Expression and Characterization of Dextransucrase from *Leuconostoc citreum* ABK-1

Mr. Kakuttapun Bulan

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Biochemistry and Molecular Biology
Department of Biochemistry
FACULTY OF SCIENCE
Chulalongkorn University
Academic Year 2021

การแสดงออกและลักษณะสมบัติของเด็กซ์แทรนซูเครสจาก *Leuconostoc citerum* ABK-1

นายกฤธภัณฑ์ บุหลัน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาชีวเคมีและชีววิทยาโมเลกุล ภาควิชาชีวเคมี
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2564

Thesis Title	Expression and Characterization of Dextransucrase from *Leuconostoc citreum* ABK-1
By	Mr. Kakuttapun Bulan
Field of Study	Biochemistry and Molecular Biology
Thesis Advisor	Assistant Professor Rath Pichyangkura, Ph.D.

Accepted by the FACULTY OF SCIENCE, Chulalongkorn University in Partial Fulfillment of the Requirement for the Master of Science

..... Dean of the FACULTY OF SCIENCE
(Professor POLKIT SANGVANICH, Ph.D.)

THESIS COMMITTEE

..... Chairman
(Associate Professor SUPAART SIRIKANTARAMAS, Ph.D.)

..... Thesis Advisor
(Assistant Professor Rath Pichyangkura, Ph.D.)

..... Examiner
(Kittikhun Wangkanont, Ph.D.)

..... External Examiner
(Wonnop Visessanguan, Ph.D.)

กกุธภัณฑ์ บุหลัน : การแสดงออกและลักษณะสมบัติของเด็กซ์แทรนซูเครสจาก *Leuconostoc citerum* ABK-1. (Expression and Characterization of Dextransucrase from *Leuconostoc citreum* ABK-1) อ.ที่ปรึกษาหลัก : ผศ. ดร.รัฐ พิชญางกูร

เด็กซ์แทรนซูเครสเป็นเอนไซม์ในตระกูลไกลโคไซด์ไฮโดรเลส 70 ที่มีเร่งปฏิกิริยาการไฮโดรไลซ์น้ำตาลซูโครสให้เป็นน้ำตาลฟรุกโตส และน้ำตาลกลูโคส นำเอาน้ำตาลกลูโคสมาสร้างสายพอลิเมอร์ของเด็กซ์แทรนโดยอาศัยการส่งถ่ายของโมเลกุลน้ำตาลกลูโคส เราพบยีนเด็กซ์แทรนซูเครสใน *Leuconostoc citreum* ABK-1 *(Lc*dextransucrase) และทำการยืนยันทางยีนว่าตรงกันกับเด็กซ์แทรนซูเครสใน *Leuconostoc citrem* DS ด้วยความเหมือนกัน 99.50% กับฐานข้อมูล NCBI ในงานนี้เราได้แสดงออกและหาลักษณะสมบัติของแด็กซ์แทรนซูเครสจาก *Leuconostoc citreum* ABK-1 รวมถึงศึกษาผลิตภัณฑ์ที่ได้ จากการทดลองเราสามารถโคลนเด็กซ์แทรนซูเครสยีนที่มีความยาวของยีน 4,935 คู่เบสเข้าไปในเวกเตอร์ pET21b โดยเชื่อมต่อเข้ากับ His-tag และแสดงออกโปรตีน โดยสภาวะที่เหมาะสมที่สุดในการแสดงออกของโปรตีน คือ ที่อุณหภูมิ 20 องศาเซลเซียส และชักนำด้วย IPTG ความเข้มข้น 0.5 mM จากการคำนวณโดย ExPASy พบว่าเด็กซ์แทรนซูเครสมีค่า pI อยู่ที่ 5.15 และมีน้ำหนักโมเลกุล 168 กิโลดาลตัน เรายืนยันการแสดงออกของโปรตีนโดยอาศัย Western blot และทำโปรตีนให้บริสุทธิ์โดยใช้ นิเกิล และ DEAE anion exchange คอลัมน์พบว่าสามารถทำให้โปรตีนบริสุทธิ์ขึ้น 80% เมื่อทำการวิเคราะห์ผลิตภัณฑ์ของเด็กซ์แทรนซูเครสโดยอาศัย TLC HPAEC-PAD และ NMR พบว่าผลิตภัณฑ์ที่ได้เป็นเด็กซ์แทรน ที่กลูโคสเชื่อมต่อกันด้วยพันธะ แอลฟา-1,6 ไกลโคซิดิก และอาจมีโซ่กิ่ง แบบแอลฟา-1,2 หรือ แอลฟา-1,3 นอกจากนี้คุณสมบัติของผลิตภัณฑ์สามารถนำไปประยุกต์ใช้ในเป็นพรีไบโอติกหรืออาหารฟังก์ชันได้

สาขาวิชา ชีวเคมีและชีววิทยาโมเลกุล ลายมือชื่อนิสิต .....

ปีการศึกษา 2564 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

6172114523 : MAJOR BIOCHEMISTRY AND MOLECULAR BIOLOGY

KEYWORD: Dextransucrase; Dextran; Protein purification; Leuconostoc citreum ABK-1

Kakuttapun Bulan : Expression and Characterization of Dextransucrase from *Leuconostoc citreum* ABK-1. Advisor: Asst. Prof. Rath Pichyangkura, Ph.D.

Dextransucrase is an enzyme in the glycoside hydrolase 70 family that catalyze the hydrolysis of sucrose to fructose and glucose, the glucose residues are transferred to synthesize dextran via transglycosylation reaction. Dextransucrase from *Leuconostoc citreum* ABK-1 (*Lc*dextransucrase), which has 99.5% homology with dextransucrase in the NCBI database, was cloned, expressed, and purified. The size of the *Lc*dextransucrase was predicted by ExPASy and the result showed that its size was 168 kDa with a pI of 5.15. Protein expression was confirmed by western blot analysis. *Lc*dextransucrase was purified through Ni-sepharose coupled with DEAE anion exchange column chromatography to approximately 80% purity. Moreover, the dextran product was analyzed by TLC, HPAEC-PAD, and NMR confirming that the product was dextran where glucose residues were mainly linked via a alpha-1,6 with some alpha-1,2 or alpha-1,3 branching. The dextran product may be applied and use as a prebiotic or functional food.

Field of Study:	Biochemistry and Molecular Biology	Student's Signature .....
Academic Year:	2021	Advisor's Signature .....

ACKNOWLEDGEMENTS

I would like to express my delight of finishing this master degree thesis. Firsly, I would like to thank my professor, Assistant Professor Dr. Rath Pichyangkura, for accepted me as his master degree student. He supported me all along the processes from start point to my finish line. He gave all beneficial advises and also his creativity to do all experiments. I deeply appreciated for his kindness and gave me an passionated lab work. Besides, I also have to say thank you to Dr. Karan Wangpaiboon for his convenient. His L. citreum ABK-1 helped me to amplified this LcDS gene. My western blot analysis would not be finished if I did not get Dr. Pawinee Panpetch help. The NMR results had been done by Assistance Professor Dr. Panuwat Padungros. If I did not get his assistance, my project would still be unclear.

Secondly I would like to thank to my backup. First people who I have to mention is Mr. Vijak Jareonsuk and Mr. Karanpat Seangsanoh. They are my rescue team. Mr. Vijak gave me his Macbook pro to finished my proceeding paper and also brought me from severe diarrhea to finish it. Mr. Karanpat, english teacher, help me to recorrect all points that reviewer need me to fix. I cannot get this proceeding paper if I did not have them. My 709 team also the best squad. Mr. Chay, Mr. Not, nong Fon, and nong Dragon tried to service me the best to finish my lab work.

Thirdly I would like to thank my friends. Oath, Ping, Petch, Pooh, and Guy that always be on my side no matter how! I can feel you love and supportive power in my suffering times. My super, dupper, and lovely sisters, Ms Pattarapon (Sai) and Ms Wanweepa (Por) who always sent their love when I was weak and also provisioned me through the thesis writing time. I would not forget all of your warmth. Thank Aom who always treated me when we eating out and Paulie who boosted my when I had no energy to work

Fourthly I would like all of my dated people and also my partners who

answered what I need. Thank Max, Alvin, Arsenio, Kevin, Ethan and the rest that I had forgotten their names.I also thanks Stefan, my German friend, who also played game with me when I had to work in late night.

Last but not least, I have to thank to my family, Pa and Mi (My dad and mom). You guys are always with me although we are in different places. When I felt failure or broken, I always felt that you are with me, always! Moreover you are my financial support, I gonna be died long time ago if you did not provide me that.

With love

Kakuttapun Bulan

Kakuttapun Bulan

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Chapter 1 Introduction

Carbohydrates polymers

Carbohydrates are vital macromolecules that can be synthesized into polymers or polysaccharides. Carbohydrate polymers are plentiful and crucial biological macromolecules on earth. They play several roles such as acting as energy sources for living organisms, ribose and deoxyribose are the carbohydrate polymers of nucleic acids, and also are involved in cell-cell communication as glycoproteins. The polymers can be categorized based on their origin of them. First are polymers that can be found in nature. These polymers can be homo- or hetero-polymers. The examples for this group are cellulose and agarose. The second is synthetic polymers. This type is based on the synthesis of polymers using carbohydrate backbone as the templates. The synthesis one could be in the ring or opening form. The last polymers are glycopolymers. Glycopolymers are polymers that consist of monosaccharides or polysaccharides as branching. These examples could state that carbohydrates are a complex macromolecule[1].

The key to the carbohydrate polymer complexity is formed by glycosidic linkages. These linkages led to a diversity of polysaccharides and provided a lot of beneficial polymers. Nowadays many reports showed that the structure of polymers is related to their functions. Here are the lists of polysaccharides functions in the present years[2, 3, 5].

1) The antioxidant

In this group, polymers exhibited from plants were produced, the important polymer which makes the antioxidant activity is mannose. The reports showed that the high content of mannose with low content of sucrose will produce ROS scavengers to destroy free radicals. Not only mannose but electrophile uronic acid also can release hydrogen to accept the free radicals and also to eradicate

them. Moreover, the reports showed the relation between the molecular weight of the polymers and their scavenging activities. They revealed that the increase of molecular weight led to a lowering the scavenging activity[6-8].

2) The immune regulators

The reports showed that plant polysaccharides can be good candidates for therapeutic agents. The main reason is their low toxicity. These polysaccharides help macrophages to be more vital. Galactose contents in polysaccharides help to promote the proliferation of macrophages. The activity is based on the recognition of polysaccharides and the receptors specifically. furthermore, the higher molecular weights provide higher repetitive receptor sites. The studies showed that the polymers with more mannoses and galactoses display higher immunomodulatory activity[9-11].

3) The antitumor activity

The antitumor activity is depending on the structure of polymers. In vitro study found that the high degrees of branching can exhibit strong antitumor activity. Mannose and glucose are essential monomers that manufacture antiproliferative activity. It could be concluded that the factors which effect on antitumor are the monosaccharide compositions, types of glycosidic linkages, molecular weights, and degrees of branching[6, 7, 12-14].

4) The biostimulator activity

In agriculture, polysaccharides play prominent roles to solve agricultural production. The polymers from algae enhance the secondary metabolites and trigger the plant defense mechanisms. Many reports showed that the polymers were used as plant growth regulators and decreased of using chemical fertilizers. The study showed that alginate oligosaccharides increased the expression level of salicylic acid, jasmonic acid, and ethylene production to inhibit the proliferation of the fungi, *Aternaria solani*.[15-17]

5) Use in the medical treatments

Marine algae produce marine polymers which are biocompatibility, biodegradability, low toxicity, and can be used as a hydrogel. Examples of these polymers are carrageenan, ulvan, and fucoidan. These polymers play biological activities such as antioxidant, anticoagulant, anticancer, antiviral, anti-allergic, and anti-inflammatory activities. [2, 18, 19]

6) Polysaccharide using in the food field

Food hydrocolloids such as agar, carrageenan, alginate, and laminarins are used as animal food supplements. They help to improve the antioxidant activity in horses and pigs. The study believed that this activity will help the food production as functional cooked food and directed to improve the human antioxidant defense.[20, 21]

Screening of Khao-tom-mud

Wangpaiboon et. al had annotated novel glucansucrase genes in *Leuconsotoc citreum* ABK-1 isolated from Khao-tom-mud (The thai dessert). Two genes were studied and characterized as alternansucrase and glucansucrase. There were two genes that were not identified. One of them was annotated as dextransucrase. Thus, we decided to study dextransucrase from *Leuconostoc citreum* ABK-1.

Leuconostoc citreum ABK-1 alternansucrase gene (*Lcalt*) was an enzyme which catalyzed the transferring of glucose moiety onto sucrose to form an alternan. *Lcalt* had been expressed in *E. coli* system. In the study found that *Lcalt* encoding 2,057 amino acid residues which produced the product containing main chain of 1,6 glycosidic linkage with the 1,3 glycosidic branching.[22]

The next gene which had been annotated was glucansucrase gene (*dex-N* gene). As its name, the product from this gene was glucansucrase enzyme which can produce glucan polymer using sucrose as substrate. The glucansucrase can

catalyzed the hydrolysis of sucrose and transfer glucose to form glucans. *Lc*dex-N enzyme produced 2 forms of products, soluble and insoluble glucans (S-dextran and I-glucan). S-dextran exhibited the main chain of 1,6 glycosidic linkage whereas I-glucan displayed 1,3 and 1,6 glycosidic linkages.[23]

Lactic Acid Bacteria

Lactic acid bacteria (LAB) are a group of gram-positive cocci or bacilli which can be both facultative anaerobes or anaerobes[24, 25]. The LAB can produce lactic acid from the fermentation of carbohydrates such as sugars. These bacteria can survive in several environment niches[26] i.e., in the soil[27], on the plants, or even in the gut of plant eater mammals[28]. The specific LAB can digest simple sugar, sucrose, and convert it into polysaccharide polymers.

Nowadays the LAB were used in food industries because of their notable characteristics e.g., the production of bacteriocins[29]. The bacteriocins are small proteins inhibiting the proliferation of pathogens[30, 31]. Bacteriocins are lantibiotic[32], nisin. It is an antibiotic containing unique amino acids such as thioether amino acids[33, 34]. The lanbiotics help the LAB to attack other gram-positive bacteria. Nisin is used for a food preservation[35].

Moreover, the other benefit of LAB is an immune regulator[36]. When the gut is invaded by other microorganisms, *Lactobacilli* LAB promote phagocytic activity[37] to kill those bacteria. They can promote host immune response by the interaction between LAB protein to specific host receptors and trigger the secretion of cytokines and chemokines[38, 39]. This benefit is used in diarrhea patients[40].

Chapter 2 Literature Reviews

Glycoside hydrolase 70 family

Glycoside hydrolase 70 family (GH70) is the family of enzymes which can hydrolyze and synthesize many types of glycosidic linkage i.e., α-1⟶2, α1⟶3, α1⟶4, α1⟶5, and α1⟶6. The special character is that the enzymes in this group create various products in terms of sizes, structures, degree of branching, and the arrangements of branches. Examples of enzymes in this family are glucansucrase, dextransucrase, and alternasucrase.[22, 41]

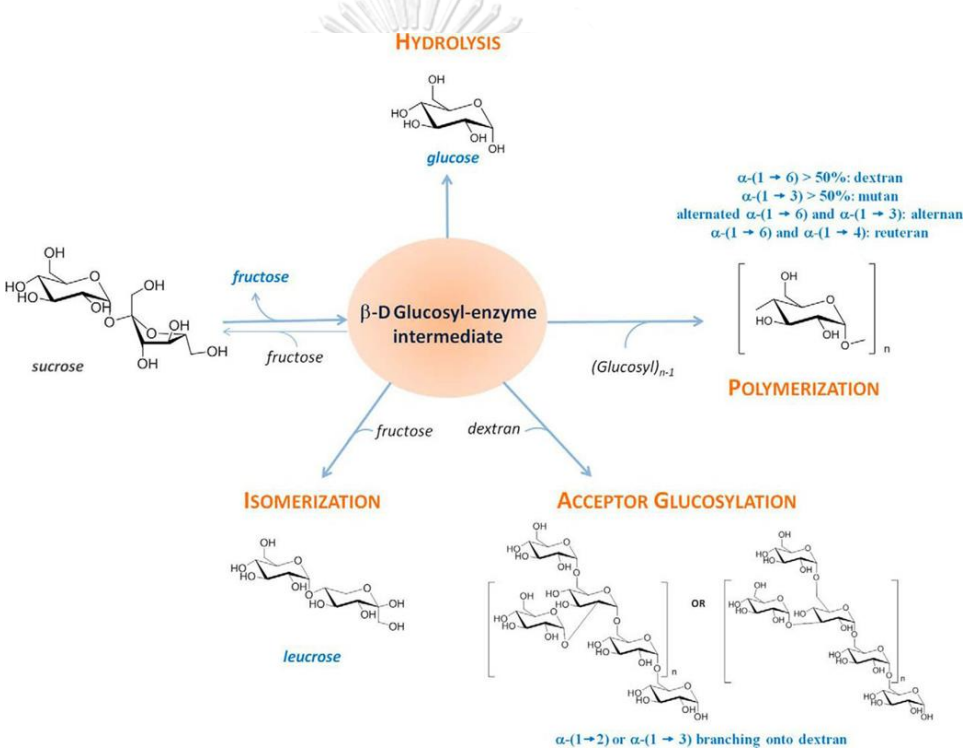


Figure 1 the activities of glycoside hydrolase 70 family (GF70) including hydrolysis and transglycosylation[42]

Dextransucrase enzyme

Dextransucrase is an enzyme in glycoside hydrolase 70 family which can catalyze sucrose into glucose and fructose before transferring glucosyl moiety to the acceptors and produce several dextrans in the nature. The dextrans were first found in *Leuconostoc mesenteroides* while this microbial created slime on the cane and beet sugar juice. After that, the slime was investigated and named it as dextrans. The

dextrans were continuously found that they could synthesize on other organisms not only in *Leuconostoc mesenteroides*. Dextrans are homopolysaccharide composing of glucose with α1→6 glycosidic linkage. For dextrans this linkage must occupied in the products more than 50% of total linkages[43-45].

Dextransucrase is secreted mainly by lactic acid bacteria (LAB). The examples of LAB are *Leuconostoc, Streptococcus*, and *Lactobacillus* spp. The enzyme appeared to have a size around 170 kDa. The proposing mechanism of this enzyme using *L. mesenteroides* NRRL and B512F and streptococcus mutans 6715 by Robyt revealed that the C-6 attacks to C-1 of the other to form 1→6-α glycosidic linkage and one of glucosyl residue is transferred to the other. The freed nucleophilic residue attacks on the nearby sucrose and form a new glucosyl intermediate which attaches to C-1 of the growing dextran chain and continuously performing polymerize activity[46, 47].

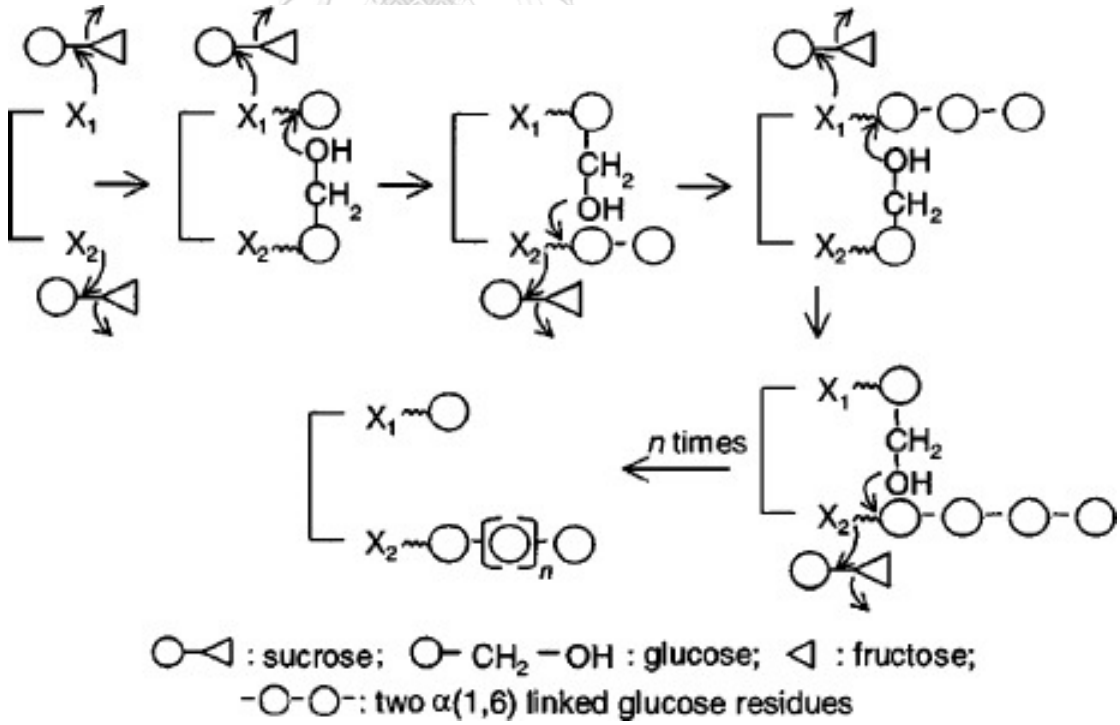


Figure 2 the dextransucrase mechanism suggested by Robyt[47]

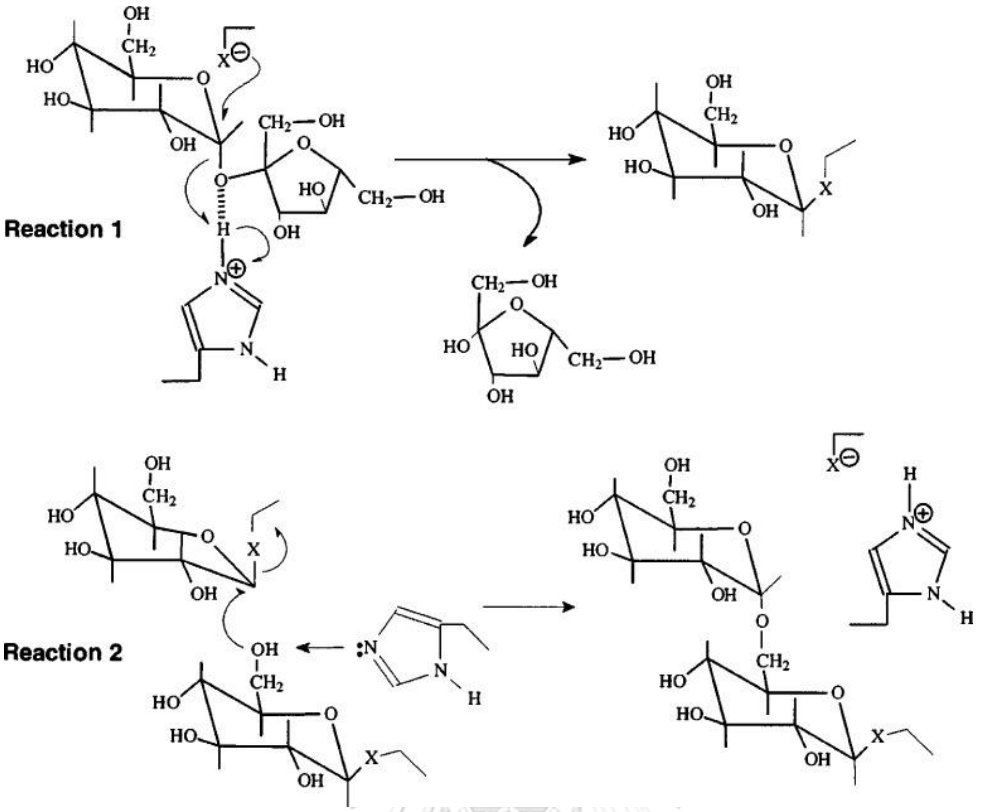


Figure 3 Hydrolysis mechanism of dextransucrase[47]

Dextransucrase of *Leuconostoc*

As we mentioned earlier in the previous topic (Dextransucrase enzyme), in general, dextransucrases work in an environment which enriches by sucrose so it can catalyze the transferring of D-glucosyl moiety to form dextran. The report of Quirasco and colleagues supported that in *Leuconostoc mesenteroides* NRRL B-512F encompassing dextransucrase gene can grow on D-glucose or D-fructose, it made the screening of dextranucrse gene became more problematic[48]. The enzymes from D-glucose and D-fructose media showed protein aggregation and also expressed low enzyme yield leading to the multiple forms of the products. These enzymes established the difficulty of enzyme purification.

The adventages of dextrans

1) **Cosmetic preparation**

Dextran was used as a binding agent to improve the distribution of the components in cosmetic inventions[49]. With the thickening behavior, dextran

generates the viscosity of the product and acts as a bulking agent. There were reports that dextran helped in antiaging activity and also deducted skin allergies. High molecular weight dextrans are commonly used in the cosmetics industries[50]. The dextrans derivatives with a molecular weight of more than 40 kDa are added to cosmetic and they can create a film-like character which can be absorbed by skin and hair[51]. Moreover, dextrans with 70 kDa tend to generate adhesive patches on wrinkled skin hence they help the skin looks younger. Besides, dextrans contain sulfate can help the blood flow thus they can activate the growth of hair and also regenerate keratinocytes.

2) Nanocarrier for active compounds agents

Since dextran has a hydroxyl group, it can conjugate other chemicals easily. For example, the modification of dextran with hydrophobic chemicals. This type of dextran exhibits low water solubility that it can as the capsule to cover the chemotherapeutic agents[52, 53]. Then it can carry those agents throughout the body as the nanocarrier[54]. Moreover, in the same situation, dextrans also can be a drug delivery. The addition of lithocholic acid into dextran can develop the hydrophobic dextran which can carry out the drug into the drug target

3) Functional food

Probiotic LAC can produce tprebiotic dextrans. The previous studies revealed that dextran produced by *Weissella cibaria* RBA12 can act as a prebiotic to grow *Bifidobacterium* and *Lactobacillus* spp., and inhibited the growth of enteric bacteria[55]. What's more *Leuconostoc mesenteroides* NRRL B-1426 had been studied and showed the same characteristics. This dextran was not digested by **α**-amylase so it can pass through the stomach and intestine. These supporting data displayed that the dextrans from probiotics such as *Weissell* and *Leuconostoc* spp. could benefit normal flora as a prebiotic[56, 57].

Chapter 3 Materials and Methods

1) Materials

1) Cloning

i) Restriction Enzymes and buffers

(1) NdeI (NEB)

(2) XhoI (NEB)

(3) 10×Cut smart buffer (NEB)

(4) T4 DNA ligase (NEB)

(5) 10×T4 DNA ligase buffer (NEB)

(6) Primestar polymerase (Takara)

(7) 5×Primestar polymerase (Mg^{2+} plus) (Takara)

(8) Forward primers (Macrogen)

(9) Reverse primers (Macrogen)

(10) 2.5 mM dNTP mixture (Takara)

ii) Bacterial host and cloning vectors

(1) pET21b vector (Novagen)

(2) pET19b vector (Novagen)

(3) *E. coli* TOP10 (Novagen)

iii) Chemiclas

(1) Deionized water

(2) Double deionized water

(3) 10×Tris-EDTA pH 8.0

(4) Phenol: Chloroform: Isoamyl alcohol

(5) LE Agarose (SBio)

iv) Antibiotic

(1) Ampicillin (Goldbio)

v) Media

(1) Luria-Bertani Media (LB)

(a) 1%(w/v) tryptone (Bio basic)

(b) 0.5%(w/v) NaCl (Univar)

(c) 0.5%(w/v) yeast extract powder (Himedia)

(d) Agar bacteriological (Difco)

vi) Kits

(1) GenepHlow™ Gel/PCR kit (Geneaid)

(2) Presto™ Mini Plasmid kit (Geneaid)

vii) Instuments

(1) T100™ Thermal cycler (Bio-rad)

(2) Minigel electrophoresis (Hercuvan)

2) Protein expression

i) Bacterial host

(1) *E. coli* BL21 star (DE3) (Novagen)

ii) Media

(1) Luria-Bertani Media (LB)

(a) 1%(w/v) tryptone (Bio basic)

(b) 0.5%(w/v) NaCl (Univar)

(c) 0.5%(w/v) yeast extract powder (Himedia)

(d) Agar bacteriological (Difco)

iii) Antibiotic

(1) Ampicillin (Goldbio)

iv) Chemical

(1) Isopropyl-ß-D-thiogalactoside (Goldbio)

(2) Phenylmethylsulfonate fluoride (PMSF) (Applichem Panreac)

3) Protein Purification

i) Beads

(1) Ni-sepharose™ 6 fast flow (GE Healthcare)

(2) DEAE-toyopreal 650M (TOSOH)

ii) Buffers

(1) 2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulfonic acid (HEPES) (Golbio)

iii) Chemicals

(1) Triton X-100 (Scharlau)

(2) $CaCl_2$ (Scharlau)

(3) Imidazole (Loba chemie)

2) Construction of dextransucrase gene from *Leuconostoc citreum* ABK-1

i) Polymerase chain reaction amplification of *LcDS* gene

Genomic DNA (gDNA) of *Leuconostoc citreum* ABK-1 had been prepared by Dr. Karan Wangpaiboon.[22] The gDNA was then used as the template in the amplification of dextransucrase gene from *Leuconostoc citreum* ABK-1. The *LcDS* gene was amplified using polymerase chain reaction (PCR). The PCR reaction was performed under the condition in table 2. Four sets of primers were used in the experiment. The restriction sites, NdeI and XhoI, were added to the primers as shown below.

Forward primer 1: 5'CCAACATATGTCAGTTACTGTCTCCAATAATTCGAATAC3'

Reverse primer 1: 5' CCTTCTCGAGAGCGACTGAGACAAAGTAACCTTGGTCATCG 3'

Forward primer 2: 5'CAAGCATATGTCAGTTACTGTCTCCAATAATTCGAATAC3'

Reverse primer 2:
5'ATCCTCGAGTTAAGCGACTGAGACAAAGTAACCTTGGTCATCGA3'

The template was diluted 100-fold before use. The reaction was set as in table1

Substances	Volume
Template	1µl
Forward primer	1.25 µl
Reverse primer	1.25 µl
Primestar polymerase	0.5 µl
5×Primestar polymerase buffer	10 µl
2.5 mM dNTP Mixture	4 µl

Table 1 the chemicals for Lcdextransucrase gene amplify using in PCR technique

States	Temperature	Time
Pre-denaturation	98°C	30 seconds
Denaturation	98°C	10 seconds
Annealing	55°C	10 seconds
Extension	72°C	4.4 minutes
Final extension	72°C	5 minutes

Table 2 the amplification of PCR products protocol

After the polymerase chain reaction had been performed, the PCR products were collected and cleaned up with GenepHlow™ Gel/PCR kit (Geneaid). The purified PCR was cleaved at the NdeI and XhoI sites by endonuclease enzymes.

ii) Plasmid preparation

pET19b and pET21b vectors were amplified by transforming them into *E. coli* TOP10 and then the transformants were spread onto LB agar containing 10 μg/ml ampicillin (working concentration). The bacteria were picked and grown in 7 ml LB broth containing 10 μg/ml ampicillin. The culture was collected by centrifugation at 14,000×G for 1 minute at room temperature and the vectors were extracted using Presto™ Mini Plasmid Kit. After the plasmids had been extracted, the plasmids were analyzed by 0.7% agarose gel. The positive clones were digested with NdeI and XhoI restriction enzymes preparing for the ligation with the *LcDS* in the next process. The digestion reaction was shown in the table. After the double restriction enzymes were digesting, the reactions were run on 0.7% agarose gel and the vectors were extracted from the gel with GenepHlow™ Gel/PCR kit (Geneaid).[58]

Chemicals	Volume
pET19b or pET21b plasmid	87 µl
NdeI restriction enzyme	1.5 µl
XhoI restriction enzyme	1.5 µl
10×Cutsmart buffer	10 µl
Final volume	100 µl

Table 3 The restiction enzyme for edonucleation of the plasmids

iii) Ligation reaction of *LcDS* gene into expression vector

Three constructs of *LcDS* gene were conducted in the previous experiment. The constructs consisted of the non-modified *LcDS* gene, modified-C-terminus histidine-tagged *LcDS* gene, and modified-N terminus histidine-tagged *LcDS* gene. The construct of non-modified *LcDS* gene was performed using the forward primer 2 and the reverse primer 2. The non-modified *LcDS* gene was ligated into pET21b vector (Novagen). The modified C-terminus histidine-tagged *LcDS* gene was performed using the forward primer 1 and reverse primer 1 and then ligated into pET21b vector (Novagen). The last construct, the modified N-terminus histidine *LcDS* gene, was amplified using the forward primer 2 and reverse primer 2. The PCR product was ligated into pET19b vector (Novagen). The ligation reactions were purified with phenol: chloroform: isoamyl alcohol.[58]

iv) The amplification of the 3 constructs of *LcDS* gene

The ligated *LcDS* gene with expression vectors was transformed into *Escherichia coli* TOP10 to propagate the clones. *E. coli* TOP10 was prepared using electrophoresis transformation competent cell preparation. 4 µl of ligation reactions were transformed into *E. coli* TOP10 competent cell. The vectors pET19b and pET21b had the ampicillin resistance gene as the gene marker. The transformants were spread on LB agar containing 100 µg/ml ampicillin. The plate was then incubated at 37°C for 16 hours.[22, 41]

v) Plasmid extraction of *LcDS* constructs

The transformants from previous experiment were grew in 7 ml LB broth containing 100 µg/ml ampicillin at 37°C, 250 rpm for 16 hours. The cultures were collected at 14,000×G for 1 minute at room temperature. The supernatant was discarded and disinfect with disinfectant for 30 minutes. The cell pellet was used to extract the plasmid using Presto™ Mini Plasmid Kit. The extracted plasmids were analyzed with 0.7% agarose gel. [22]

vi) Analyzing of *LcDS* constructs

The extracted plasmids were analyzed by restriction enzyme analysis. The plasmids were incubated as in the table4.

Chemicals	Volume
Plasmids	3 µl
NdeI restriction enzyme	0.5 µl
XhoI restriction enzyme	0.5 µl
10× Cutsmart buffer	2 µl
Double distilled water	14 µl
Final volume	20 µl

Table 4 the resticition enzymes using in the analysis of recombinant plasmid

The reactions were run on 0.7% agarose gel and the positive clones were sent for sequencing with Barcode Tagged Sequencing (U2Bio Thailand, Co. ltd.)

3) Protein expression of *Lc*DS enzyme

i) Preparation of *E. coli* BL21 Star (DE3)

E. coli BL21 Star (DE3) (Invitrogen) was streak on LB agar and grown at 37°C for 16 hours. The single colony was picked and grown on LB broth at 37°C, 250 rpm for 16 hours as starter. Then 1% of starter was transferred into 1 liter LB broth and grown at 37°C, 250 rpm until the OD_{600} reached 0.4. When the OD_{600} had reached 0.4, the cultured was immediately shaking on ice for 20 minutes, vigorously. The

culture was transferred into prechilled 250 ml bottles. After the culture had been transferred, it was centrifuged at 4,000×G, 4°C for 10 minutes. The supernatant was discarded and the pellet was resuspended in prechilled $MgCl_2$-$CaCl_2$ solution (80mM $MgCl_2$, 20 mM $CaCl_2$). The dissolved pellet was spun down again at 4,000×G, 4°C for 10 minutes. After spun down, the supernatant was discarded and the pellet was resuspended in 0.1 M $CaCl_2$. 50 µl of cell was aliquot into 1.5 ml tubes and suddenly flash freeze with liquid N_2. [43]

ii) Transformation of *LcDS* gene into *E. coli* BL21 Star (DE3) as the expression host

All three constructs of *LcDS* gene were transformed into *E. coli* BL21 Star (DE3) as the expression host. 1 µl of plasmids was transferred into *E. coli* BL21 Star (DE3) and chilled on ice for 20 minutes. Then heat shock the competent cell at 42°C for 45 seconds. The transfected cell was immediately chilled on ice for 2 minutes. 0.9 ml LB broth was added to the cell and incubated at 37°C for an hour. 100 µl of culture was spread on LB agar containing 100 µg/ml ampicillin.

iii) Optimization of *Lc*DS expression in *E. coli* system

(1) Temperature optimization

The *Lc*DS transformant within *E. coli* BL21 star (DE3) was picked and grown in 7 ml LB broth containing 100 µg/ml ampicillin at 37°C, 250 rpm for 16 hours as the starter. 1% of starter was transferred into 50 ml LB broth containing 100 µg/ml ampicillin and incubated at 37°C, 250 rpm until OD_{600} reached 0.5. After the OD_{600} reached 0.5, the culture was incubated at 20, 30, and 37°C with 0.5 mM IPTG induction for optimize the temperature for 6 hours.

(2) Optimization of IPTG concentration and time coarse analysis

The *Lc*DS transformant within *E. coli* BL21 star (DE3) was picked and grown in 7 ml LB broth containing 100 µg/ml ampicillin at 37°C, 250 rpm for 16 hours as the starter. 1% of starter was transferred into 50 ml LB broth containing 100 µg/

ml ampicillin and incubated at 37°C, 250 rpm until OD_{600} reached 0.5. After the OD_{600} reached 0.5, the culture was incubated at optimal temperature and induced with 0, 0.1, 0.2, 0.4, 0.5, 0.8, and 1 mM IPTG for 0, 3, 6, 9, and 18 hours, respectively.

(3) Protein expression of *Lc*DS enzyme

The *Lc*DS transformant within *E. coli* BL21 star (DE3) was picked and grown in 7 ml LB broth containing 100 µg/ml ampicillin at 37°C, 250 rpm for 16 hours as the starter. 1% of the starter was transferred into 1 l LB broth containing 100 µg/ ml ampicillin. and incubated at 37°C, 250 rpm until OD_{600} reached 0.5. Then the culture was incubated with 0.5 mM IPTG and incubated at 20°C, 200 rpm for 6 hours.

4) *Lc*DS activity assay

An *Lc*DS was expressed in *E. coli* BL21 star (DE3) with optimal conditions. The culture was collected and removed the media by centrifugation at 8,000×G for 10 minutes. The cell then was lysed with lysis buffer (25 mM HEPES buffer pH 7.4, 500 mM NaCl, 0.1% Triton X-100 or 25 mM HEPES buffer pH 6.0, 1 mM $CaCl_2$, 0.1% Triton X-100) and sonicated with pulse 1×4 second for 1.30 minutes. The cell lysate was collected by centrifugation again at 10,000×G for 10 minutes. The supernatant was collected as a soluble protein and the pellet was collected as inclusion bodies. The *Lc*DS was tested for an activity with 3,5-dinitrosalicylic acid (DNS assay). The DNS was turned into 3-amino-5-nitrosalicylic acid when it was incubated with reducing sugar, fructose. The enzymatic reaction contained 250 mM sucrose, 1 mM $CaCl_2$, and 50 mM acetate buffer pH 5.5. 50 µl of the enzyme was added to the reaction and then incubated at 30°C for 15 minutes. The reaction was terminated by adding 500 µl of DNS and boiled for 10 minutes. The reaction measured the absorption at OD 540 nm. One unit of *Lc*DS activity was defined as the releasing 1 µM of fructose per minute.

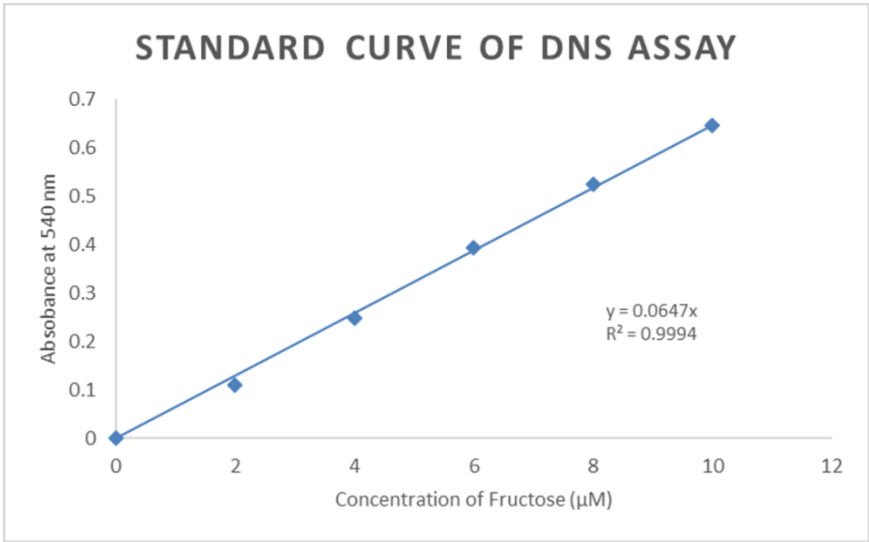


Figure 4 standard curve of DNS assay detection of fructose

5) The purification of *Lc*DS enzyme

i) Ni-sepharose column chromatography

An *Lc*DS was expressed in *E. coli* BL21 star (DE3). Then the cell culture was centrifuged at 8,000×G for 10 minutes. The supernatant was discarded and the pellet was resuspended in lysis buffer (25 mM HEPES buffer pH 7.4, 500 mM NaCl, 10%Triton X-100). The resuspension was sonicated with a pulse of 1×4 seconds for 1.30 minutes. The cell lysate was spun down at 10,000×G for 10 minutes. The supernatant was collected as *Lc*DS. The *Lc*DS was purified through 2 ml Ni-sepharose gravitationally. In the purification, 2 buffers were used in the experiment. The first buffer, buffer A, was 25 mM HEPES buffer pH 7.4 and the second one, buffer B, was 25 mM HEPES buffer pH 7.4, 1 M NaCl. Buffer A was used to equilibrate the system until the baseline was 0. Then the *Lc*DS was loaded into the system and collected as a flowthrough. After all, the enzyme had been loaded, the system was washed with buffer B for 5 times the column volume. Then the enzyme was eluted against 10-50 mM imidazole. The system was washed again with a 5-time column volume of buffer B.

ii) DEAE anion exchange

An *Lc*DS was expressed in *E. coli* BL21 star (DE3). Then the cell culture was centrifuged at 8,000×G for 10 minutes. The supernatant was discarded and the pellet was resuspended in lysis buffer (25 mM HEPES buffer pH 6.0, 500 mM NaCl, 10%Triton X-100). The resuspension was sonicated with a pulse of 1×4 seconds for 1.30 minutes. The cell lysate was spun down at 10,000×G for 10 minutes. The supernatant was collected as *Lc*DS. The *Lc*DS was purified by 2 ml of Toyopearl DEAE-650M bead. The column was purified gravitationally. In the purification, 2 buffers were used in the experiment. The first buffer, buffer A, was 25 mM HEPES buffer pH 6.0, and the second one, buffer B, was 25 mM HEPES buffer pH 6.0, 500 mM NaCl. Buffer A was used to equilibrate the system until reaching baseline. Then the *Lc*DS was loaded to the system and collected as flowthrough. After all, the enzyme had been loaded, the system was washed with 5 times column volume buffer. Then the enzyme was washed again with diluted buffer B to 0.1 M NaCl final concentration. The enzyme was eluted against diluted buffer B to 0.2 M NaCl final concentration.

iii) The purification of *Lc*DS using ultrafiltration

The LcDS enzyme, which was purified against the Ni-Sepharose column, was loaded to AMICON (MWCO 100 kDa) spin column. The purpose of this experiment was to remove nonspecific protein with a size lower than 100 kDa to make the protein cleaner. After the Ni-sepharose protein had been loaded, buffer B for DEAE anion exchange was used to exchange the buffer preparing for next step purification.

In this experiment, we were also required to detect protein aggregation. We decided to compare the result of the addition and non-addition of triton X-100. The result was analyzed using 8% SDS PAGE gel.

iv) The coupling purification method using Ni-sepharose and DEAE anion exchange

The *Lc*DS was purified using Ni-sepharose as in topic i) then the purified protein was clean up by AMICON MWCO 100 kDa ultrafiltration as described in topic iii). After that the protein was purified against DEAE Toyopearl 650M as in topic ii).

6) The analysis of *L*cdextran product

i) Thin-layer chromatography technique

1 unit of the crude enzyme means that dextransucrase can hydrolyze sucrose into 1 μM fructose in a minute. Then if we incubate 60 μM of sucrose, the enzyme can convert it into the product in an hour. So, we incubate 20% of sucrose with tan excessive amount of enzyme (140 U) overnight. Then the product was used for analysis by TLC. The 0.5 μl of products were spotted on TLC plate. The standards which were used in this technique were monomer of glucose from G1-G7, fructose-sucrose standard (FS), standard containing 1→6 α glycosidic linkage, GF_n is standard for sucrose (GF_1), 1-kestose (GF_2), and nystose (GF_3). Then the orcinol-sulfuric acid was used to visualize the product's patterns[22, 41]

ii) HPAEC-PAD analysis of *Lc*dextran product

The product heated for 10 minutes to remove the contaminated proteins and then the product was precipitated in 1:1 acetone. The polymer was precipitated and take all oligosaccharides away by resuspending the pellet with distilled water for 50 ml. Again, with the centrifugation at 10,000×G for 20 minutes and repeat the step 3 times. Then the polymer was partially hydrolyzed in 1 M HCl and neutralization with saturated Na_2CO_3. After that the *Lc*dextran was lyophilized. 200 mg of *Lc*dextran was dissolved in 3 ml distilled water. The product was sonicated at 50% amplitude for 5 minutes. Next, 5% of *Lc*dextran

was desalted by mix bed resin. And the 0.5 % desalted product was filtered through 0.45 μm nylon membrane and analyzed with HPAEC-PAD.

iii) NMR analysis of product

We sent the dextran polymer to be analyzed with ^{1}H and ^{13}C NMR 500 MHz with Assistant Professor Dr. Panuwat Padungros's laboratory, Mahamakut Building, faculty of Chemistry, Chulalongkorn University.

Chapter 4 Results and Discussion

1) *LcDS* cloning

The construction of *LcDS* gene had constructed into 3 constructs the first construct was the *LcDS* with C-terminus histidine-tagged. The gene was inserted into pET21b vector. The *LcDS* gene size is 4,935 base pair. The vector, pET21b, size is 5,443 base pair. The result showed 0.7% agarose gel analyzed comparing cut and uncut plasmid of *LcDS* within pET21b. the upper band showed the pET21b vector while the lower band showed insert fragment of *LcDS* (Figure5). The positive clone was sent for sequencing to confirm the sequence. The rest two constructs were simultaneously cloned into the vectors. After the confirmation of restriction enzyme analysis showed that the insert fragment was ligated into the vector, further confirmation by DNA sequencing had been done also. The corrected recombinant plasmid was collected in -20°C and used for the next step which was protein expression in *E. coli* system.

Here are the DNA sequences of *LcDS* gene:

TTGATTAAAGAGAGAAATGTACGAAAAAAGCTCTACAAGTCTGGTAAGAGTTGGGTTATTG
GGGGACTCATTTTATCGACAATTATGCTGTCTATGACCGCTACTTCACAAAATGTTAATGCAGAT
AGCACAAACACAGTGACGGATAAGTTCAGTTACTGTCTCCAATAATTCGAATACAACCAATCAA
CACGATACTGTCGTTGACCAACAAACGACACCTGTCAAAAATAACCAAACAACACAACAAATCG
CCGCAAATGCCAACCAAGCAGAAAAAGTAAAAGCATCAGACACAACGACTGATACGCAAAAGCA
AGCTGAAACGCCAAACAACACTAACAAGGATTCGATAGATAATCTCACCAAGCAGTTGCCGACT
GTTACGCCAACAGCTAATCAAAAAACTGGTTATCTGGAAAAAGATGGTAAATGGTACTATGTAA
CCAGTGATAACACACTTGCTAAGGGGTTGACTACTGTTGACAACCACAAGCAGTATTTTGACAA
CAATGGCGTGCAGGCAAAAGGCCAATTCGTTACTGATAACAGTAAAACATACTATCTCGATTCT
AACTCCGGTAACGCAGTAACCGGGATACAACAAATTGGCTCACAAACATTAGCCTTCAATGACA
ACGGTGAACAAGTTTTTGCTGATTTCTATACAGCGCCAGATGGCAAAACTTATTATTTTGACGAT
AAAGGACAAGCAACTATTGGTCTAAAGGCCATTAATGGGCACAATTATTACTTCGATAGTTTGG

GACAACTAAAAAAAGGATTTACCGGTGTCATTGACGGTCAAGTACGCTATTTTGATCAAGAATC

AGGACAAGAGGTATCAACAACCGACTCACAAATCAAAGAAGGTTTAACTTCTCAGACAACAGAC

TATACAGCACATAATGCCGTTCACAGCACCGATAGCGCTGATTTCGACAATTTTAATGGTTATTT

GACTGCTTCTTCATGGTATCGCCCTAAAGATGTTTTAAGAAATGGTCAACACTGGGAAGCAACA

ACAGCTAATGACTTCCGGCCCATTGTGTCAGTTTGGTGGCCTAGCAAGCAAACACAAGTAAATT

ACCTAAACTACATGTCTCAAATGGGACTCATTGACAATCGTCAGATGTTCTCGCTAAAAGACAA

TCAAGCCATGTTGAATATTGCTTGCACAACAGTCCAACAAGCAATTGAAACAAAAATCGGTGTG

GCTAATAGTACAGCATGGCTTAAAACAGCCATTGATGATTTCATTCGTACACAGCCACAATGGA

ACATGTCGAGTGAAGATCCCAAAAATGATCATTTACAAAACGGCGCTTTGACTTTCGTCAACAG

TCCATTGACACCAGATACTAACTCTAATTTCAGACTATTAAATCGCACACCAACAAACCAAACAG

GTGTGCCAAAATATACAATTGATCAATCTAAGGGTGGTTTTGAACTCTTACTCGCTAATGATGTA

GACAACTCTAATCCTGTTGTGCAAGCTGAGCAGTTAAATTGGTTACACTATTTGATGAATTTTGG

TAGCATCACAGCAAACGATTCTGCTGCTAATTTTGATGGGATACGTGTCGATGCTGTCGATAAT

GTTGACGCTGATTTGCTCCAGATTGCAGCAGATTATTTCAAAGCTGCTTATGGTGTTGATAAAA

ATGACGCAACAGCAAATCAACATCTTTCAATTCTTGAAGATTGGAGCCATAACGACCCTGAATA

CGTGAAGGATTTTGGTAATAATCAACTCACAATGGATGATTACATGCATACCCAGTTAATCTGG

TCGTTGACTAAAGATATGCGTATGCGTGGTACCATGCAACGCTTCATGGACTATTACCTCGTCA

ATCGCAATCACGATAGTACCGAAAACACTGCCATTCCAAATTACAGCTTTGTTCGCGCACACGA

TAGTGAAGTACAAACAGTCATTGCTCAAATTATTTCTGAGTTACATCCCGACGTAAAAAATAGTT

TGGCACCAACAGCAGACCAGCTAGCCGAAGCCTTTAAAGTTTATAATAACGATGAAAAACAGGC

GGATAAGAAATATACACAATACAACATGCCTAGCGCCTATGCGATGCTGTTAACTAATAAAGAT

ACAGTACCGCGCGTTTATTATGGTGATTTATACACCGATGATGGTCAATATATGGCAAATAAGT

CCCCTTATTTTGATGCCATCAACGGCTTGCTAAAGTCACGTATCAAATATGTTGCTGGTGGTCA

GTCAATGGCTGTTGATCAAAACGATATCCTGACAAATGTTCGTTATGGTAAAGGTGCCATGAGT

GTGACAGATAGCGGTAATGCAGACACACGAACACAAGGTATTGGTGTGATTGTCAGTAATAAAG

AAAATCTGGCCTTAAAATCAGGCGACACGGTGACATTACACATGGGTGCCGCTCACAAAAATCA

AGCATTCAGATTATTATTAGGGACAACTGCTGATAATTTGTCTTATTATGATAATGACAACGCCC

CAGTAAAGTACACCAATGATCAGGGCGATTTAATCTTTGATAATACTGAAATCTATGGTGTCCG

TAACCCGCAAGTCTCTGGCTTCTTAGCTGTTTGGGTGCCTGTTGGAGCTGACAGCCATCAAGAC
GCGCGTACTTTGTCTGACGACACAGCCCATCATGATGGCAAAACCTTCCACTCAAATGCTGCTT
TAGATTCTCAGGTTATTTACGAAGGTTTTTCAAATTTCCAAGCTTTTGCCACAAACACTGAAGAC
TATACAAACGCTGTCATTGCAAAAAATGGTCAGTTATTCAAAGATTGGGGTATCACAAGTTTCC
AGTTGGCACCACAATATCGTTCAAGCACCGATACCAGTTTCTTAGATTCAATTATCCAAAATGGT
TATGCCTTTACAGATCGTTATGATTTGGGCTACGGTACACCAACAAAATATGGCACAGTTGACC
AGTTACGCGATGCCATCAAGGCTCTGCACGCAAATGGCATCCAAGCAATCGCTGACTGGGTACC
CGACCAAATTTATAATTTACCGGGTCAAGAATTAGCGACCGTCACCCGAACAAACTCTTATGGT
GATAAAGACACTAACTCAGATATTGATCAGTCACTATATGTCATACAAAGTCGTGGTGGTGGTA
AATACCAAGCACAGTATGGCGGTGCCTTCTTATCTGATATCCAGAAAAAATATCCAGCGCTTTT
CGAAACAAAACAAATTTCTACAGGGCTACCTATGGATCCTAGTCAGAAAATAACAGAATGGTCT
GGTAAGTACTTTAATGGCTCAAATATTCAAGGCAAAGGGGCTGGCTATGTCTTAAAAGACAGTG
GTACCGATCAATACTATAAGGTTACATCAAACAATAATAATCGTGACTTCTTACCAAAACAGTTA
ACAGATGACTTATCTGAAACAGGATTTGTCCGCGATAACATTGGTATGGTCTACTACACATTGA
GTGGCTATCTAGCTCGAAACACCTTTATACAAGATGATAATGGCAATTATTATTACTTTGATAGC
ACCGGCCATCTCGTTACTGGCTTCCAGAATATTAATAACCATCACTATTTCTTCCTACCAAACGG
TATTGAACTCGTTCAATCTTTCTTGCAGAATGCTGACGGTTCAACGATTTATTTTGACCAAAAAG
GGCGTCAAGTATTTAATCAATACATTACTGACCAAACCGGTACCGCCTATTACTTCCAGAATGA
TGGCACAATGGTCACTTCTGGCTTCACTGAAATCGATGGCCATAAGCAATACTTCTACAAGAAC
GGCACACAAGTCAAAGGGCAATTTGTATCAGACACTGATGGTCACGTTTTCTACTTAGAAGCTG
GTAACGGCAACGTGGCGACACAAAGATTTGCACAAAATAGTCAAGGTCAATGGTTCTATTTGGG
TAATGATGGCATTGCCTTGACTGGTTTGCAAACAATCAATGGTGTTCAAAATTATTTCTACGCCG
ATGGTCATCAAAGTAAGGGTGATTTTATTACGATACAAAATCACGTATTATATACTAACCCACTA
ACTGGCGCTATAACGACAGGTATGCAACAAATTGGTGACAAGATTTTTGTCTTTGACAATACGG
GCAACATGTTGACCAATCAATACTATCAAACACTAGATGGCCAATGGTTACATTTAAGTACTCAA
GGTCCAGCAGACACTGGTTTGGTAAACATTAATGGTAATTTGAAATATTTCCAAGCTAATGGTC
GGCAAGTGAAAGGTCAATTTGTGACTGATCCTATCACGAACGTGAGTTATTATATGAATGCCAC
TGATGGTTCGGCAGTATTTAATGACTACTTTGCCTATCAAGGCCAATGGTATTTAACGGATAGT

AATTATCAACTCGTCAAAGGATTTAAAGTTGTTAATAATAAGCTACAACATTTTGATGAAATAAC
AGGCGTACAAACTAAATCAGCTCATATCATCGTTAATAATCGAACATACATTTTCGATGACCAAG
GTTACTTTGTCTCAGTCGCTTAA

The gene was annotated using BLAST showed that it was identical to *Leuconostoc citreum* DS gene with 99.49% identity.

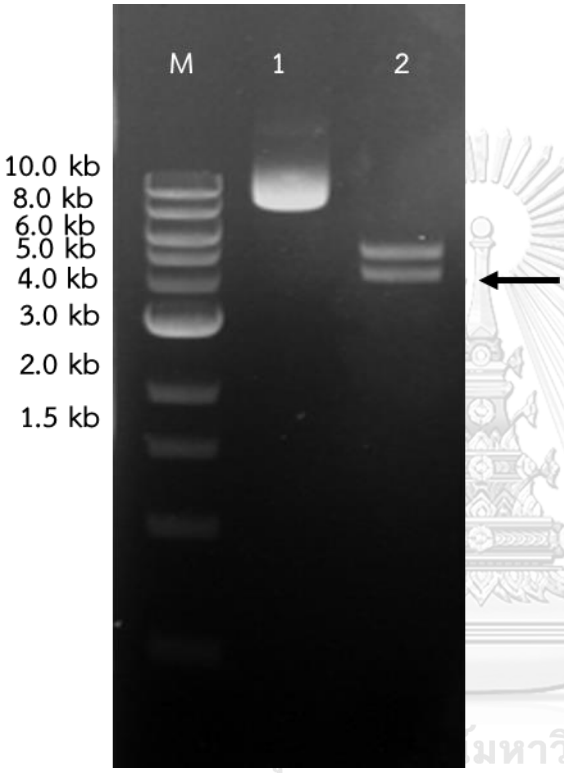


Figure 5 0.7% agarose gel analysis of c-terminus histidine tagged LcDS gene. M indicated 1 kb DNA marker, lane 1 was uncut LcDS gene with c-terminus histidine tagged, lane 2 was cut LcDS gene with c-terminus histidine tagged with upper band, pET21b vector, and lower band, insert fragment.

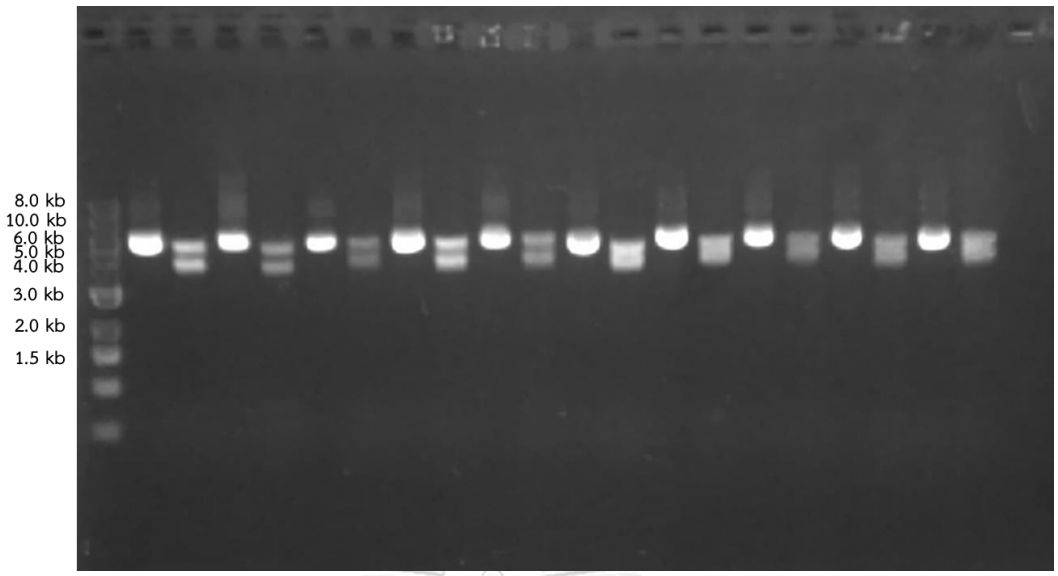


Figure 6 showed 0.7% agarose gel analysis of LcDS gene with N-terminus and without any histidine tagged.
M was 1kb DNA marker, odd lanes (1-19) are uncut recombinant plasmids, even lanes (2-20) are cut recombinant plasmids

2) *Lc*DS enzyme expression in *E. coli* system

i) Homology modelling of *Lc*DS enzyme

The amino acid sequences of LcDS enzyme were translated using LcDS gene sequences as showed below

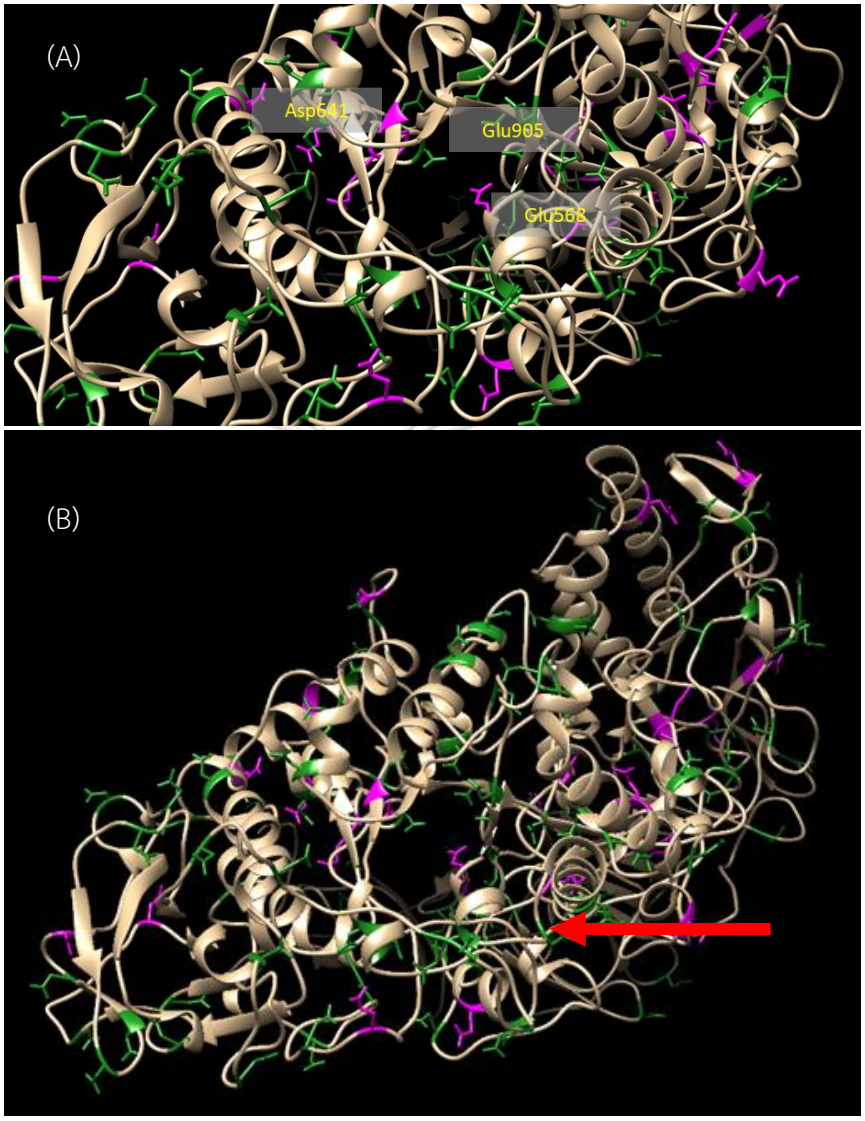
MIKERNVRKKLYKSGKSWVIGGLILSTIMLSMTATSQNVNADSTNTVTDKSVTVSNNS
NTTNQHDTVVDQQTTPVKNNQTTQQIAANANQAEKVKASDTTTDTQKQAETPNNTNKD
SIDNLTKQLPTVTPTANQKTGYLEKDGKWYYVTSDNTLAKGLTTVDNHKQYFDNNGVQA
KGQFVTDNSKTYYLDSNSGNAVTGIQQIGSQTLAFNDNGEQVFADFYTAPDGKTYYFDDK
GQATIGLKAINGHNYYFDSLGQLKKGFTGVIDGQVRYFDQESGQEVSTTDSQIKEGLTSQT
TDYTAHNAVHSTDSADFDNFNGYLTASSWYRPKDVLRNGQHWEATTANDFRPIVSVWW
PSKQTQVNYLNYMSQMGLIDNRQMFSLKDNQAMLNIACTTVQQAIETKIGVANSTAWLK
TAIDDFIRTQPQWNMSSEDPKNDHLQNGALTFVNSPLTPDTNSNFRLLNRTPTNQTGVPK
YTIDQSKGGFELLLANDVDNSNPVVQAEQLNWLHYLMNFGSITANDSAANFDGIRVDAVD
NVDADLLQIAADYFKAAYGVDKNDATANQHLSILEDWSHNDPEYVKDFGNNQLTMDDYM
HTQLIWSLTKDMRMRGTMQRFMDYYLVNRNHDSTENTAIPNYSFVRAHDSEVQTVIAQIIS

ELHPDVKNSLAPTADQLAEAFKVYNNDEKQADKKYTQYNMPSAYAMLLTNKDTVPRVYY
GDLYTDDGQYMANKSPYFDAINGLLKSRIKYVAGGQSMAVDQNDILTNVRYGKGAMSVTD
SGNADTRTQGIGVIVSNKENLALKSGDTVTLHMGAAHKNQAFRLLLGTTADNLSYYDNDN
APVKYTNDQGDLIFDNTEIYGVRNPQVSGFLAVWVPVGADSHQDARTLSDDTAHHDGKTF
HSNAALDSQVIYEGFSNFQAFATNTEDYTNAVIAKNGQLFKDWGITSFQLAPQYRSSTDTS
FLDSIIQNGYAFTDRYDLGYGTPTKYGTVDQLRDAIKALHANGIQAIADWVPDQIYNLPGQE
LATVTRTNSYGDKDTNSDIDQSLYVIQSRGGGKYQAQYGGAFLSDIQKKYPALFETKQISTG
LPMDPSQKITEWSGKYFNGSNIQGKGAGYVLKDSGTDQYYKVTSNNNNRDFLPKQLTDDL
SETGFVRDNIGMVYYTLSGYLARNTFIQDDNGNYYYFDSTGHLVTGFQNINNHHYFFLPNG
IELVQSFLQNADGSTIYFDQKGRQVFNQYITDQTGTAYYFQNDGTMVTSGFTEIDGHKQYF
YKNGTQVKGQFVSDTDGHVFYLEAGNGNVATQRFAQNSQGQWFYLGNDGIALTGLQTIN
GVQNYFYADGHQSKGDFITIQNHVLYTNPLTGAITTGMQQIGDKIFVFDNTGNMLTNQYY
QTLDGQWLHLSTQGPADTGLVNINGNLKYFQANGRQVKGQFVTDPITNVSYYMNATDGS
AVFNDYFAYQGQWYLTDSNYQLVKGFKVVNNKLQHFDEITGVQTKSAHIIVNNRTYIFDDQ
GYFVSVA

The homology modelling of *Lc*DS enzyme was predicted using Swiss model server revealed the protein structure of the *Lc*DS enzyme manipulating crystal structure of *Leuconostoc citreum* NRRL B-1299 N-terminally truncated dextransucrase DSR-M with 53.11% identity. The catalytic cleavage showed the pattern of $(\alpha/\beta)_8$ barrel (Figure 7 and 8). The predicted structure presented crucial amino acids which were Glu-568, Asp-641, and Glu-905. These amino acids helped the catalyze of dextrans production by they played the role of attacking the nucleophile position on sucrose and cleaving it into glucose and fructose. Then the glucose moiety had created the bond between 1-C of glucose with positively charge amino acids. The nearby positive charged amino acids transferred glucose moiety forming 1⟶6 α glycosidic linkage to another glucose sticking to positively charge amino acid. After the transferring of glucose, the amino acid with be recharged and

can attack the other sucrose again to polymerize the dextrans chain. Moreover, we also did the computation for the molecular weight and pI of the enzyme were 168.08 kDa and 5.03 respectively. We also computed for LcDS containing c-terminus 6×His and the protein had 168.91 kDa with pI 5.15.

As *Leuconostoc citreum* ABK-1 is lactic acid bacteria, the pI of the enzyme should be acid and the result showed that our enzyme had a weak acid property. This experiment facilitated us for the protein purification experiment. We can create the protonated state of the enzyme by using these data.


(A)
Asp641
Glu905
Glu568
(B)

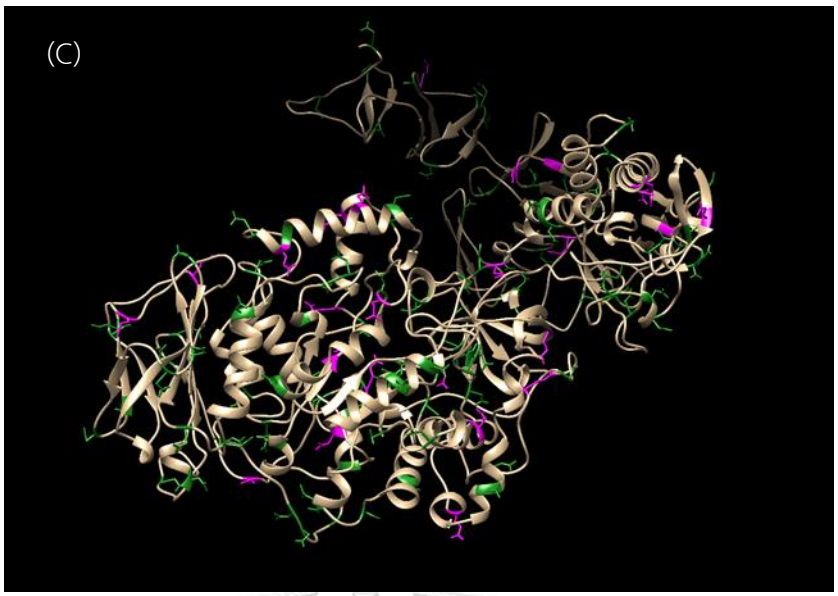
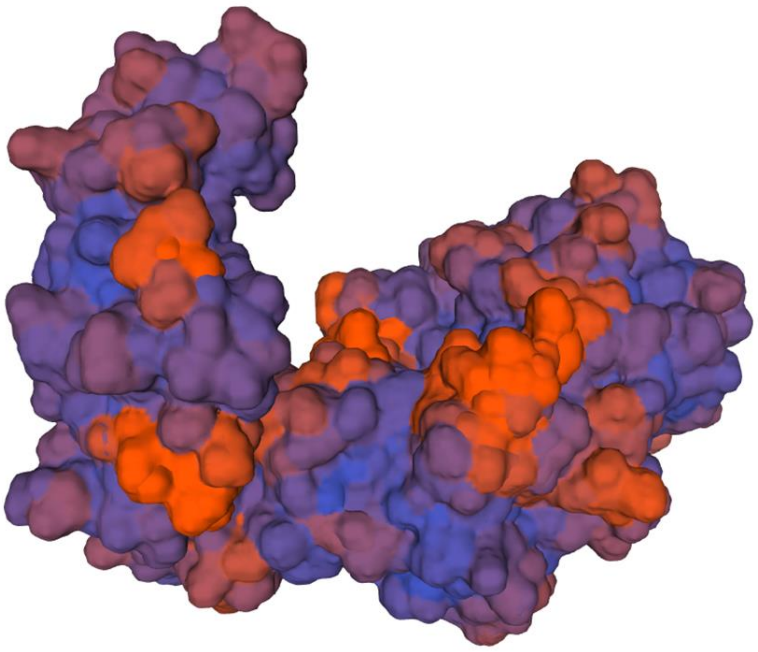


Figure 7 The homology modelling of LcDS enzyme using Swiss-model server. (A) displayed the cartoon presentation of the LcDS catalytic cleavage. The green color indicated for Glu and the purple indicated for Asp (B) and (C) presented the sideview of LcDS model. The catalytic cleavage exposed $(\alpha/\beta)_8$ *barrel.*



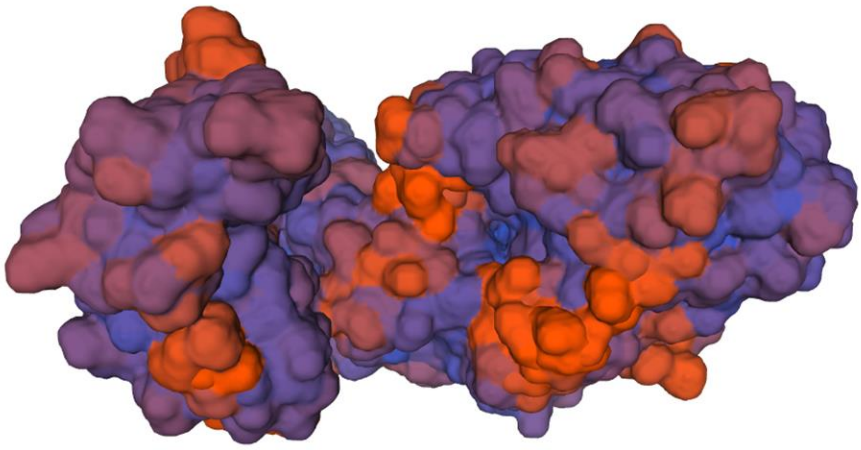


Figure 8 The homology modelling of LcDS enzyme using Swiss-model server. The protein was represented in surface representation showed the catalytic cleavage of LcDS enzyme.

ii) The analysis of *Lc*DS protein expression

The successful constructs were optimized the protein expression in *E. coli* system against various conditions including the concentrations of IPTG, temperatures, and times. The protein expression was analyzed by the activity of the *Lc*DS enzyme. As we knew that dextransucrase can catalyzed sucrose into glucose and fructose. And then the enzyme can polymerize dextran using D-glucose moiety. The fructose in the environment will be using to detect the enzyme activity. DNS assay was used in this detection. The conversion of DNS when it faced with reducing sugar, fructose, turned into 3 – amino - 5 nitrosalicylic acid containing orange color.

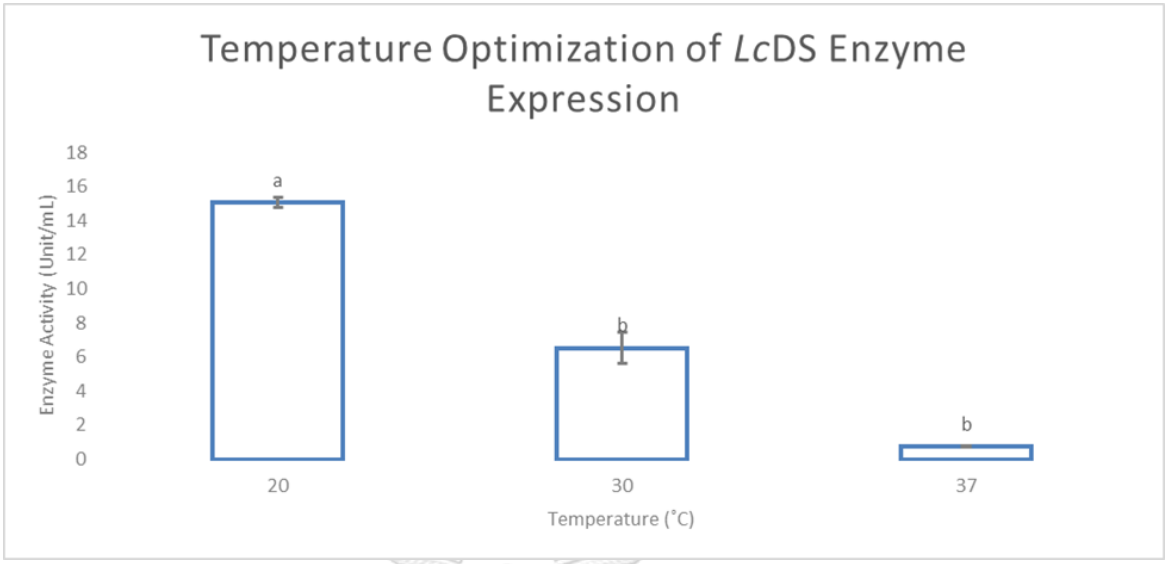
(a) Temperature optimization

*Lc*DS gene which had been transformed into expression host, BL21 star (DE3) was grown on 50 ml LB agar containing 100 µg/ml ampicillin for 6 hours with the induction of 0.5 mM IPTG. We had selected 3 temperature points

for this experiment. The temperatures were 20, 30, and 37°C. The result showed that the highest enzyme activity was optimized at 20°C (Figure 9).

In this experiment, we conducted the experiment into 2 parts. For the first part, we want to clarify the optimal temperature thus we fixed the IPTG concentration and the expression time with 0.5 mM IPTG and 6 hours of the expression. We measured the protein expression by using the enzymatic activity. The cell was collected and lysed with 25 mM HEPES buffer pH 7.4 and followed by sonicated with 27% amplitude for 1×4 seconds. The soluble protein was used for analysis. The 50 µl of the soluble crude enzyme was incubated with 50 mM sucrose and let the activity start in 25 mM HEPES buffer pH 5.5 at 30 °C for 15 minutes. The activity was deliberated by DNS assay detected for the fructose releasing. The 1 unit of enzyme means the releasing of 1 µM of fructose in 50 µl of enzyme. The result showed that the optimal temperature of this enzyme expression was 20°C. The activity of enzyme at 20°C was 15 unit/ml enzyme whilst at 30 and 37 °C divulged the activity were 6.5 and 0.77 unit/ml, respectively. The expression level at 20°C exhibited significantly different comparing to 30 and 37°C with $P>0.01$ using Tukey test.

In the first part we can confer that at low temperature, the protein can fold slower but in corrected conformation and also increasing the product yield. In our experiment also reflected the same perspective that the enzyme exhibited higher activity compared to higher temperatures.



	Temperature (˚C)		
	37	30	20
Activity (U/mL)	0.768±0.302	6.527±0.914	15.1±0.023*

Figure 9 showed the relative of temperature on protein expression.

(b) Time coarse analysis and the optimization of IPTG concentration

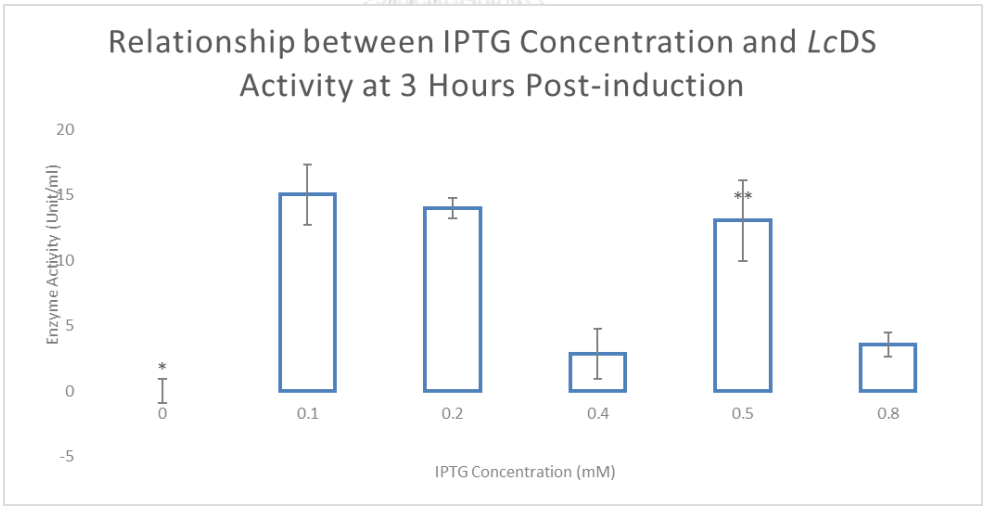


Figure 10 showed the protein expression at 3 hours post-induction in various IPTG concentration.

The 4 time points was selected for this experiment including 3-, 6-, 9-, and 18-hours post-induction. The IPTG concentrations was co-investigated here with

6 concentrations comprising 0, 0.1, 0.2, 0.4, 0.5, 0.8 mM IPTG. The results showed that the optimized time was 6 hours post-induction with 0.5 mM IPTG (Figure11).

At 3 hours post-induction, we found that the protein expression had been observed the highest activity when inducing with 0.5 mM IPTG was14 U/ml while the other IPTG concentrations showed lower activity as 0, 11, 11, 6, and 4 U/ml respectively (Figure 10).

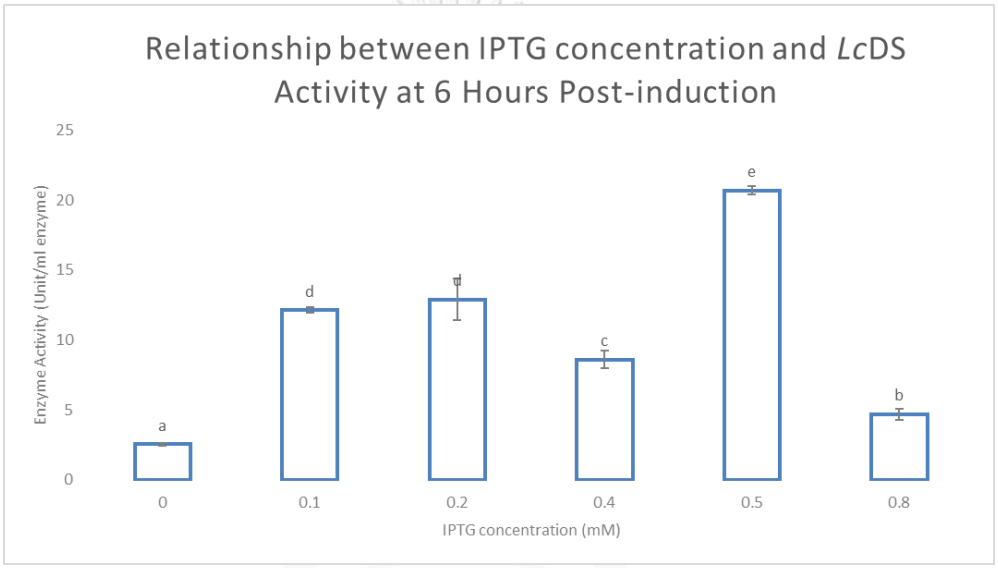


Figure 11 showed the protein expression at 6 hours post-induction in various IPTG concentration

The expression at 6 hours after induction showed the highest enzyme activity exhibited at 0.5 mM IPTG induction. The activity was measure as 21 U/ml while the induction with 0, 0.1, 0.2, 0.4, and 0.8 mM IPTG were 2, 12, 13, 9, and 4 U/ml respectively. However, this time point also showed that the enzyme activity also detected even without the addition of IPTG. At 0 mM IPTG we can observe the enzyme activity with 2 U/ml. this could reflect that the promoter started to leak at 6 hours (Figure 11). [59]

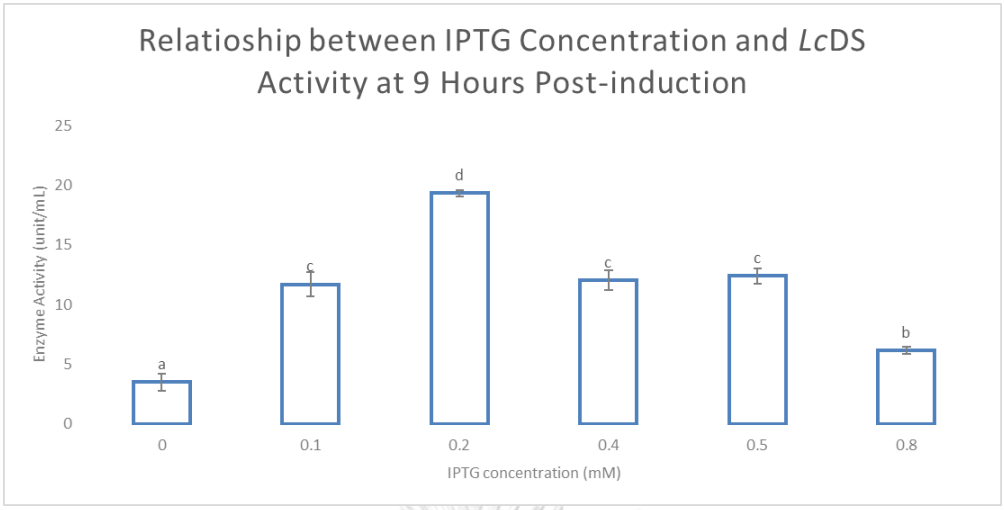


Figure 12 showed the protein expression at 9 hours post-induction in various IPTG concentration

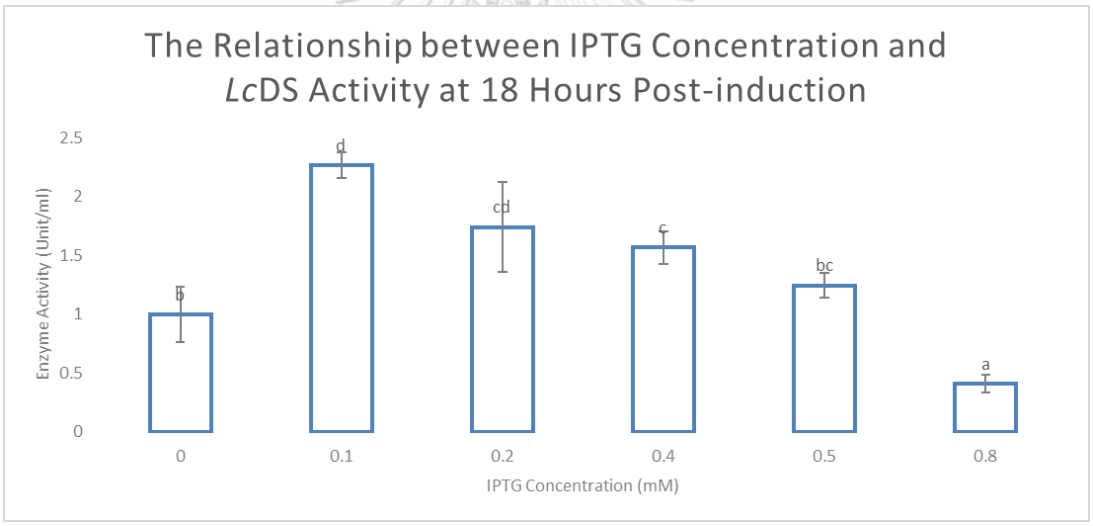


Figure 13 showed the protein expression at 18 hours post-induction in various IPTG concentration

The time analysis at 9 hours post induction showed the highest protein expression at the induction with 0.2 mM IPTG with the activity of enzyme 19 U/ml (Figure 12).

The expression level at 18 hours post induction showed the lowest activity in every IPTG concentrations. The highest expression level in this time point showed only 2.4 U/ml in the induction with 0.1 mM IPTG. when compared

to the other time point, we can see that the activity mostly dropped and this can confer that *Lc*DS enzyme cannot express overnight (Figure13).

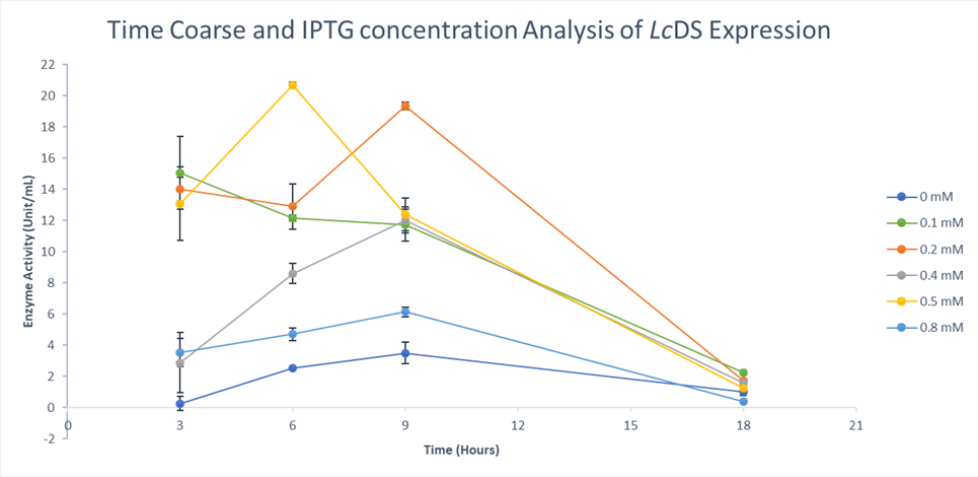


Figure 14 showed the relative between IPTG concentrations and protein expression at different time points.

The second experiment was the optimization of IPTG concentrations and time coarse analysis. In this experiment, we aimed to elucidate the concentrations of IPTG which can promote the *Lc*DS enzyme expression. We selected 0 mM as uninduced and also internal control. The concentration of IPTG which were used here were 0.1, 0.2, 0.4, and 0.8 mM or the multiple two of the concentrations and added 0.5 mM IPTG because of the usage in the previous study. The expression time we interested in this experiment were 3-, 6-, 9-, and 18-hours post-induction. Each time points were multiple of 3 so the time would be linearized. The result showed that the trend of protein expression with 0, 0.4, and 0.8 mM IPTG induction exhibited the same. At 3 to 9 hours post-induction, the protein expressed higher continuously despite the activity of the enzyme dropped after the time reached 9 hours. The maximum activity of 0, 0.4, and 0.8 mM IPTG were 3.5, 12, and 9 unit/ml, respectively. Differ to the first 3 concentrations, the activity of the induction with 0.1 mM IPTG showed a declined trend. The activity reached the maximum since the protein expression reached 6 hours exhibiting 15 unit/ml then the activity of

the enzyme lowered against the time. The induction of 0.2 mM IPTG showed a declined trend at the first 6 hours after that the enzyme activity increased to 19 unit/ml at 9 hours post-induction. Then the activity was released again against the time. The last concentration, 0.5 mM IPTG, presented the activity of the enzyme with the highest activity that can be detected at 6 hours post induction and then the activity decayed over the time. The highest activity can be measured as 20 unit/ml (Figure 14).

When we compared all the results, we can infer that the optimal time and IPTG concentration was 0.5 mM IPTG at 6 hours. Also, we can conclude that our enzyme cannot express over the night because all of the results showed that the enzyme lost its activity at 18 hours post-induction. In this experiment, we can see the differences in the trend lines which can separate into 3 trends. The first was increasing protein expression until it reached 9 hours of expression then the protein expression declined. The second was a slow expression at the first 6 hours then increased the expression at 9 hours. After 9 hours, the protein expression dropped. And the last one was the increase of expression level at the first 6 hours. After that, the protein lost its activity. Interestingly, various concentrations of IPTG showed different optimization. We suggested that it was not extraordinary for the reason that Silaban, et. al also faced the same situation that their soluble enzyme expressed only specific concentration of IPTG while the rest of IPTG decreased the protein expression[60]. In this experiment, we found that the optimal time for *Lc*DS enzyme expression was at 6 hours after induction by adding 0.5 mM IPTG as an inducer. The activity of enzyme exhibited as 20 U/ml or 40 total unit while in the other time points with the same IPTG concentration showed lower activity[61] as 13 U/ml, 12 U/ml, and 1 U/ml for 3-, 9-, and 18-hours postinduction, respectively (Figure14).

ii) The analysis of protein expression using western blot analysis

The *Lc*DS enzyme was expressed in *E. coli* system with 0.5 mM IPTG, for 6 hours at 20°C. 20 µl of the enzyme was loaded into 8% SDS gel and then transferred onto nitrocellulose and polyvinylidene fluoride (PVDF). The *Lc*DS enzyme had calculated the size and pI using ExPASy server. The computed size and pI were 168 kDa and 5.15, respectively. The result on the western blot showed the interaction between histidine-tagged and anti-histidinde tagged antibody at the size 168 kDa. To confirm the interaction between His-tagged and Anti-his tagged antibody, the external protein was loaded as control (Figure15).

C-terminus histidine tagged *Lc*DS was analyzed using western blot analysis. As we computed the size of this enzyme being 168.92 kDa, the western blot analysis of *Lc*DS expression had been performed. The detection of histidine tags was visualized on the membrane. Besides, 8% SDS gel also exhibited that our protein of interest overexpressing compared to the uninduced protein. However, much more protein of the same size was expressed in inclusion bodies.

From this experiment, we suggested that if we want to produce more soluble protein, we should adjoin our recombinant gene co-transformed with chaperones to facilitate the soluble protein expression yet we need to be aware of unwanted protein production as well. Some reports showed the consequence of negative feedback from chaperone i.e., increasing of proteolytic activity which can damage or degrade our protein of interest.[62]

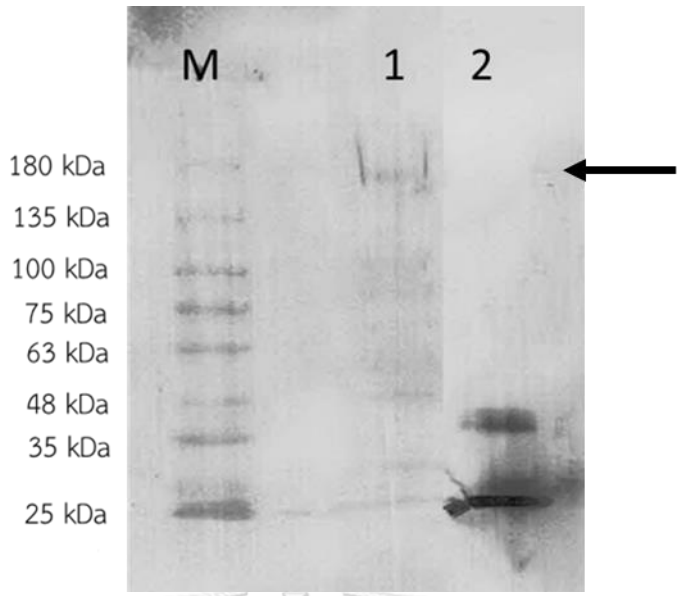


Figure 15 western blot analysis of Lcdextransucrase enzyme expression, M is marker, 1 is Lcdextransucrase enzyme, 2 is Lcdextransucrase enzyme, Ext. is external control. Protein of interest is at the blue aero

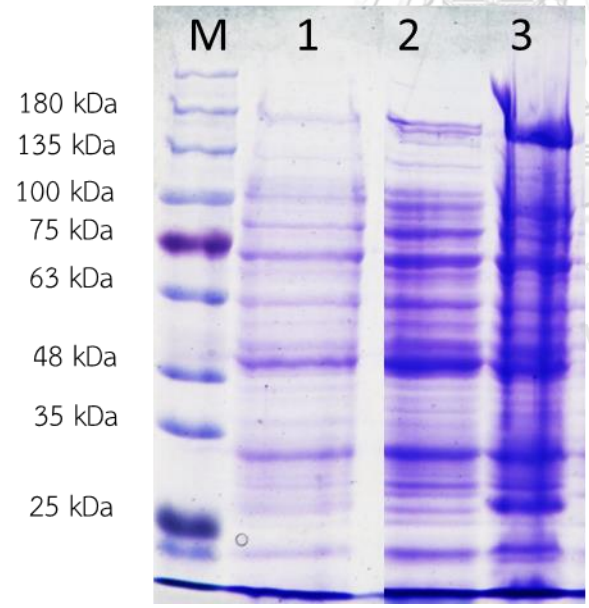


Figure 16 The 8% of SDS PAGE analysis of LcDS expression. M was protein Ladder Lane 1 was uninduced protein, Lane 2 was soluble crude enzyme and Lane 3 was inclusion bodies protein

3) *Lc*dextransucrase enzyme purification

i) Ni column chromatography

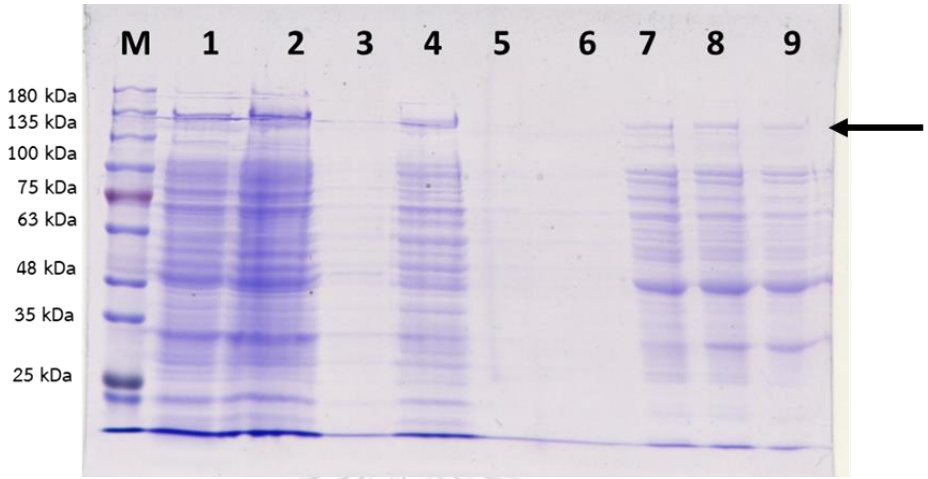


Figure 17 8% SDS gel analysis of LcDS purification using Ni-sepharose chromatograpy

M is markers, 1 was soluble crude protein, 2 was concentrated crude protein, 3 was the flowthrough from spin column, 4 was the flowthrough from Ni-sepharose column, 5-6 were wash fractions, 7-9 were elution fraction using 10 mM of imidazole pH 7.4

Because the crude *Lc*DS had been tagged with 6×His tagged so the protein was purified against 2 ml of Ni-sepharose bead. 50 ml of the crude was loaded into the column. The flowthrough was collected and then the protein was washed with wash buffer followed by eluted with elution buffer. At that time, each fraction was analyzed by DNS assay and the fraction with enzyme activity was analyzed again with 8% SDS gel (Figure17). The stepwise elution was performed under the concentration of 10 to 50 mM imidazole pH 7.4.

The result showed that the protein was eluted by 10 mM imidazole pH 7.4. We thought that this phenomenon might come from the positive charge of $CaCl_2$ in the lysis buffer. The Ca^{2+} ion may disturb the interaction between Ni^{2+} and histidine tags. The report of Pabst et., al. proved that the low concentration

of calcium ion (Upto 125 mM $CaCl_2$) not effect on the binding activity between positively charge protein and the Ca^{2+} ion[63].

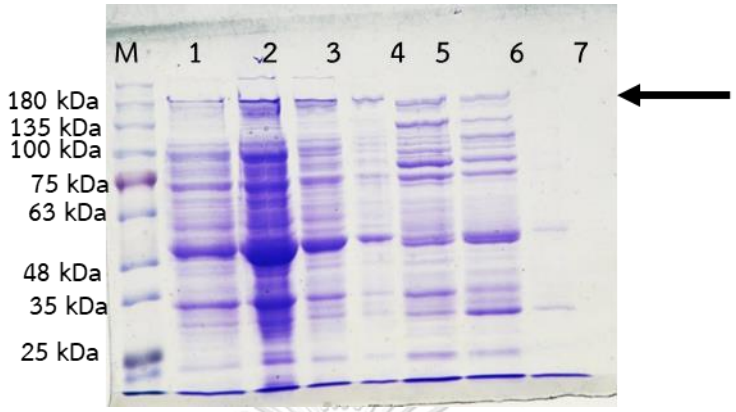


Figure 18 8% SDS gel analysis of LcDS purified using Ni-sepharose column chromatography.
M is marker, 1 is elution fraction number 3, 2 is elusion fraction number 4, 3 is elusion fraction number 5, 4 is elusion fraction number 6, 5 is elusion fraction number 7, 6 is elusion fraction number 8, 7 is elusion fraction number 11

After purified, the protein showed activity in the elusion fraction; however, the protein was not fully purified (Figure18).

ii) Ultrafultration using Vivaflow MWCO 100 kDa

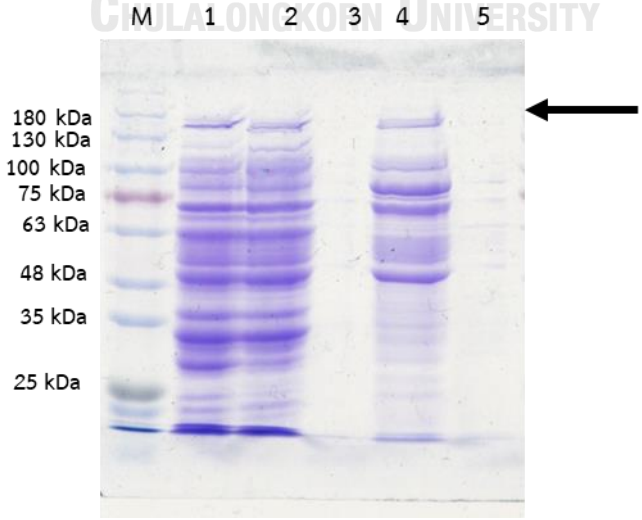


Figure 19 8% SDS gel analysis of ultrafiltration through Vivaflow MWCO 100K

M is marker, 1 is crude LcDS, 2 is concentrated LcDS, 3 is flowthrough of vivaflow spin, 4 is DEAE-Toyopearl 650M purified LcDS, 5 is flowthrough of DEAE-Toyopearl 650M chromatohgraphy

Our hypothesis in this experiment was small protein can be aggregated, thus we added triton X-100 into the protein to remove the protein aggregation and the small protein would be detached out of the purification system through ultra-purification method. The result showed that only some small proteins was getting rid of but a lot of them were still existed and the purification with DEAE anion exchange showed the more purified ability to discard small proteins (Figure20).

iii) Comparison of ultrafiltration spin column AMICON MWCO 100K in Triton X-100 to remove protein aggregation

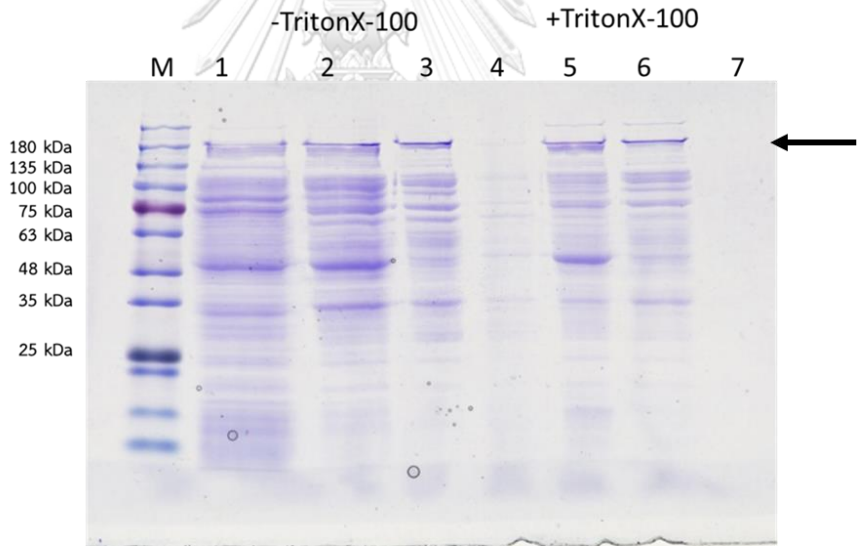


Figure 20 The protein purification using AMICON ultrafiltration
M is marker, 1 is crude 2 is DEAE-purified Lcdextransucrase, 3 is DEAE-purified Lcdextransucrase via ultrafiltration, 4 is flowthrough of spin column, 5 is DEAE-purified Lcdextransucrase, 6 is DEAE-purified Lcdextransucrase via ultrafiltration spin column against buffer containing Triton X-100, 7 is flowthrough of DEAE-purified Lcdextransucrase via ultrafiltration spin column against buffer containing Triton X-100

Because the purification with the Ni-sepharose column can remove some of none participating protein. We purified protein against the Ni-sepharose column and then used the ultrafiltration method with AMICON spin column MWCO 100kDa. the results showed that when comparing to the crude and DEAE-purified protein, some of the small proteins were removed and these results also showed that this way is better than using only vivaflow column (Figure 19). Moreover, we proved the hypothesis that bigger proteins were aggregated protein because of the high hydrophobicity of *Lc*DS enzyme. Thus, we added triton X-100 to the system and cleaned them up by spin column. The result showed that with or without the addition of triton X-100 shared the same pattern of protein elution meaning that there was no protein aggregation (Figure 20).

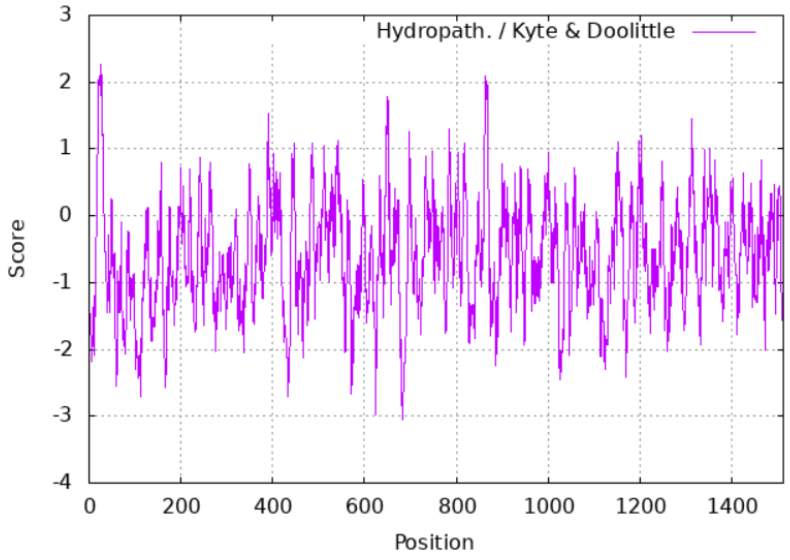


Figure 21 The hydropathicity of LcDS enzyme using ProScale calculator exhibited the hydrophobicity

iv) Ni Sepharose coupling with -DEAE anion exchange chromatography

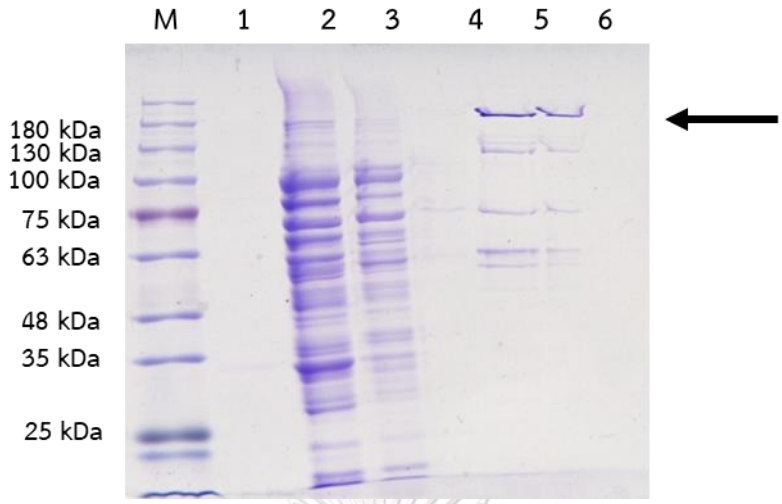


Figure 22 8% SDS gel analysis of Lcdextransucrase enzyme purification via Ni sepharose coupling with DEAE anioexchange

M is marker, 1 was wash fraction,

2 was protein precipitation of 750 µl elution fraction 2 eluted with 100 mM NaCl,

3 was protein precipitation of 750 µl elution fraction 3 eluted with 100 mM NaCl,

4 was protein precipitation of 750 µl elution fraction 5 eluted with 100 mM NaCl,

5 was protein precipitation of 750 µl elution fraction 7 eluted with 200 mM NaCl,

6 was protein precipitation of 750 µl elution fraction 8 eluted with 200 mM NaCl

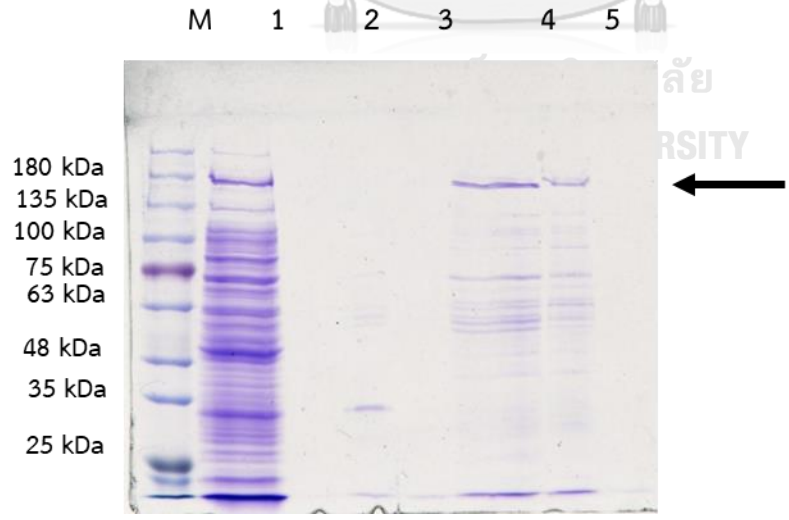


Figure 23 8% SDS gel analysis of Ni sepharose coupling with DEAE anion exchager LcDS purification

M was marker, 1 was crude, 2 was wash fraction without NaCl, 3 was wash fraction with 100 mM NaCl, 4 was protein precipitation of elution fraction number 1 eluted

with 200 mM NaCl, 5 was protein precipitation of elution fraction number 2 eluted with 200 mM NaCl

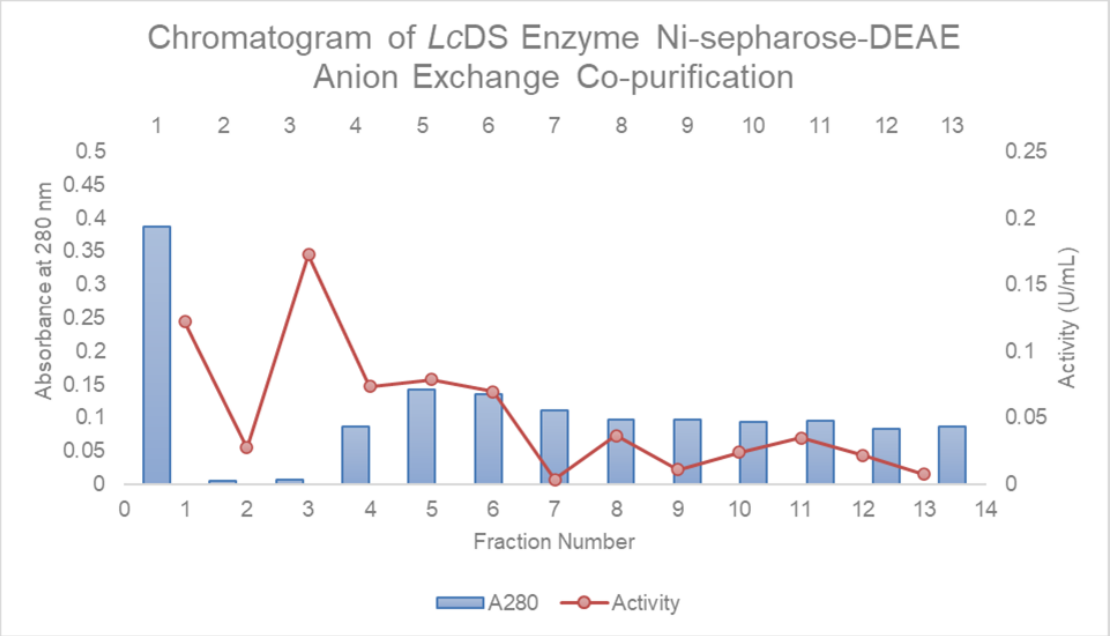


Figure 24 Chromatogram showed the relationship between protein concentration and protein activity

We coupling Ni-sepharose and DEAE column purification methods and found that if the protein was purified against DEAE column, most of the protein were lost the activity (95% of the activity were deducted). Then we decided to purify *Lc*DS against Ni-sepharose and followed by DEAE column, the protein had been purified as the major band and still presented the activity. The activity *Lc*DS enzyme via Ni-sepharose coupling with DEAE anion exchange exhibited the activity at 0.14 U/ml with final 2 ml protein or 0.28 U. Crude enzyme before purification had activity at 1.9 U/ml with 25 ml of protein or 47.5 U (Figure22, Figure29, Figure24).

4) Biochemical Characterization of *Lc*DS enzyme

i) LcDS pH optimization

We studied the activity of *Lc*DS enzyme. The optimization of an enzyme was based on its pI (pI = 5.15). We observed that purified enzyme worked and survived at pH 5.0 and 5.5, equally (Figure 25). The activity decreased when the enzyme worked against basic condition. The activity of the enzyme dropped around 60% comparing to the activity at pH 5.0.

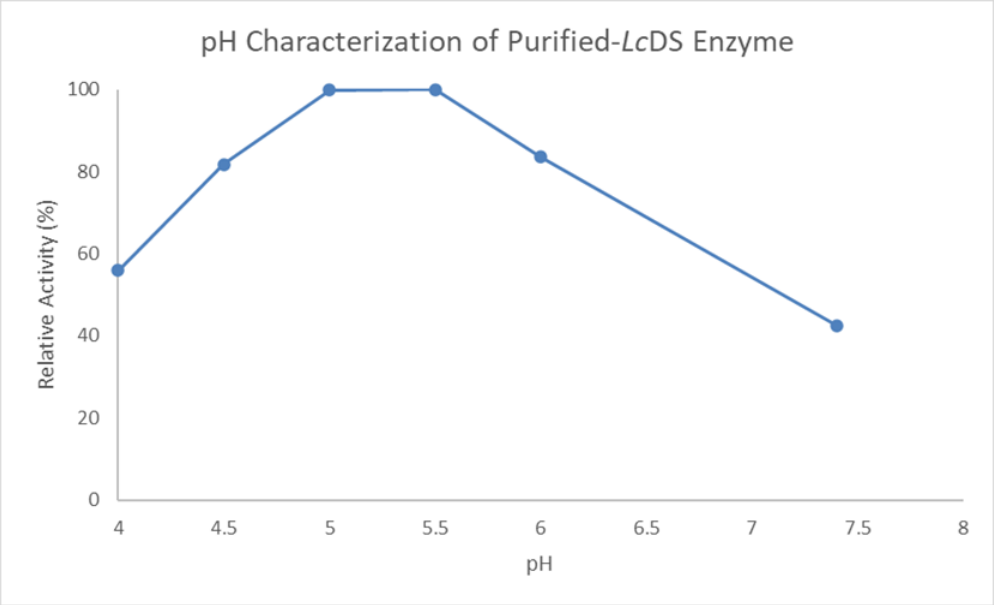


Figure 25 the pH optimization of Lcdextransucrase enzyme

ii) *Lc*DS temperature optimization

The crude enzyme had been studied for its temperature tolerance. Temperatures were optimized before *Lc*DS enzyme had been used to produce the products. We found that enzyme can work better at 30 and 37 °C with the same activity, 5.7 U/ml. The activity dropped lower to 0.57 U/ml when the enzyme was incubated at 60°C (Figure14).

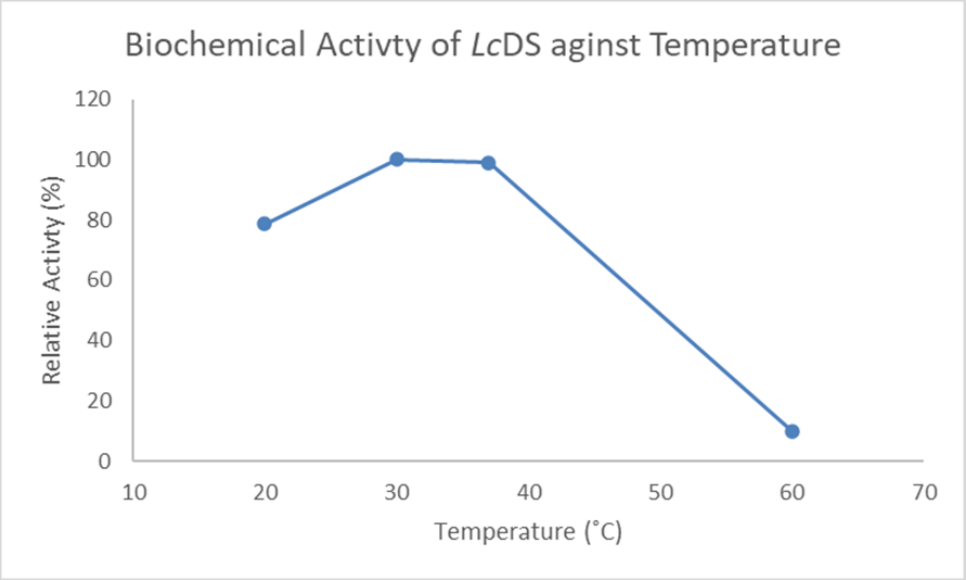


Figure 26 the temperature optimization of Lcdextransucrase enzyme

5) Kinetic of the *Lc*DS enzyme

The kinetic of *Lc*DS enzyme cannot be tested because the purified protein against the coupling column reduced the protein yields and also the activity of the enzyme. Hence, we skipped this part but we added the data of the purification table of this protein shown in the table below to prove that our protein lost during the purification technique. As in table 5, the recovery yield of our enzyme was 5.17% suggesting that the enzyme was not enough for further analysis. The report recommended that the proteolytic had an effect during purification causing degradation of protein[64], in this case we can prove that proteolytic might have less effect on our purification step because we added PMSF in lysis buffer to prevent that phenomenon. As a result of using PMSF, another perspective which can be the problem is the half-life of PMSF. We purified our protein for two days in row furthermore PMSF has half-life is 35 minutes (*CSH protocol*)

Enzyme	Volume (mL)	Total protein (mg)	Activity (U/mL)	Total activity (U)	Specific activity (U/mL)	Purification fold	%yield
Crude *Lc*DS	25	0.0142	1.884	47.09	3322	1	100
Purified *Lc*DS	4	0.0131	0.609	2.437	185.4	0.05	5.17

Table 5 The specific activity of LcDS enzyme before and after purification steps.

6) Product analysis of *Lc*dextran

i) TLC analysis of *Lc*dextran pattern

In this experiment, we visualized the polymer pattern which was the product of the enzyme on TLC plate. The sample shared the character of mostly polymers form and showed some character of oligomers but differed from the pattern in hydrolyzed commercial dextrans. The TLC visualized that none of sucrose or the substrate had left in the system. We can conclude that it had highly trans glycosylation activity as well as it produced some dimers products but it was not contained $\alpha 1 \rightarrow 6$ linkage (Figure 27).

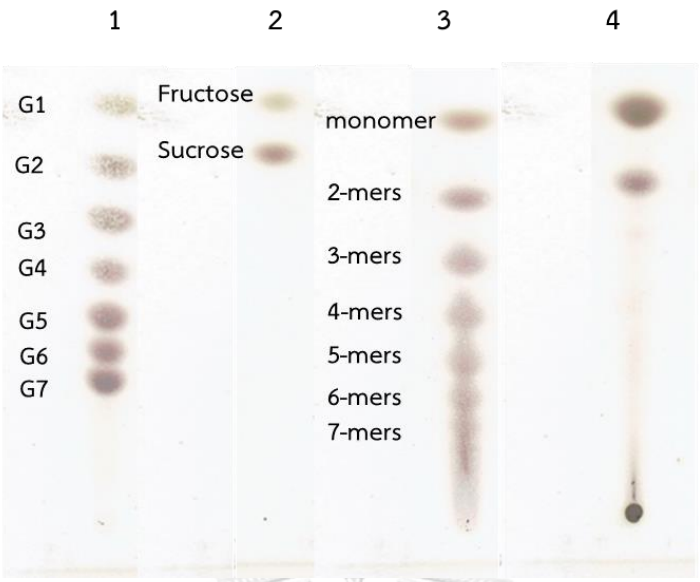


Figure 27 TLC visualization of Lcdextran pattern,

1 was G1-G7 standard, 2 was fructose and glucose standard, 3 is standard for $\alpha 1 \rightarrow 6$ *glycosidic linkage, and 4 is Lcdextran*

ii) Partial hydrolysis of *Lc*dextran polymer preparing for NMR

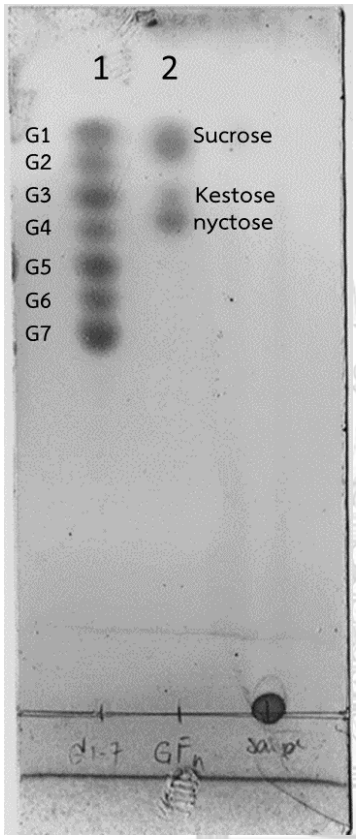


Figure 28 TLC anaylsis of partial hydrolysis Lcdextran
G1-G7 is standard glucose monomer, GF_n is standard for sucrose (GF_1), 1-kestose (GF_2), and nystose (GF_3), Sample

In this experiment we intended to remove some glucose from the system, as a consequence, we did the partial hydrolysis of *Lc*dextran. The glucose may affect the interpretation of HPAEC-PAD analysis. The result of TLC presented that after the product was partial hydrolysis in 1 M HCl, only the polymer existed in the system (Figure26). We dissolved the *Lc*dextran in DMSO and sent it to analyze for ^{1}H NMR and ^{13}C NMR (Figure 28).

iii) NMR analysis of *Lc*dextran

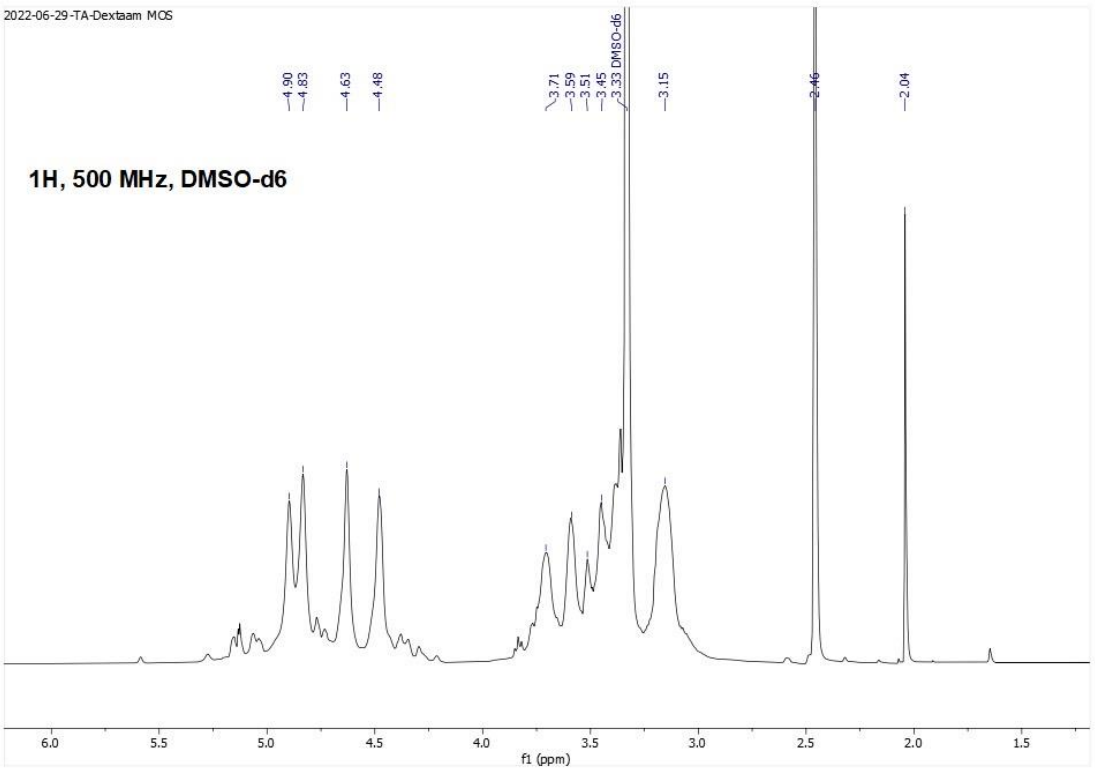


Figure 29 ^{1}H NMR spectrum (500 MHz, DMSO-D6) of Lcdextran

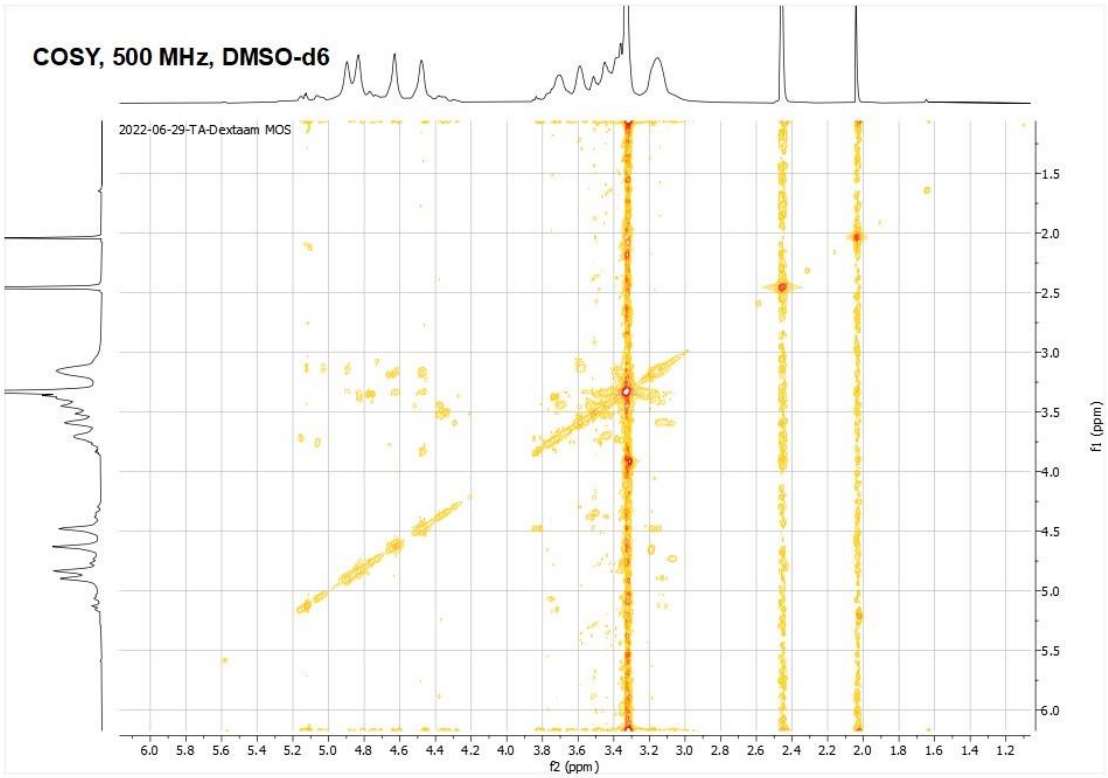


Figure 30 COSY NMR spectrum (500 MHz, DMSOD6) of Lcdextran showed 2D expected structure of Lcdextran

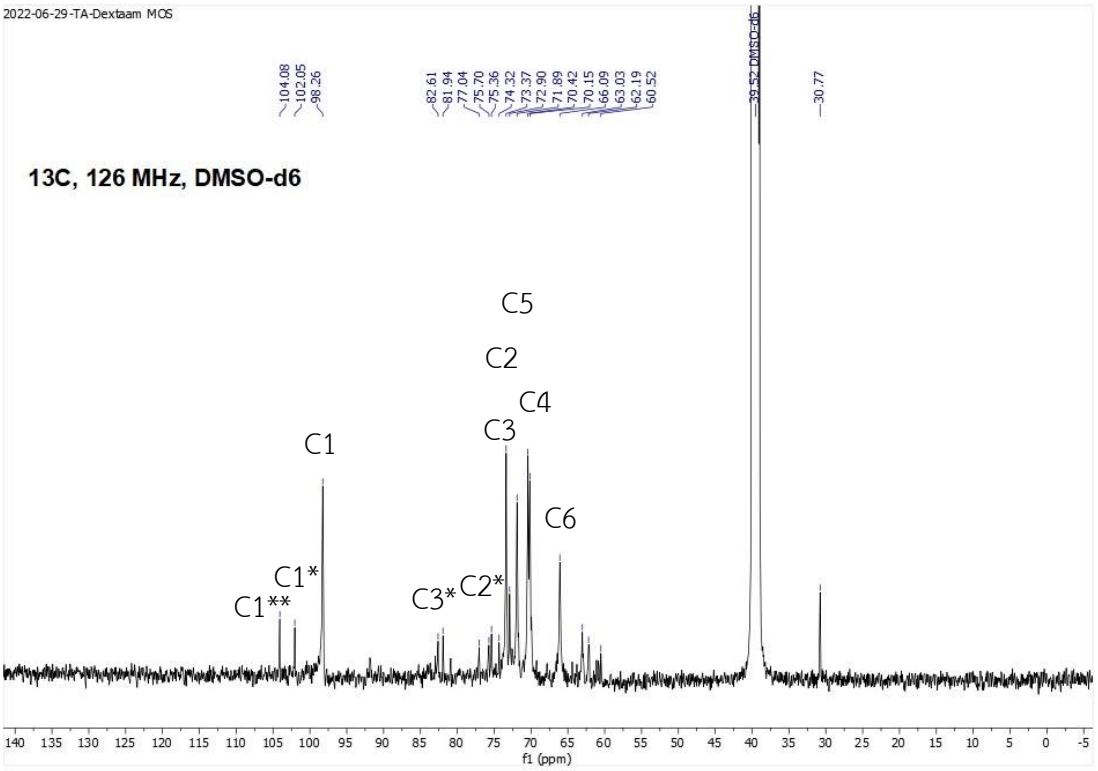


Figure 31 ^{13}C NMR spectrum (500mHz, DMSO-D6) of Lcdextran

The 1H NMR could not explain much about the C-linkage but studying of ^{13}C NMR revealed that at the peak 98.26 ppm showed the anomeric carbon. The chemical shift at 70.42 ppm revealed the C-6[65-68], it could state that our product consisted of 1→6-α glycosidic linkage which was a dextran chain. Besides, it was occupied as a major product (mainly more than 50%). Interestingly that our product also included other linkages at 102.05 and 104.08[66, 67] ppm of ^{13}C NMR (Figure27) but the linkage seemed not clear to interpret. As signals at 75.70, 75.36, 74.23, and 72.90 ppm. Our product might compose 1→2 glycosidic linkage (102.05 with 77.04 ppm) or 1→3 glycosidic linkage (104.08 with 82.16 ppm). Because the signals were not too strong so it was hard to confer what types of glycosidic linkage were exhibited there (Figure 29-31)

iv) HPAEC-PAD analysis of *Lc*dextran

The HPAEC-PAD result showed that the partially hydrolyzed *Lc*dextran polymer shared the same product pattern as the dextran commercial but it showed some fructose signals. We think that the fructose might be at the beginning of product polymerization. When merging the TLC analysis data of partial hydrolyzed *Lc*dextran, in which there was none of the monosaccharides, the fructose must stick at the start point on polymerization. Moreover, we found that our product contains a low amount of other linkage but we cannot state what should be the linkage because of the weak signal (Figure 32).

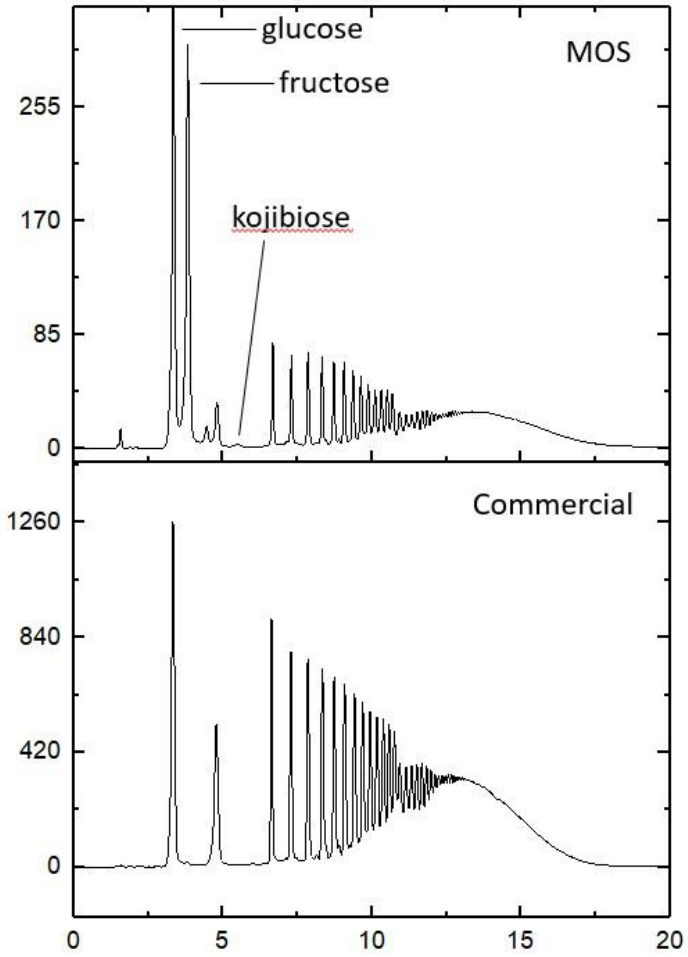


Figure 32 HPAEC-PAD result show the shared pattern between comercial dextrans and Lcdextran

Chapter 5 Conclusion

Dextransucrase gene was newly discovered in *Leuconostoc citreum* ABK-1. We preliminarily annotated the gene and found that *Lcdextransucrase* was 99.50% identical to the dextransucrase gene in *Leuconostoc citreum* DS. This work aimed to clone, express, and purify the dextransucrase from *Leuconostoc citreum* ABK-1. In this project, we successfully cloned the dextransucrase gene of *Leuconostoc citreum* ABK-1, *LcDS*. The *LcDS*, 4,935 base pairs, was subcloned into pET21b. The recombinant plasmid was then transformed into *Escherichia coli* BL21 (star) DE3 for protein expression with C-terminus histidine-tagged. The computed size of the *Lc*DS enzyme was performed by ExPASy and the result showed that its size was 168 kDa with a pI of 5.15. The optimization of protein expression was observed at 20°C for 6 hours after being induced with 0.5 mM IPTG. SDS PAGE analysis was used to investigate the expression of the *Lc*DS enzyme. We found that the enzyme was an intracellular expression. To additional confirm the protein expression, western blot analysis was performed and detected the signal of the anti-6×histidine antibody. The *Lc*DS was purified against Ni-sepharose column chromatography and then the eluted proteins with 10 mM imidazole pH 7.4 were pooled and cleaned up by ultrafiltration via AMICON spin column with MWCO 100K. The concentrated protein was again purified in DEAE-Toyopearl 650 M anion exchange. The purified protein was analyzed by 8% SDS PAGE analysis and also detected for its activity with DNS assay for 0.28 U while the protein before purification exhibited 47.5 U. Crude enzyme was incubated with 20% sucrose and the dextran product was analyzed by TLC, HPAEC-PAD, and NMR confirming that the product was dextran.

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p. 30-35.

VITA

NAME	Kakuttapun Bulan
DATE OF BIRTH	17 April 1993
PLACE OF BIRTH	Hatyai, Songkhla, Thailand
INSTITUTIONS ATTENDED	B.Sc. Mahidol University
PUBLICATION	Bulan, K. and R. Pichyangkura (2021). "Cloning and expression of dextransucrase from Leuconostoc citreum ABK-1." Proceeding in 7th International Conference on Biochemistry and Molecularbiology