HFA, which showed similar thermo-stability profile. Immobilized LsRN by covalently linking it to the matrix may result in an increase of the enzyme stability by multiple point attachment. This would lead to the increase in stability at higher temperatures

The immobilized LsRN-N251Y and Y246W by covalently cross-linking on supporting matrices showed stable more than 30% relative activity at 4 ^{o}C for 3 mo and more than 50% for Y246S. The decrease in immobilized LsRN activity was explained as a time dependent natural loss in enzyme activity, and this was prevented to a significant degree by immobilization. Also the immobilization of LsRN by covalently cross-linking brings another advantage by increasing enzyme stability as demonstrated by storage stability experiments.

The residual activity of LsRN after each reaction cycle was measured. LsRN-Y246S immobilized on chitosan beads retained over 75% of its activity after 10 cycles of repeated use and over 60% activity after 17 cycles of repeated use. In contrast, Sepabead EC-EP and Sepabead EC-HFA lost 67% and 70% of its activity after only 5 cycles of repeated use. Moreover, CTS bead could retain activity more than 40% after used for 10 and 12 cycles of N251Y and Y246W, respectively. These results demonstrated that CTS beads could help increase the stability of LsRN upon usage. The porous fibrous surface of CTS beads may help prevent the denaturation of the enzyme bound on the surface from shearing forces during the reaction process. This result could explain by the inactivation of levansucrase caused by the denaturation of the protein, and the leakage of protein from Sepabead EC-EP and Sepabead EC-HFA. This clearly showed the advantage of CTS bead over other immobilization matrices.

Effect of substrate and immobilized LsRN activity ratio on the production of L-FOS was measured as L-FOS products and residual sucrose. L-FOS was produced approximately 7.1 g, using the substrate over LsRN ratio of 1:20 which no significant difference comparing to the ratio of 1:50. This demonstrates that we have an excess substrate over enzyme ratio once we exceed the ratio of 1:20.

Effect of sucrose concentrations on the sucrose conversion to L-FOS revealed similar trend of sucrose conversion of all LsRN derivatives. When the sucrose concentration was increase from 10% to 50% the production of L-FOS was pronouncedly increased. However, when the sucrose concentration was increased over 50% up to 70% the production of L-FOS increased only slightly. The ability of the immobilized LsRN to tolerate higher concentration of sucrose was preferable for high yield of L-FOS production.

The production of L-FOS by immobilized LsRN derivatives showed similar trend of reaction in all LsRN derivatives. In the first 3 h the sucrose conversion for the production of L-FOS was lagging. However, at 3 to 5 h, the reaction rate enhanced substantially, resulting in significant increasing the amount of L-FOS products. The reaction reached equilibrium after the reaction was prolonged over 5 h.

The reusability of immobilized LsRN was studied, N251Y immobilized on CTS bead could be reused for 5 cycles and 66% efficiency for L-FOS production was retained from the first cycle. The efficiency for L-FOS production of immobilized

Y246S and Y246W was retained about 75% and 71%, respectively after repeated use for 5 cycles. This decreasing of L-FOS content of all LsRN derivatives after repeated use over 5 cycles might be caused by the immobilizes LsRN beads being crushed. This crushed CTS beads disrupt the flow which could affect the conversion rate. Moreover, the natural loss in LsRN activity may also occur.

Process design and optimization of L-FOS production by immobilized LsRN

Effect of substrate feed direction on the production of L-FOS was performed. The results indicated that the upward flow had higher efficiency than the downward flow substrate feed. Approximately 3.57 g of L-FOS was observed after 3 h and slightly increased to 7.93 g at 8 h when feed substrate with upward flow direction. This could be due to the even and thoroughly exposure of the substrate with the immobilized LsRN beads as the substrates passed through into the column and resulting in higher reaction rate. In contrast, in the downward flow direction, the substrate with higher density may pass through the void volume between the beads without being exposed evenly with the immobilized LsRN, resulting in poorer catalytic activity than the upward flow direction.

Effect of flow pattern on the production of L-FOS was performed. The result showed that the L-FOS products were produced 5.47 g at 12 h when feed sample with single pass flow. In contrast, the multiple pass flow could produce 7.93 g of L-FOS products at 8 h. Therefore, multiple pass flow was suitable for the production of L-FOS. The column dimension was 3 x 14 cm, had an approximately total volume of 99 mL and estimated void volume of 20 mL (20% of column volume). This column had the capacity of approximately 9,900 beads containing 500 U of levansucrase activity.

At single pass flow, flow rate was investigated at 0.34 mL/min, based on the calculation that the substrate would be completely catalyzed in a single pass, considering the total enzyme units contained within the reaction column. However, the substrates did not completely catalyzed in a single pass, this was unexpected. However, when the product mixture from the first pass was again feed into the column L-FOS products increased. Therefore the reaction column needs to be primed for optimum catalysis. This may due to the dilution effect of the water within the CTS beads or the reactant composition at each of the plane within the column. In contrast, with multiple

pass flow, the 25 g of substrates (73 mmole of sucrose) was completely catalyzed with 500 U of immobilized LsRN in 146 min. However, the immobilized l LsRN did not contact with substrate in all the time. The substrates were re-circulated into the column at the rate of 4 mL/min. L-FOS products were produced at 7.93 g in 8 h, not in the 146 min calculated production time based on the immobilized LsRN activity.

The effect of flow rate of samples on the production of L-FOS was studied. At the flow rates ranged from 2-8 mL/min, there were no significant difference in the amount of L-FOS products. Interestingly, when the flow rate was increased to 10 mL/min, the reaction rate significantly increased. At flow rate of 10 mL/min, the mixture of samples, including of new substrates and acceptor molecules, was feed into the column as multiple pass flow with higher number of cycle than that in the case of flow rates between 2-8 mL/min, leading to higher yield of L-FOS.

The operational production of L-FOS by using packed immobilized LsRN reactor was performed by measuring the efficiency of the immobilized LsRN after repeated use. L-FOS products were produced at 7.83 g in the first cycle and decreased to 5.22 g in fifth cycles by LsRN-N251Y, 8.36 g and 6.26 g for LsRN-Y246S and 8.14 g and 4.56 g, respectively. The efficiency of immobilized LsRN-N251Y, LsRN-Y246S and LsRN-Y246W for the production of L-FOS were of 67%, 75% and 56%, respectively, after 5 cycle of repeated used. These results demonstrated that the LsRN-Y246W lost its activity more than LsRN-N251Y and LsRN-Y246S. The immobilized enzyme might have been affected by the long chain of L-FOS products from production of LsRN-Y246W. The long chain L-FOS products attached on the CTS-bead, masking the surface of the bead as well as causing the CTS-bead to crack and losing of catalytic activity.

Scale up production of L-FOS was performed by using packed immobilized LsRN reactor, the column dimension about 4.5 x 17 cm had approximately volume 270 mL. This column can hold about 27,000 beads of immobilized LsRN, with 1,890 U of LsRN activity. Theoretically, 50% sucrose (w/v) 500 ml containing 250 g sucrose (730 mmol) was used as substrate, therefore 1,890 U of LsRN activity should completely the reaction in 387 min (6 h 27 min). The amount of L-FOS products at 12 h of production time was 118.39 g and 130.45 g for LsRN-Y246S and LsRN-Y246W, respectively. However, when the production time was extended to 24 h, the L-FOS products slightly

increased, 7.09 g and 4.84 g for LsRN-Y246S and LsRN-Y246W, respectively. LsRN-N251Y could produce 102.93 g L-FOS in 24 h of production time. These results indicated that, more than 100 g of medium and long chain of L-FOS could be produced in 12 h with LsRN-Y246S and LsRN-Y246W, respectively. However, more than 100 g short chain of L-FOS could be produced in 24 h with LsRN-N251Y. L-FOS products were purified by Biogel P2 column chromatography. The result demonstrated that size exclusion chromatography with Biogel P2 could be purify L-FOS for DP1 – DP9 size products. Biogel P2 could separate with high purity of L-FOS for DP1 (MW. 180.16 Da) – DP9 (MW. 1477.28 Da).

CHAPTER V

CONCLUSION

The recombinant *ls*RN gene from *B. licheniformis* RN-01 in *E.coli* was successfully over expressed under the regulation of its putative endogenous promoter using by the 3X concentration of the LB medium and successfully purified had a MW of about 52 kDa and revealed a high degree of apparent homogeneity.

The optimum pH and temperature of all LsRN derivatives were 6.0 and 40 ^{o}C, respectively. Cu^{2+}, Zn^{2+}, Fe^{3+} ions SDS and EDTA were inhibited the activity of LsRN derivatives, interestingly Ca^{2+} and Mn^{2+} significantly enhanced LsRN activity. The ionic strength negatively affected all LsRN derivatives activity when increasing the NaCl concentration to 2 M. 40% of sucrose concentration was shown the highest activity of all LsRN derivatives. All LsRN derivatives were stabilized at temperature of 40 ^{o}C around 2-3 h, retained about 50%. The storage stability at 4 ^{o}C for one month of all LsRN derivatives retained around 70% - 80%.

We have identified a suitable immobilization matrix for immobilizing LsRN by covalently binding. CTS beads that was cross-linked with 2% GTA had superior characteristics providing large surface area, high immobilization yield, and enhance the stability and increase the reuse/recycle property of levansucrase for the production of L-FOS.

The optimum pH and temperature of all immobilized LsRN derivatives were 6.0 and 50 ^{o}C, respectively. Immobilization of LsRN on CTS bead could slightly protect LsRN from the inactivation by Cu^{2+}, Zn^{2+} and Fe^{3+}, as well as SDS and EDTA, as trace activity could be detected.

The CTS beads could help increase the stability of LsRN upon usage. The LsRN-Y246S immobilized on chitosan beads retained over 75% of its activity after 10 cycles of repetitive used. CTS bead could retain activity more than 40% after repeated used for around 10 - 12 cycles of all LsRN derivatives.

LsRN derivatives immobilized on CTS-bead shown ability of tolerate higher concentration of sucrose is preferable for high yield of L-FOS production. The

production of L-FOS using by a high concentration of sucrose brings to probably a high yield of L-FOS. The production of L-FOS by immobilized LsRN showed similar trend of reaction in all LsRN derivatives. The operational production of L-FOS by immobilized LsRN was indicated that, N251Y immobilized on CTS bead could reuse for 5 cycles and the efficiency for production of L-FOS retained 66% from the first cycle. According to the efficiency for L-FOS production of immobilized Y246S was retained about 75% repetitively used for 5 cycles. And 71% efficiency of repetitively used for 5 cycles of Y246S was observed.

The upward flow direction of substrate had higher efficiency than downward flow direction. The multiple pass flow of samples mixture was suitable for the production of L-FOS. The optimum flow rate of samples on the production of L-FOS was 10 mL/min. The efficiency of repeated use of immobilized LsRN-N251Y, Y246S and Y246W for the production of L-FOS were of 67%, 75% and 56%, respectively after fifth cycle of repeated used.

We successfully produced the L-FOS more 100 g using packed immobilized LsRN derivative reactor. The short chain L-FOS was produced from N251Y for 103 at 24 h. The medium and long chain of L-FOS was produced from Y246S and Y246W for 118.39 and 130.45 g, respectively at 12 h. L-FOS products could purify by Biogel P2 column chromatography with DP1 – DP9.

REFERENCES

REFERENCES

1. Hayashi, S., et al., **Production of fructosyl-transferring enzyme by *Aureobasidium sp.* ATCC 20524**. *Journal of Industrial Microbiology*, 1990. **5**: p. 395-400.
2. Yun, J.W., et al., **Semibatch production of fructo-oligosaccharides from sucrose by immobilized cells of *Aureobasidium pullulans***. *Applied Biochemistry and Biotechnology*, 1990. **24/25**: p. 299-308.
3. Yun, J.W., et al., **Continuous production of fructo-oligosaccharides from sucrose by immobilized cells of *Aureobasidium pullulans***. *Journal of Microbiology and Biotechnology*, 1992. **2**: p. 98-101.
4. Rhee, S.K., et al. **Levan: Biopolymers Online**. Wiley-VCH Verlag GmbH & Co. KGaA, 2005.
5. Vandamme, E.J. and D.G. Derycke, **Microbial inulinase: fermentation process, properties, and applications**. *Advances in Applied Microbiology*, 1983. **29**: p. 139-176.
6. Sangeetha, P.T., M.N. Ramesh, and S.G. Prapulla, **Maximization of fructooligosaccharide production by two stage continuous process and its scale up**. *Journal of Food Engineering*, 2005. **68**: p. 57-64.
7. Hidaka, H., M. Hirayama, and N. Sumi, **A fructooligosaccharide-producing enzyme from *Aspergillus Niger* ATCC-20611**. *Agricultural and biological chemistry*, 1988. **52**: p. 1181-1187.
8. Park, H.E., et al., **Enzymatic synthesis of Fructosyl oligosaccharides by levansucrase from *Microbacterium laevaniformans* ATCC 15953**. *Enzyme and Microbial Technology*, 2003. **32**: p. 820-827.
9. Euzenat, O., A. Guibert, and D. Combes, **Production of fructo-oligosaccharides by levansucrase from *Bacillus subtilis* C4**. *Process Biochemistry*, 1997. **32**(3): p. 237-243.
10. Hao, L.H., et al., **Separation, purification and structure of burdock oligosaccharide**. *Chemical Journal of Chinese University*, 2005. **26**: p. 1242-1247.

11. Allen, P.J. and J.S.D. Bacon, **Oligosaccharides formed from sucrose by fructose-transferring enzymes of higher plants**. *Biochemical Journal* 1956. **63**: p. 200-206.
12. Edelman, J. and A.G. Dickerson, **The metabolism of fructose polymers in plants transfructosylation in tubers of *Helianthus tuberosus* L**. *Biochemical Journal*, 1966. **98**: p. 787-794.
13. Henry, R.J. and B. Darbyshire, ***S*ucrose:sucrose fructosyltransferase and fructan:fructan fructosyltransferase from *Allium cepa***. *Phytochemistry*, 1980. **19**: p. 1017-1020.
14. Shiomi, N., J. Yamada, and M. Izawa, **Isolation and identification of fructooligosaccharides in roots of asparagus (*Asparagus oficinalis* L.)**. *Agricultural and Biological Chemistry*, 1976. **40**: p. 567-575.
15. Nandra, K.S. and I.S. Bhatia, ***In vivo* biosynthesis of glucoftuctosans in *Agave americana***. *Phytochemistry*, 1980. **19**: p. 965-966.
16. Kida, M., et al., **Formation of fructooligosaccharides from sucrose catalyzed by immobilized *β*-fructofuranosidase originated from *Aspergillus oryzae***. *Nippon Kagaku Kashi*, 1988. **11**: p. 1830-1835.
17. Dickerson, A.G., **A *β*-D-fructofuranosidase from *Claviceps purpurea***. *Biochemical Journal*, 1972. **129**: p. 263-272.
18. Patel, V., G. Saunders, and C. Bucke, **Production of fructooligosaccharides by *Fusarium oxysporum***. *Biotechnology Letter* 1994. **11**: p. 1139-1144.
19. Hayashi, S., et al., **Observation of the chemical structure of fructooligosaccharide produced by an enzyme from *Aureobasidium sp.* ATCC20524**. *Current Microbiology*, 1989. **19**: p. 175-177.
20. Leibovici, J. and Y. Stark, **Increase in cell permeability to a cytotoxic agent by the polysaccharide levan**. *Cell and Molecular Biology*, 1985. **31**: p. 337-341.
21. Calazans, G.M.T., et al., **Molecular weight and antitumour activity of *Zymomonas mobilis* levans**. *International Journal of Biological Macromolecules*, 2000. **27**: p. 245-247.

22. Vina, I., et al., **Influence of some physicochemical factors on the viscosity of aqueous levan solutions of *Zymomonas mobilis***. *Acta Biotechnology*, 1998. **18**: p. 167-174.

23. Clarke, M.A., E.J. Roberts, and P.J. Garegg, **New compounds from microbiological products of sucrose**. *Carbohydrate Polymer*, 1997. **34**: p. 425.

24. Guo, J.H., et al., **Pharmaceutical applications of naturally occurring water-soluble polymers**. *Pharmaceutical Science and Technology Today*, 1998. **1**: p. 254-261.

25. Imam, G.M. and N.M. Abd-Allah, **Fructosan, a new soil conditioning polysaccharide isolated from the metabolites of *Bacillus polymyxa* AS-1 and its clinical applications**. *Egyptian journal of botany*, 1974. **17**: p. 19-26.

26. Esawy, M.A., et al., **Production of levansucrase from novel honey *Bacillus subtilis* isolates capable of producing antiviral levans**. *Carbohydrate Polymers*, 2011. **86**(2): p. 823-830.

27. Dahech, I., et al., **Oral administration of levan polysaccharide reduces the alloxan-induced oxidative stress in rats**. *International Journal of Biological Macromolecules*, 2011. **49**(5): p. 942-947.

28. Dahech, I., et al., **Antidiabetic activity of levan polysaccharide in alloxan-induced diabetic rats**. *International Journal of Biological Macromolecules*, 2011. **49**(4): p. 742-746.

29. Müller, M. and W. Seyfarth, **Purification and substrate specificity of an extracellular fructanhydrolase from *Lactobacillus paracasei ssp. paracasei* P4134**. *New Phytologist*, 1997. **136**: p. 89-96.

30. Yamamoto, Y., et al., ***In vitro* digestibility and fermentability of levan and its hypocholesterolemic effects in rats**. *Journal of Nutritional Biochemistry*, 1999. **10**: p. 13-18.

31. Marx, S.P., S. Winkler, and W. Hartmeier, **Metabolization of *β*-(2,6)-linked fructose-oligosaccharides by different bifidobacteria**. *FEMS Microbiology Letters*, 2000. **182**: p. 163-169.

32. Kang, S.K., et al., **Physiological effects of levanoligosaccharide on growth of intestinal microflora**. *Journal of the Korean Society of Food Science and Nutrition*, 2000. **29**: p. 35-40.

33. Kang, S.A., et al., **Anti-obesity and hypolipidemic effects of dietary levan inhigh fat diet-induced obese rats**. *Journal of Microbiology and Biotechnology*, 2004. **14**(4): p. 796-804.

34. Semjonovs, P. and P. Zikmanis, **An influence of levan on the fermentation of milk by a probiotic ABT-type starter**. *Journal of Food Technology*, 2007. **5**: p. 123-130.

35. Semjonovs, S.P., et al., **Development of *Bifidobacterium lactis* Bb 12 on *β*-(2,6)-linked fructan-containing substrate**. *Engineering in Life Sciences*, 2004. **4**(5): p. 433-437.

36. Kim, Y.Y., et al., **Effect of types of levan with or without phytic acid on intestinal environment and mineral absorption in rats**. *Journal of Foodscience and Biotechnology*, 2004. **14**(3): p. 450-454.

37. Rairakhwada, D., et al., **Dietary microbial levan enhances cellular non-specific immunity and survival of common carp (*Cyprinus carpio*) juveniles**. *Shell and fish Immunology*, 2007. **22**(5): p. 477-486.

38. Kasapis, S., et al., **Solution properties of levan polysaccharide from *Pseudomonas syringae pv. phaseolicola*, and its possible primary role as a blocker of recognition during pathogenesis**. *Carbohydrate Polymer*, 1994. **23**: p. 55-64.

39. Chung, B.H., et al., **Novel polyethylene glycol/levan aqueous two-phase system for protein partitioning**. *Biotechnology Techniques*, 1997. **11**: p. 327-329.

40. Vijn, I. and S. Smeekens, **Fructan: more than reserve carbohydrates**. *Plant Physiology*, 1999. **120**: p. 351-359.

41. Pilon-Smits, E.A.H., et al., **Improved performance of transgenic fructan-accumulating tobacco under drought stress**. *Plant Physiology*, 1995. **107**: p. 125-130.

42. Park, Y.I., S.K. Rhee, and K.H. Paek, **Transgenic tobacco plants expressing the bacterial levansucrase gene show enhanced tolerance to osmotic stress**. *Journal of Microbiology and Biotechnology*, 1999. **9**: p. 213-218.

43. Rhee, S.K. and K.B. Song, **Levansucrase**. *Handbook of Food Enzymology*. CRC Press. 2002.

44. Hettwer, U., et al., **Cloning, nucleotide sequence, and expression in *Escherichia coli* of levansucrase genes from the plant pathogens *Pseudomonas syringae pv. glycinea* and *P. syringae pv. phaseolicola***. *Applied and Environmental Microbiology* 1998. **64**: p. 3180–3187.

45. Chambert, R., G. Treboul, and R. Dedonder, **Kinetic studies of levansucrase of *Bacillus subtilis***. *European Journal of Biochemistry*, 1974. **41**(2): p. 285-300.

46. Hernandez, L., et al., **Isolation and enzymic properties of levansucrase secreted by *Acetobacter diazotrophicus* SRT4, a bacterium associated with sugar cane**. *Biochemical Journal*, 1995. **309**(Pt1): p. 113-118.

47. Ananthalakshmy, V.K. and P. Gunasekaran, **Overproduction of levan in *Zymomonas mobilis* by using cloned *sacB* gene**. *Enzyme and Microbial Technology* 1999. **25**: p. 109–115.

48. Song, K.B., et al., **Levansucrase of *Rahnella aquatilis* ATCC33071: Gene cloning, expression, and levan formation**. *Annals of the New York Academy of Sciences*, 1998. **864**(1): p. 506-511.

49. Tajima, K., et al., **The production of a new water-soluble polysaccharide by *Acetobacter xylinum* NCI 1005 and its structural analysis by NMR spectroscopy**. *Carbohydrate Research*, 1997. **305**(1): p. 117-122.

50. Geel-Schutten, G.H.V., et al., **Biochemical and structural characterization of the glucan and fructan exopolysaccharides synthesized by the *Lactobacillus reuteri* wild-type strain and mutant strains**. *Applied and Environmental Microbiology* 1999. **65**: p. 3008–3014.

51. Kang, H.K., et al., **Cloning and expression of levansucrase from *Leuconostoc mesenteroides* B-512FMC in *Escherichia coli***. *Biochimica et Biophysica Acta - Gene Structure and Expression*, 2005. **1727**(1): p. 5-15.

52. Homann, A., et al., **Insights into polymer versus oligosaccharide synthesis: mutagenesis and mechanistic studies of a novel levansucrase from *Bacillus megaterium***. *Biochemical Jouranal*, 2007. **407**(2): p. 189-198.

53. Gupta, M. and B. Mattiasson, **Unique applications of immobilized proteins in bioanalytical systems**. *Methods of Biochemical Analysis*, 1992. **36**: p. 1-34.

54. Mattiasson, B. and R. Kaul, **Determination of coupling yields and handling of labile proteins in immobilization technology**, in *Protein immobilization. Fundamentals and Applications*. 1991. p. 161-179.

55. Taylor, R.F., **Commercially available supports for protein immobilization**, in *Protein immobilization. Fundamentals and Applications* 1991. p. 139-160.

56. Woodward, J., **Immobilized enzymes: adsorption and covalent coupling**, in *Immobilized Cells and Enzymes*. 1985. p. 3-17.

57. Porath, J., **Salting-out adsorption techniques for protein purification**. *Biopolymers*, 1987. **26**: p. 193-204.

58. Caldwell, K., et al., **Immobilization of enzymes based on hydrophobic interaction. I. Preparation and properties of a betaamylase adsorbate**. *Biotechnology and Bioengineering*, 1976. **18**: p. 1573-1588.

59. Cashion, P., et al., **Enzyme immobilization on trityl-agarose: Reusability of both matrix and enzyme**. *Biotechnology and Bioengineering*, 1982. **24**: p. 1221-1224.

60. Solomon, B., et al., **Use of monoclonal antibodies for the preparation of highly active immobilized enzymes**. *Methods in Enzymology*, 1987. **135**: p. 160-170.

61. Cabral, J.M.S. and J.F. Kennedy, **Covalent and coordination immobilization of proteins**, in *Protein immobilization Fundamentals and Applications*. 1991. p. 73-138.

62. Gemeiner, P., **Materials for enzyme engineering**, in *Enzyme Engineering*. 1992. p. 13-119.

63. Muzzarelli, R.A.A., **Natural chelating polymers**, in *Pergamon Press*. 1973.

64. Zikakis, J.P., ***Chitin***, in *Chitosan and Related Enzymes Academic Press* 1984.

65. Dutta, P.K., et al., **Use of chitosan amine oxide gel as drug carrier**. *Journal of Polymer Materials*, 1997. **14**: p. 351.

66. Dutta, P.K. and M.N.V.R. Kumar, **Chitosan-amine oxide: thermal behavior of new gelling system**. *Indian Journal of Chemical Technology*, 1999. **6**: p. 55.

67. Kumar, M.N.V.R., P. Singh, and P.K. Dutta, **Effect of swelling on chitosan-amine oxide gel in extended drug delivery**. *Indian Drugs*, 1999. **36**: p. 393.

68. Apocella, A., et al., **Poly(ethylene oxide) PEO and different molecular weight PEO blends as monolithic devices for drug release**. *Biomaterials*, 1993. **14**: p. 83-90.

69. Ida, G., et al., **Influence of glutaraldehyde on drug release and mucoadhesive prop erties of chitosan microspheres**. *Carbohydrate Polymers*, 1998. **36**: p. 81-82.

70. Hayashi, T. and Y. Ikada, **Protease immobilization onto porous chitosan beads**. *Journal of Applied Polymer Science*, 1991. **42**: p. 85-92.

71. Chandy, T. and C.P. Sharma, **Chitosan beads and granules for oral sustained delivery of nifedipine: in vitro studies**. *Biomaterials*, 1992. **13**: p. 949-952.

72. Chandy, T. and C.P. Sharma, **Chitosan matrix for oral sustained delivery of Ampicillin**. *Biomaterials*, 1993. **14**: p. 939-944.

73. Kost, J. and R. Langer, **Hydrogels in medicine and pharmacy**, in *CRC Press* 1987.

74. Graham, N.B., **Controlled drug delivery systems**. *Chemistry and Industry*, 1990. **15**: p. 482.

75. Yao, K.D., et al., **pH dependent hydrolysis and drug release of chitosan/polyether interpenetrating polymer network hydrogel**. *Polymer International*, 1994. **34**: p. 213-219.

76. Sakiyama, T., et al., **Preparation of polyelectrolyte complex gel from chitosan and κ-carrageenan and its pH sensitivity swelling**. *Journal of Applied Polymer Science*, 1993. **50**: p. 2021-2025.

77. Bronsted, J. and J. Kopecek, **Hydrogels for site specific oral drug delivery synthesis and characterization**. *Biomaterials*, 1991. **12**: p. 584-592.

78. Kost, J., **Pulsed and self-regulated drug delivery**. *CRC Press*. 1990.

79. Moor, C.P.D., L. Doh, and R.A. Siegel, **Long term structural changes in pH-sensitive hydrogels**. *Biomaterials*, 1991. **12**: p. 836-840.

80. Torres, R., et al., **A novel heterofunctional epoxy-amino sepabeads for a new enzyme immobilization protocol: Immobilization-stabilization of β-galactosidase from *Aspergillus oryzae***. *Biotechnology Progress*, 2003. **19**: p. 1056−1060.

81. Shin, H.J., J.M. Park, and J.W. Yang, **Continuous production of galacto-oligosaccharides from lactose by *Bullera singularis* β-galactosidase immobilized in chitosan beads**. *Process Biochemistry*, 1998. **33**: p. 787-792.

82. Yun, J.W., et al., **Production of inulo-oligosaccharides from inulin by immobilized endoinulinase from *Pseudomonas sp***. *Journal of Fermentation and Bioengineering*, 1997. **84**: p. 369-371.

83. Blibech, M., et al., **Production of manno-oligosaccharides from locust bean gum using immobilized *Penicillium occitanis* mannanase**. *Journal of Molecular Catalysis B: Enzymatic*, 2011. **73**: p. 111- 115.

84. Esawy, M.A., D.A.R. Mahmoud, and A.F.A. Fattah, **Immobilisation of *Bacillus subtilis* NRC33a levansucrase and some studies on its properties**. *Brazilian Journal of Chemical Engineering*, 2008. **25**: p. 237-246.

85. El-Refai, H., A. Abdel-Fattah, and F. Mostafa, **Immobilization and properties of *Bacillus circulans* levansucrase**. *Acta Pharmaceutica Sciencia*, 2009. **51**: p. 149-156.

86. Sambrook, J. and D.W. Russell, **Molecular cloning**. 3 ed. **A laboratory manual**. 2000, *New York*: *Cold Spring Harbor Laboratory Press*.

87. Miller, G.L., **Use of dinitrosalicylic acid reagent for determination of reducing sugar**. *Analytical Chemistry*, 1959. **31**: p. 426-428.

88. Bradford, M.M., **Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding**. *Analytical Biochemistry*, 1976. **72**: p. 248-254.

89. Laemmli, U.K., **Cleavage of structural protein during the assembly of the head of bacteriophage T4**. *Nature*, 1970. **227**: p. 680-685.

90. Hijum, S.A.v., et al., **Purification of a novel fructosyltransferase from *Lactobacillus reuteri* strain 121 and characterization of the levan produced**. *FEMS Microbiology Letters*, 2001. **205**(2): p. 323-328.

91. Hijum, S.A.v., et al., **Biochemical and molecular characterization of a levansucrase from *Lactobacillus reuteri***. *Microbiology*, 2004. **150**(Pt 3): p. 621-630.

92. Hettwer, U., M. Gross, and K. Rudolph, **Purification and characterization of an extracellular levansucrase from *Pseudomonas syringae pv. phaseolicola***. *Journal of Bacteriology*, 1995. **177**(10): p. 2834-2839.

93. Song, D.D. and N.A. Jacques, **Purification and enzymic properties of the fructosyltransferase of *Streptococcus salivarius* ATCC 25975**. *Biochemical Journal*, 1999. **341**(Pt 2): p. 285-291.

94. Oseguera, M.A.P., L. Guereca, and A. Lopez-Munguia, **Properties of levansucrase from *Bacillus circulans***. *Applied Microbiology and Biotechnology*, 1996. **45**(4): p. 465-471.

95. Tian, F., L. Inthanavong, and S. Karboune, **Purification and characterization of levansucrases from *Bacillus amyloliquefaciens* in intra- and extracellular forms useful for the synthesis of levan and fructooligosaccharides**. *Bioscience, Biotechnology and Biochemistry*, 2011. **75**(10): p. 1929-1938.

96. Mäntsälä, P. and M. Puntala, **Comparison of levansucrase from *Bacillus subtilis* and from *Bacillus amyloliquefaciens***. *FEMS Microbiology Letters*, 1982. **13**(4): p. 395-399.

97. Nakapong, S., et al., **High expression level of levansucrase from *Bacillus licheniformis* RN-01 and synthesis of levan nanoparticles**. *International Journal of Biological Macromolecules*, 2013. **54**: p. 30-36.

98. Baciu, I.E., et al., **Investigations of the transfructosylation reaction by fructosyltransferase from *B. subtilis* NCIMB 11871 for the synthesis of the sucrose analogue galactosyl-fructoside**. *Journal of Biotechnology*, 2005. **116**(4): p. 347-357.

99. Yanase, H., et al., **Expression of the extracellular levansucrase and invertase genes from *Zymomonas mobilis* in *Escherichia coli* cells**. *Bioscience, Biotechnology and Biochemistry*, 1998. **62**(9): p. 1802-1805.

100. Ozimek, L.K., et al., **Mutational analysis of the role of calcium ions in the *Lactobacillus reuteri* strain 121 fructosyltransferase (levansucrase and inulosucrase) enzymes**. *FEBS letters*, 2005. **579**(5): p. 1124-1128.

101. Chambert, R. and M.F. Petit-Glatron, **Reversible thermal unfolding of *Bacillus subtilis* levansucrase is modulated by Fe^{3+} and Ca^{2+}**. *FEBS Lett* 1990. **275**(1-2): p. 61-64.

102. Crittenden, R.G. and H.W. Doelle, **Identification and characterization of the extracellular sucrases of *Zymomonas mobilis* UQM 2716 (ATCC 39676)**. *Applied Microbiology and Biotechnology*, 1994. **41**(3): p. 302-308.

103. Ohtsuka, K., et al., **Characterization of levansucrase from *Rahnella aquatilis* JCM-1683**. *Bioscience, Biotechnology and Biochemistry*, 1992. **56**(9): p. 1373-1377.

104. Cetinus, A.S. and H.N. Oztop, **Immobilization of catalase into chemically crosslinked chitosan beads**. *Enzyme and Microbial Technology*, 2003. **32**: p. 889-894.

105. Jang, K.H., et al., **Levan production by use of the recombinant levansucrase immobilized on titanium activated magnetite**. *Process Biochemistry*, 2001. **37**: p. 339-343.

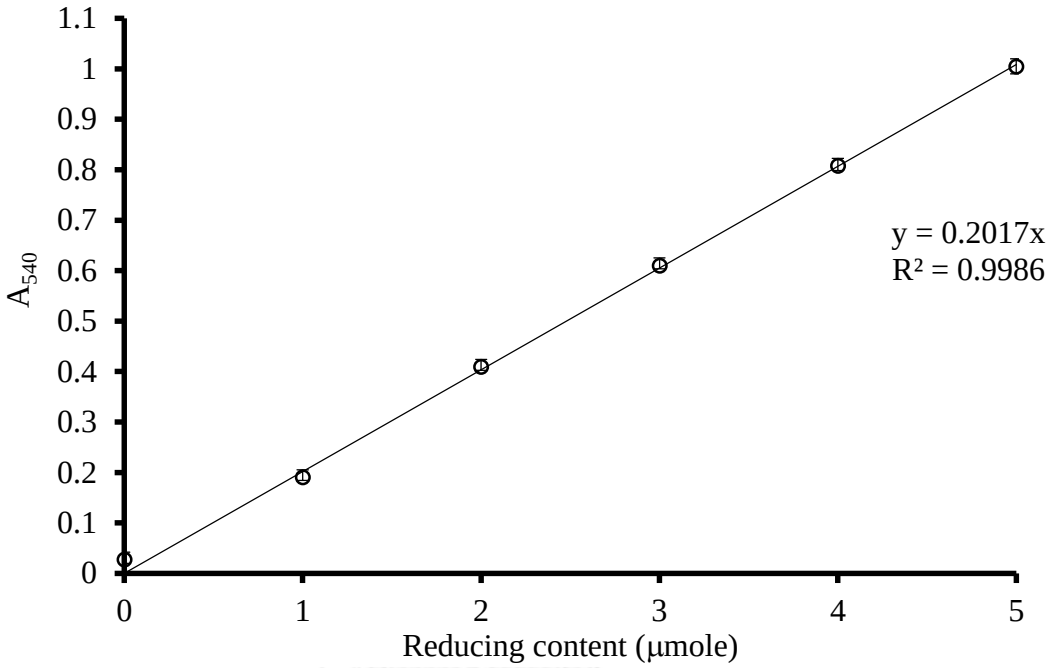
106. Mostafa, F.A.A., **Biochemical studies on bacterial fructosyltransferase**, in *Ph.D Thesis, Cairo, Egypt, Faculty of Science, Ain-Shams University*. 2006.

107. Gottschalk, N. and R. Jaenicke, **Authenticity and reconstitution of immobilized enzymes: characterization and denaturation/renaturation of glucoamylase II**. *Biotechnology and Applied Biochemistry*, 1991. **14**: p. 324-335.

APPENDIX

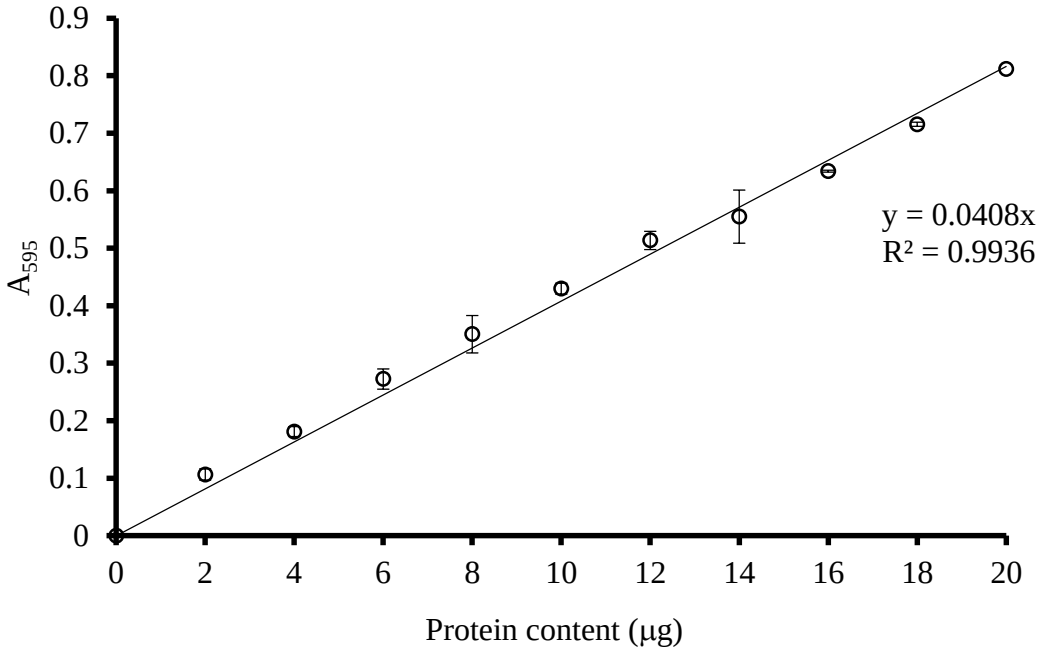
APPENDIX A

Standard calibration curve of reducing sugar by DNS's method



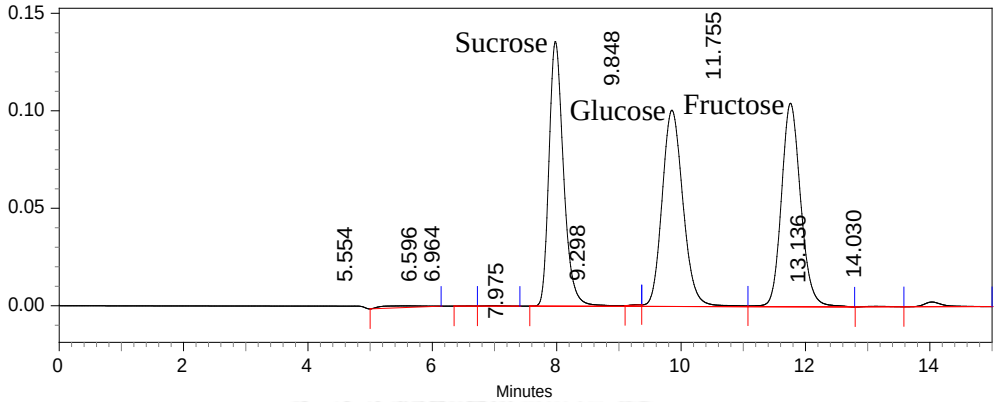
APPENDIX B

Standard calibration curve of protein by Bradford's reagent



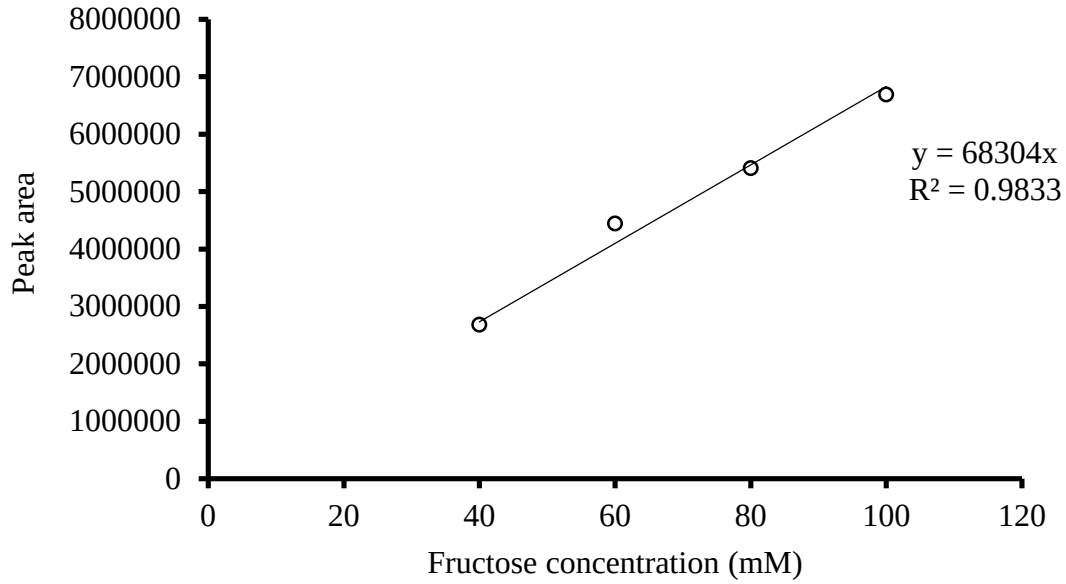
APPENDIX C

HPLC chromatogram of retention time of sucrose, glucose and fructose



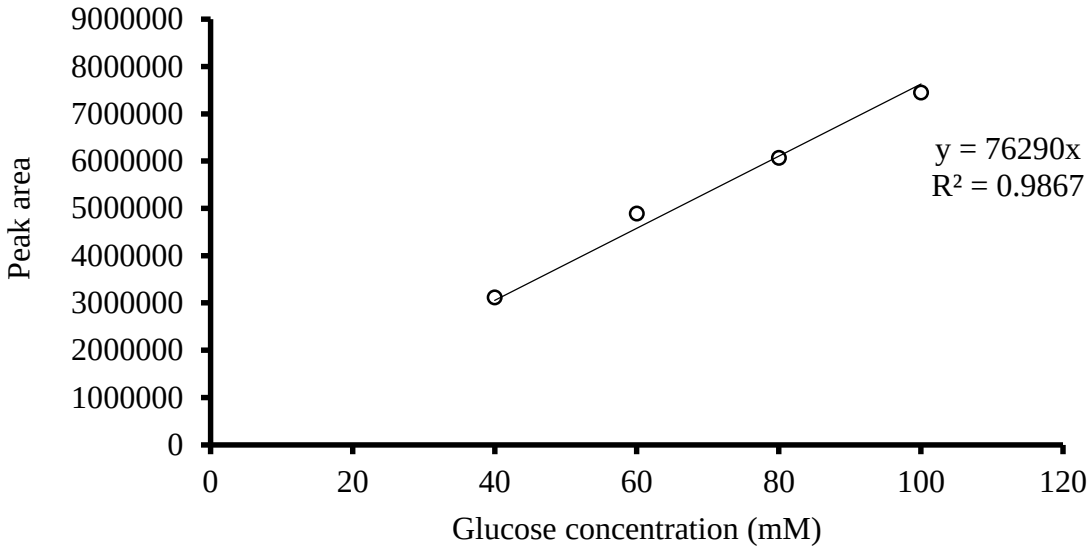
APPENDIX D

Standard calibration curve of fructose by HPLC



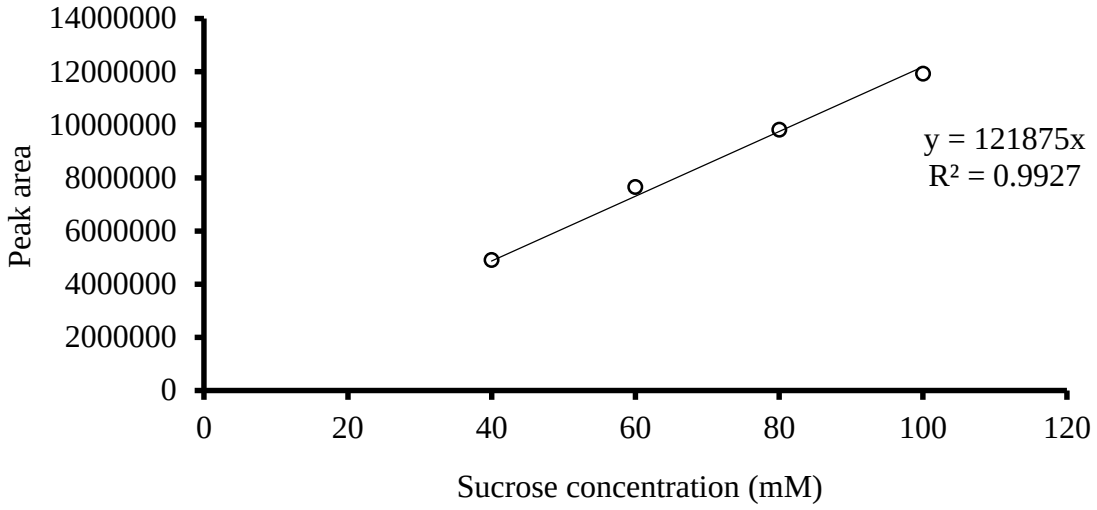
APPENDIX E

Standard calibration curve of glucose by HPLC



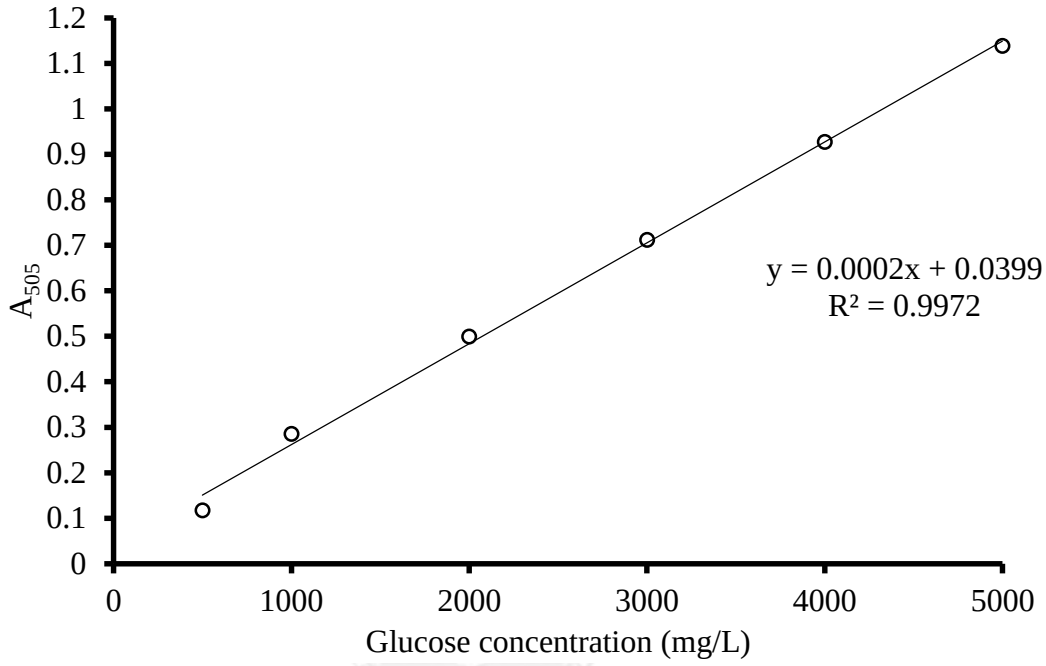
APPENDIX F

Standard calibration curve of sucrose by HPLC



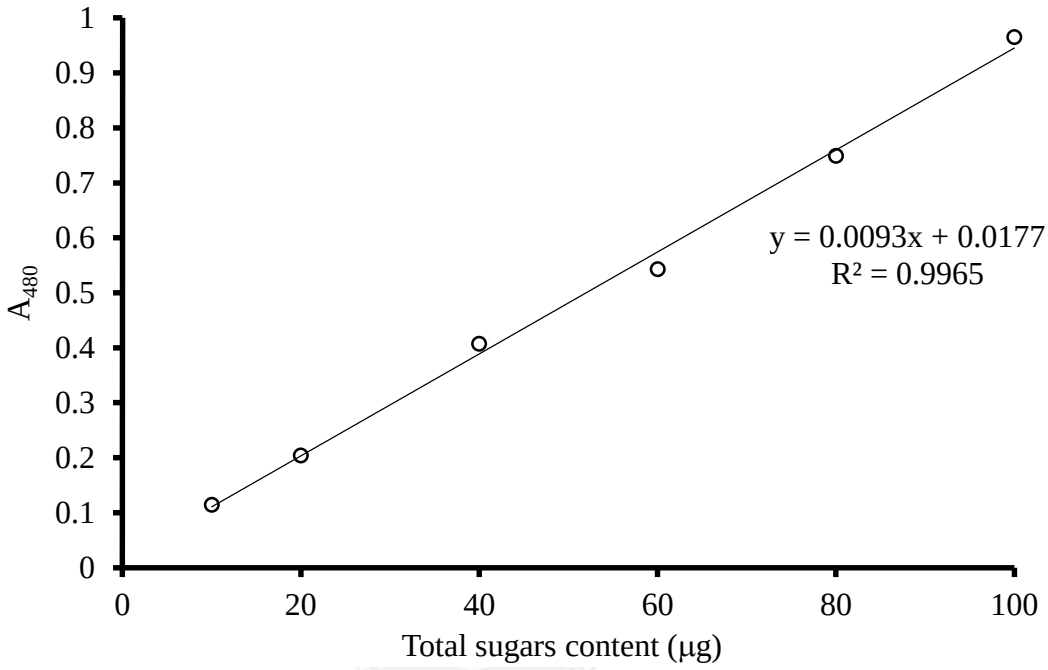
APPENDIX G

Standard calibration curve of glucose by glucose oxidase method



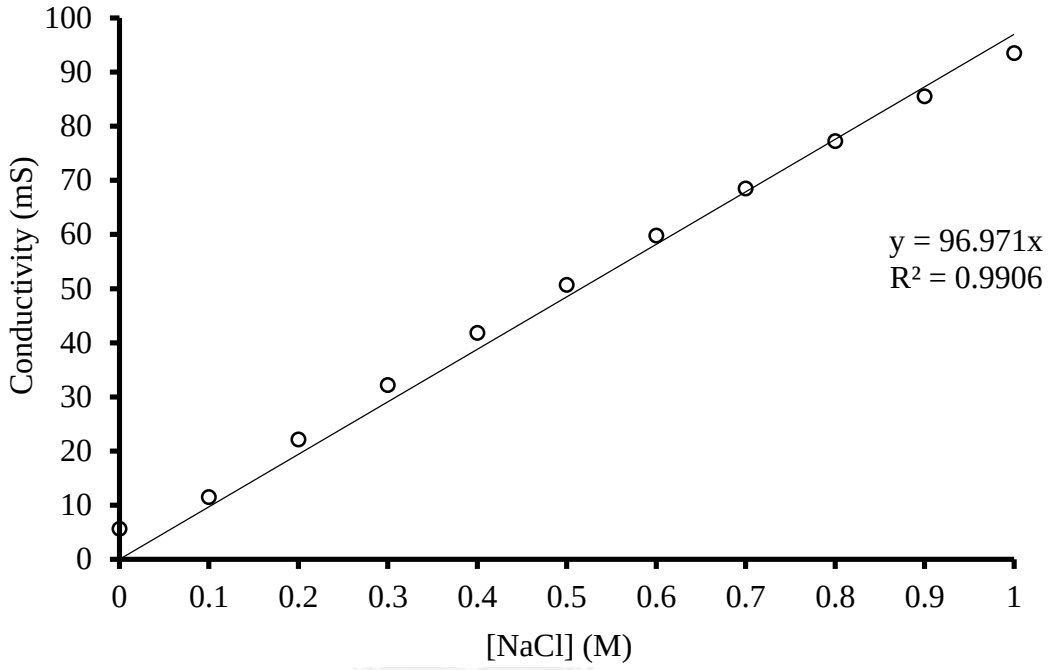
APPENDIX H

Standard calibration curve of total sugar phenol-sulfuric method



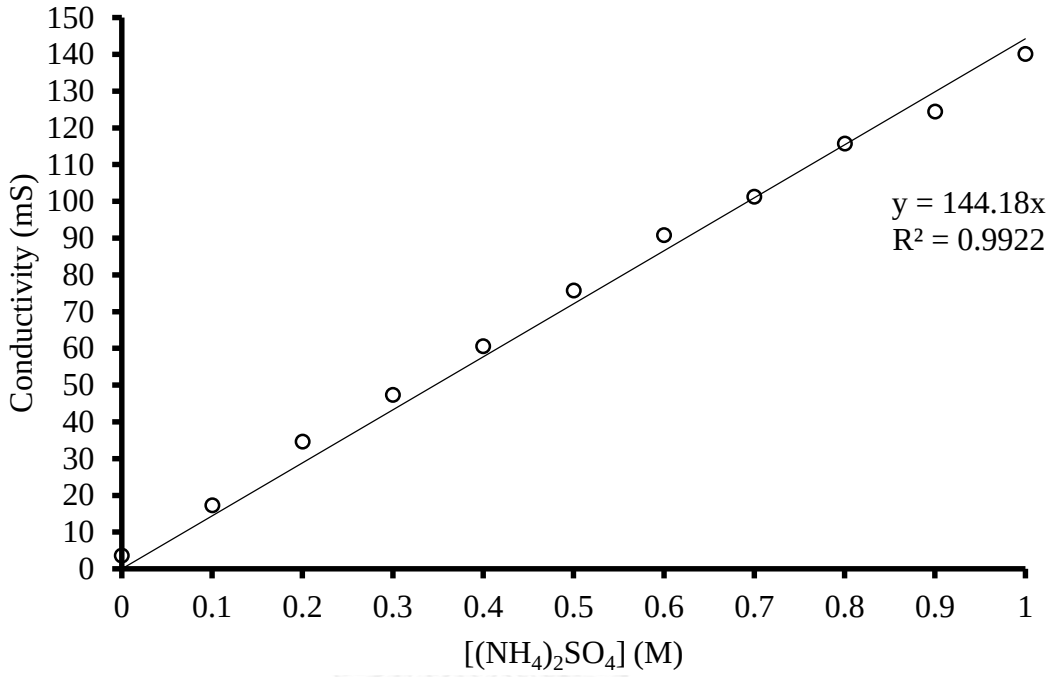
APPENDIX I

Standard calibration curve of NaCl concentration by conductivity measurement



APPENDIX J

Standard calibration curve of $(NH_4)_2SO_4$ concentration by conductivity measurement



VITA

Mr. Surawut Sangmanee was born on January 20th 1982 in Bangkok, Thailand. He graduated with a Bachelor Degree in Biotechnology, Faculty of Science, Ramkhamhaeng University in 2004 and a Master Degree in Biotechnology, Faculty of Science, Chulalongkorn University in 2006. After which, he worked at Greater Pharma Manufacturing Company Limited for 2 Years. He has enrolled in the Ph.D. in Biotechnology Program, Faculty of Science, Chulalongkorn University in 2009. He participated in the 15th International Biotechnology Symposium and Exhibition (IBS 2012) conference on 16th – 21th 2012 and the 1st International Graduate Research Conference (IGRC 2013) conference on 20th 2013.