CHAPTER II

MATERIALS AND METHODS

2.1 Equipments

Autoclave: Model HA 30, Hirayama Manufacturing Cooperation, Japan

Autopipette: Multipette plus, Eppendorf, Germany

Autopipette: Nichipet EX, Nichiryo, Japan

Balance: AB204-S, Mettler Toledo, Switzerland

Balance: PB303-S, Mettler Toledo, Switzerland

Bench Top Refrigerated Centrifuge: Model HARRIER 18/80, Sanyo Gellenkamp PLC, UK

Centrifuge, refrigerated: Model J-21C, Beckman Instrument Inc, USA

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

Electrospray Ionization-Time of Flight Mass Spectrometry (ESI-TOF MS): micrOTOF, Bruker, Germany

Evaporator: BÜCHI Rotavapor R-200, Switzerland

Fourier Transform Nuclear Magnetic Resonance (FT-NMR): INOVA-500, Varian, USA

Fraction collector: Frac-100, Pharmacia Biotech, Sweden

High Performance Liquid Chromatography: Model LC-3A Shimadzu, Japan

Incubator: Memmert, Germany

Incubator shaker: Innova 4000, New Brunswick Scientific, USA

Laminar flow: Model BVT-124, International Sciencetific Supply Co., USA

Magnetic stirrer: Model Fisherbrand, Fisher Scientific, USA

NH_2 (Amino) Column: Luna 5u NH_2 100A, Phenomenex,Inc., EU

Peristaltic pump: pump p-1, Pharmacia Biotech, Sweden

Power supply: Model EC 135-90-LVD CE, EC Apparatus Inc., USA

pH meter: pH900, Precisa, Switzerland

Syringe: Holder 13 mm SST Swinney Syringe, Millipore, USA

TLC plates: Silica gel 60 F_{254}, Merck, Germany

UV-VIS Spectrophotometer: DU650 Spectrophotometer, Beckman, USA

Vortex: Model K-550-GE, Scientific Industries, USA

Water bath: Memmert, Germany

2.2 Chemicals

Acetonitrile (HPLC grade): LAB- SCAN Analytical Science, Ireland

Acrylamide: Merck, USA

Ammonium persulfate: Pharmacia fine chemicals, Sweden

Ammonium sulfate: Carlo Erba reagent, Germany

AnaeroPack: Mitsubishi Gas Chemical Co., Inc., Japan

Agar: Merck, Germany

Bacto-peptone: Difco Laboratories, USA

Beef extract: Difco Laoratories, USA

Bovine serum albumin (BSA): Sigma, USA

D(+)-Cellobiose: Fluka, Slovakia

Coomassie brilliant blue G-250, R-250: Sigma, USA

DEAE- Celluose resin: DE 32, Whatman Biosystems Ltd., England

Dialysis tubing: Sigma, USA

Egg albumin soluble: Difco Laboratories, USA

Ethylenediamine tetraacetic acid (EDTA): Fluka, Switzerland

Ethyl acetate: Scharlau, Spain

D(-)-Fructose: Sigma, USA

Glacial acetic acid: BDH, England

D (+)-Glucose: Sigma, USA

Glycerol: Scharlau, Spain

Glycine: Sigma, USA

High molecular weight marker protein: Sigma, USA

Hydrochloric acid: Merck, Germany

Lactose: Sigma, USA

Lactulose: Fluka, Italy

Magnesium sulphate 7-hydrate: BDH, England

Maltose: Sigma, USA

Maltotriose, Maltotetraose , Maltohexaose, Maltoheptaose: Sigma,USA

Maltopentaose: Wako, Japan

Manganese sulphate 4-hydrate: BDH, England

Melibiose: Sigma,USA

2-Mercaptoethanol: Scharlau, Spain

Methanol: Merck, Germany

N, N′-Methylene-bis-acrylamide: Sigma, USA

Palatinose: Sigma,USA

Peptone from meat: Merck, USA

Potassium dihydrogen phosphate: Merck, Germany

n- Propyl Alcohol: Carlo Erba reagent, Germany

D(+)-Raffinose pentahydrate: Nacalaitesque Inc., Japan

Sephadex G-100, Sephadex LH-20: GE Healthcare, Sweden

Sodium acetate: BDH, England

Sodium chloride: USB, USA

Di-Sodium hydrogenphosphate: Fluka, Switzerland

Sodium hydroxide: Carlo Erba, Italy

Soluble starch, potato: Sigma, USA

Soluble starch, cassava: Flomax, Thailand

Sucrose: Bio Basic Inc., Canada

Sulphuric acid: BDH, England

TEMED (*N, N, N', N'*-tetramethylene-ethylenediamine): Fluka, Germany

Tri-Ammonium Citrate: BDH, England

2, 3, 5 Triphenyltetrazolium chloride (TTC) : Kanto Chemical Co Inc., Japan

Tris (hydroxymethyl)-aminomethane: USB, USA

Tween 80: Fluka, Italy

Yeast extract: Scharlau, Spain

2.3 Bacteria

Bacillus sp. TH 4-2, a thermotolerant bacteria isolated from soil in Thailand, was screened for levansucrase activity (Ammar *et al.*, 2002). The organism was identified as *Bacillus licheniformis* by investigating its biochemical and physiological properties and 16S rRNA gene (Nakapong, unpublished data).

Lactobacillus acidophilus TISTR No. 450, a probiotic bacteria, was obtained from Thailand Institute of Scientific and Technological Research (TISTR)

2.4 Media Preparation

2.4.1 Enrichment medium

Enrichment medium consisted of 10 % sucrose, 0.5 % peptone, 0.1 % KH_2PO_4 and 0.05 % $MgSO_4.7H_2O$. The pH of medium was adjusted to 7.0 (Ammar *et al.*, 2002). For solid medium, 1.5 % agar was added.

2.4.2 Enzyme production medium

Enzyme production medium consisted of 1 % beef extract, 1 % peptone, 0.4 % ovalbumin, 0.5 % NaCl and 20 % soluble starch (potato). The pH of medium was adjusted to 6.5 (Ammar *et al.*, 2002).

2.5 Optimization of cultivation condition for high glucansucrase production

2.5.1 Starter inoculum

Bacillus licheniformis TH 4-2 was streaked on solid Enrichment medium and incubated at 45 °C for 12 hours. A single colony was inoculated into 3 ml of liquid enrichment medium and grown until A_{600} reached 0.4-0.6.

2.5.2 Optimum carbon source

Starter inoculum of *Bacillus licheniformis* TH 4-2 was 0.5 % transferred into 100 ml of Enzyme production medium consisted of 1 % beef extract, 1 % peptone, 0.4 % ovalbumin, 0.5 % NaCl and 5 % of variable carbon source (soluble starch ; potato / cassava and sucrose) (modified from Ammar *et al.*, 2002). The cell culture was cultivated with 250 rpm shaking at 45 °C for 36 hours and growth was spectrophotometry determined at the absorbance 600 nm. Cells were removed by centrifugation at 5,000 rpm at 4 °C for 30 min. Culture broth with crude glucansucrase was collected for activity assay.

2.5.3 Optimum concentration of carbon source

Starter inoculum of *Bacillus licheniformis* TH 4-2 was 0.5 % transferred into 100 ml of Enzyme production medium consisted of 1 % beef extract, 1 % peptone, 0.4 % ovalbumin, 0.5 % NaCl and the best carbon source from section 2.5.2. The concentrations of carbon source were 2, 5 and 10 % .The cell culture was cultivated with 250 rpm shaking at 45 °C for 48 hours and growth was spectrophotometry determined at the absorbance 600 nm. Cells were removed by centrifugation at 5,000 rpm at 4 °C for 30 min. Culture broth with crude glucansucrase was collected for activity assay.

2.5.4 Optimum cultivation temperature

Starter inoculum of *Bacillus licheniformis* TH 4-2 was 0.5 % transferred into 100 ml of the best medium obtained from section 2.5.3. The cell culture

were cultivated with 250 rpm shaking at 45 °C and 50 °C for 48 hours. Growth was spectrophotometry determined at the absorbance 600 nm. Cells were removed by centrifugation at 5,000 rpm at 4 °C for 30 min. Culture broth with crude glucansucrase was collected for activity assay.

2.5.5 Optimum cultivation time

Starter inoculum of *Bacillus licheniformis* TH 4-2 was 0.5 % transferred into 100 ml of the best medium obtained from section 2.5.3. The cell culture was cultivated with 250 rpm shaking at the best of temperature from 2.5.4 for 72 hours and growth was spectrophotometry determined at the absorbance 600 nm. Cells were removed by centrifugation at 5,000 rpm at 4 °C for 30 min. Culture broth with crude glucansucrase was collected for activity assay.

2.6 Production of Glucansucrase

2.6.1 Starter inoculum

The starter inoculum from section 2.5.1 was 0.5 % inoculated into 100 ml of liquid enrichment medium and grown until A_{600} reached 0.4-0.6.

2.6.2 Enzyme production

The starter inoculum from section 2.6.1 was 0.5 % transferred into 200 ml of Enzyme production medium from section 2.5.3 in 500 ml Erlenmeyer flask (10 flasks; 2 liter) and cultivation at 45 °C with 250 rpm shaking for 42 hours (the

result from section 2.5.5). Cells were removed by centrifugation at 5,000 rpm at 4 °C for 30 min. Culture broth with crude glucansucrase was collected and kept at 4 °C for further purification.

2.7 Purification of glucansucrase

Glucansucrase from the culture broth of *Bacillus licheniformis* TH 4-2 was purified by three steps of purification ; Ammonium sulfate precipitation, DEAE-cellulose chromatography column and Sephadex G-100 chromatography column.

2.7.1 Ammonium sulfate precipitation

The precipitation of crude extract was performed by slowly adding fine solid ammonium sulfate to 30 % saturation with gentle stirring by magnetic stirrer. After that, the supernatant was collected by centrifugation at 5,000 rpm for 45 min and then adjusted to final concentration of 60 % saturation with solid ammonium sulfate. The solution was left for 3 hours on ice with continuous stirring and centrifuged again. The precipitate was dissolved in 20 mM sodium acetate buffer, pH 6.0. The protein solution was dialyzed against 100 volume of the same buffer, at least 4 hours for 3 times before determination of the enzyme activity and protein concentrations described in 2.8 and 2.9 respectively.

2.7.2 DEAE –cellulose chromatography

DEAE–cellulose was activated by washing sequentially with excess volume of 0.1 N HCl for 3 hours followed by distilled water until pH was about 7.0 and then washed with 0.1 N NaOH for 3 hours followed by distilled water until pH was about

7.0. The activated cellulose was equilibrated with 20 mM sodium acetate buffer pH 6.0. The prepared DEAE–cellulose was packed in the column (1.5 x 27 cm) and equilibrated with the same buffer for 5-10 column volume at flow rate of 45 ml/hours.

The dialyzed protein solution from section 2.7.1 was applied to DEAE-cellulose column. The unbound proteins were eluted from the column with the same buffer. Normally, keep washing until the absorbance at 280 nm of eluent decreased to almost nil. After the column was washed thoroughly with the same buffer, the bounded proteins were eluted from the column with linear salt gradient of 0 to 0.3 M sodium chloride in the same buffer. The fractions of 5.0 ml were continuously collected. The elution profile was monitored for protein by measuring the absorbance at 280 nm. The enzyme activity was determined as described in section 2.8. The fractions showing enzyme activity were pooled for further purification step.

2.7.3 Sephadex G-100 chromatography

The Sephadex G-100 was packed in the column (1.9 x 90 cm) and equilibrated with the same buffer for 5-10 column volume at flow rate of 20 ml/hours to allow stabilization of bed volume of column. Blue dextran 2000 and potassium dichromate were used to determine the void volume and the total volume of the column. An aliquot (2 ml) of the concentrated enzyme from DEAE-cellulose column chromatography was loaded into the column and eluted with elution buffer at flow rate of 20 ml/hours. Fractions of 2 ml were collected. The elution profile was monitored for protein and enzyme activity as described in section 2.8 and 2.9 respectively.

2.8 Enzyme assay

Glucansucrase (sucrose hydrolysis) activity was determined from reducing sugars by Somagyi-Nelson 's method.(1990).

The reaction containing 500 μl of 20 % (w/v) sucrose dissolved in 20 mM sodium acetate buffer pH 6.0, 480 μl of the buffer and 20 μl of enzyme were incubated at 45 °C for 10 min. The reaction was stopped with 1 ml of alkaline copper reagent. The solution was heated for 10 min in boiling water bath, then the tubes were cooled in cold water for 5 min. Then 1 ml of ammonium molybdate reagent was added. After mixing, the solution was left for 15 min at room temperature. The mixture was adjusted to a final volume of 8 ml with distilled water. The absorbance at 520 nm was measured.

One unit of glucansucrase was determined as the amount of enzyme that produced 1 μmole of fructose per min under experimental condition.

2.9 Protein determination

Protein concentration was determined by Bradford's method (1976), using bovine serum albumin as the standard protein (see Appendix 3).

One hundred microlitres of sample was mixed with 1 ml of Coomassie blue reagent and left for 5 minutes before recording the absorbance at 595 nm. One litre of Coomassie blue reagent contained 100 mg of Coomassie blue G-250, 50 ml of 95 % ethanol, 100 ml of 85 % H_3PO_4 and distilled water.

2.10 Polyacrylamide Gel Electrophoresis (PAGE)

Two types of PAGE, non-denaturing and denaturing gels, were employed for analysis of the purified protein. The gels were visualized by coomassie blue staining. For non-denaturing gel, glucansucrase activity staining was also undertaken.

2.10.1 Non-denaturing polyacrylamide gel electrophoresis (Native-PAGE)

Discontinuous PAGE was performed according to Rojtinnakorn (1994) on slab gels (10 x 8 x 0.75 cm), of 7.5 % (w/v) separating gel, and 5.0 % (w/v) stacking gels. Tris-glycine buffer pH 8.3 (25 mM Tris and 192 mM glycine) was used as electrode buffer. Preparation of solution and polyacrylamide gels was described in Appendix 1. The enzyme was mixed with 5x sample buffer (312.5 mM Tris-HCl, pH 6.8, 50 % (v/v) glycerol and 0.05 % (w/v) bromophenol blue) by the ratio of 5:1 and loaded onto the gel. The electrophoresis was run from cathode towards anode at constant current of 20 mM per slab gel. For activity staining, the experiment was done at 4 ^{o}C. After electrophoresis, the gel was developed by protein and activity staining.

2.10.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The SDS-PAGE system was performed according to the method of Bollag *et al.*, 1996. The slab gel system consisted of 0.1 % (w/v) SDS in 10 % (w/v) seperating gel and 5 % (w/v) stacking gel. Tris-glycine (25 mM Tris, 192 mM glycine and 0.1 % (w/v) SDS), pH 8.3 was used as electrode buffer. The gel preparation was described in Appendix 1. The enzyme was mixed with 5x sample buffer (60 mM Tris-HCl pH

6.8, 25 % (v/v) glycerol, 2 % (w/v) SDS, 0.1 % (w/v) bromophenol blue and 14.4 mM β-mercaptoethanol) by the ratio of 5:1 and boiled for 5 minutes before loading to the gel. The electrophoresis was run from cathode towards anode at constant current 20 mM per slab gel at room temperature. The molecular weight marker proteins were β-galactosidase (116,000 Da), bovine serum albumin (66,200 Da), ovalbumin (45,000 Da), lactate dehydrogenase (35,000 Da), restriction endonuclease (25,000 Da), β-lactoglobulin (18,400 Da) and lysozyme (14,400 Da). After electrophoresis, the gel was stained with Coomassie blue as described in section 2.10.3.1.

2.10.3 Dectection of proteins

2.10.3.1 Coomassie blue staining

The gel was stained with Coomassie staining solution (1 % (w/v) Coomassie Blue R-250, 45 % (v/v) methanol, and 10 % (v/v) glacial acetic acid) for 30 minutes on the shaker. The stain solution was poured out and the Coomassie destaining solution (10 % (v/v) methanol and 10 % (v/v) glacial acetic acid) was added. The gel was gently destained for several times until gel background was clear.

2.10.3.2 Activity staining (Gabriel and Wang, 1967)

The gel was washed twice in 10 volumes of 100 mM sodium acetate pH 6.0 for 15 min at room temperature and then incubated for 30 min at 37 °C in 5 % (w/v) sucrose in the same buffer. The gel was then immersed in 1 N sodium hydroxide containing 0.1 % (w/v) 2, 3, 5 Triphenyltetrazolium chloride (TTC) and incubation in the dark. Red bands corresponding to glucansucrase activity (reduction

of TTC) became visible within 5 min. The reaction was stopped by placing the gel in 7.5 % acetic acid.

2.11 Characterization of Glucansucrase

2.11.1 Determination of molecular weight

After SDS-polyacrylamide gel electrophoresis, the protein bands in gel were visualizaed by coomassie blue stain. The molecular weight of purified glucansucrase was determined from calibration curve obtained from Rf and molecular weight of standard proteins; β-galactosidase (116,000 Da), bovine serum albumin (66,200 Da), ovalbumin (45,000 Da) and lactate dehydrogenase (35,000 Da).

2.11.2 Effect of pH on the enzyme activity

The effect of pH on the activity was determined under the enzyme activity assay condition as described in section 2.8 at various pH. The pH range of reaction mixture was 5-9. The buffers used were sodium acetate buffer (pH 5.0-6.0), phosphate buffer (pH 6.0-8.0), borate buffer (pH 8.0-9.0). The percentage of relative activity was plotted against the pH used for the activity assay.

2.11.3 Effect of temperature on the enzyme activity

The effect of temperature on the activity was determined under the enzyme activity assay condition as described in section 2.8 at optimum pH and various

temperatures from 30 °C to 60 °C. The percentage of relative activity was plotted against the temperature used for the activity assay.

2.11.4 Kinetic studies of glucansucrase

2.11.4.1 Determination of K_m and V_{max} for sucrose substrate

Purified glucansucrase from Sephadex G-100 column was used for kinetic study. Sucrose substrate was varied at the concentrations of 5, 10, 50, 100, 200, 300 and 400 mM. The reaction was incubated for 10 min at optimum pH and temperature . The activity was determined under the enzyme activity assay condition as described in section 2.8.

2.11.4.2 Determination of K_m and V_{max} for melibiose acceptor

Purified glucansucrase from Sephadex G-100 column was used for kinetic study. Sucrose substrate was used at the fixed concentration of 150 mM. Melibiose acceptor was varied in the concentration range of 100 - 500 mM. The reaction was incubated for 180 min at optimum pH and temperature. The transglucosylation activity were analyzed by HPLC as described in section 2.12.2.2 and then determination of peak area of the product was determined.

2.12 Synthesis and detection of oligosaccharide products

2.12.1 Acceptor specificity

Oligosaccharides (OS) were prepared by incubation of purified glucansucrase (0.5 unit/ml) in acetate buffer,pH 6.0 with 5 % sucrose as glucosyl donor and 5 % various acceptors (G1; Glucose to G7; maltoheptaose, lactose, melibiose, cellobiose, raffinose, palatinose and lactulose) at 45 ^{o}C for 24 hours. The products were analyzed by thin-layer chromatography (TLC).

2.12.2 Detection of products

2.12.2.1 Thin layer chromatography (TLC)

The products were analyzed by thin-layer chromatography (TLC) using silica gel 60. Solvent system was n-propanol : ethyl acetate : water (7:1:2, v/v) by volume. Spots were detected by spraying with concentrated sulfuric acid : ethanol (1:9, v/v) (Kennedy and Pagliuca,1994). Followed by heating at 110 ^{o}C for 15 min.

2.12.2.2 High performance liquid chromatography (HPLC)

The HPLC system was a Shimadzu LC- 3A equipped with Luna-NH2 column (250 x 4.6 mm) and using Shimadzu RID-3A refractometer as detector. The reaction mixture of oligosaccharide synthesis was filtered through a13 mm Nylon 0.45 μm disc filter before injection and eluted with acetonitrile : water (70 : 30, v/v) using a flow rate of 1 ml/min at 40 ^{o}C .The oligosaccharide peaks were identified by

comparing the retention times with those of standard glucose, fructose, sucrose, acceptor (40 μg/ml). Product yield was calculated from peak area.

2.12.3 Determination of transglucosylation efficiency

Efficiency was judged by transglucosylation yield, which could be determined from the product yield directly. The yield of transglucosylated products was calculated from the ratio of peak area of product to the initial peak area of acceptor in HPLC profile, using the equation:

$$\text{Product yield (\%)} = \frac{\text{Peak area of product}}{\text{Peak area of acceptor at } t_o} \times 100$$

2.13 Optimization of transglucosylation reaction

2.13.1 Effect of acceptor concentration

The reaction was performed by incubation of glucansucrase 0.5 unit/ml with various acceptor (melibiose) concentrations (0, 1, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20, 22.5 %, w/v) and 5 % (w/v) sucrose in the 20 mM acetate pH 6.0 at 45 °C for 24 hours. The reaction was stopped by boiling for 5 minutes, and then analyzed by HPLC. The optimum melibiose concentration was judged from the determination of peak area of products.

2.13.2 Effect of donor concentration

The reaction was performed by incubation of of glucansucrase 0.5 unit/ml with various donor (sucrose) concentrations (0, 1, 2.5, 5, 7.5, 10 and 15 %, w/v) and optimum melibiose concentration(15 %, w/v) in the 20 mM acetate pH 6.0 at 45 °C for 24 hours. The reaction was stopped by boiling for 5 minutes, and then analyzed by HPLC. The optimum donor concentration was judged from the determination of peak area of products.

2.13.3 Effect of enzyme concentration

The reaction was performed by incubation of optimum concentration of melibiose acceptor and optimum concentration of sucrose donor at various enzyme concentrations (0, 0.1, 0.25, 0.5, 1.25, 2.5, 5.0, 7.5, 10.0 unit/ml) in the 20 mM acetate pH 6.0 at 45 °C for 24 hours. The reaction was stopped by boiling for 5 minutes, and then analyzed by HPLC. The optimum enzyme concentration was judged from the determination of peak area of products.

2.13.4 Effect of pH

The reaction was performed by incubation of appropriate concentration of glucansucrase with optimum concentration of melibiose acceptor and sucrose donor in 20 mM buffer in the pH range of 4.0 to 9.0 at 45 °C for 24 hours. The buffers used were sodium acetate (pH 5.0-6.0), potassium phosphate (pH 6.0-8.0) and borate (pH 8.0-9.0) (see Appendix 2). The reaction was stopped by boiling for 5 minutes, and

then analyzed by HPLC. The optimum pH was judged from the determination of peak area of products.

2.13.5 Effect of temperature

The reaction was performed by incubation of appropriate concentration of glucansucrase with optimum concentration of melibiose acceptor and sucrose donor at optimum pH at various temperatures (30, 40, 45, 50, 55, and 60 °C) for 24 hours. The reaction was stopped by boiling for 5 minutes, and then analyzed by HPLC. The optimum temperature was judged from the determination of peak area of products.

2.13.6 Effect of incubation time

The reaction was performed by incubation of appropriate concentration of glucansucrase with optimum concentration of melibiose acceptor and sucrose donor at optimum pH and temperature for various times (0, 3, 6, 12, 24, 36 and 48 hours). The reaction was stopped by boiling for 5 minutes, and then analyzed by HPLC. The optimum incubation time was judged from the determination of peak area of products.

2.14 Larger scale preparation and isolation of OS products

In the initial experiments: to prepare OS products and to optimize the transglucosylation reaction (2.13) and to determine transglucosylation efficiency, (2.12) small scale reaction mixture of 0.5 ml was used. To prepare higher amount of products for characterization, larger scale preparation (10 ml) of reaction mixture using optimum condition for transglucosylation as obtained from section 2.13 was

performed. After stop the reaction, the enzyme invertase (20 unit/ml) was added to the reaction mixture and incubated at 37 ^{o}C for 3 hours. After treated with invertase, the reaction mixture of 0.5ml was applied on Sephadex LH-20 column (1.2 x 120 cm) equilibrated with 70 % (v/v) n-propanol. Then OS products were separated from sugars and enzyme by elution of the column with 70 % (v/v) n-propanol (10 ml/hours, fraction size 1 ml). The fractions containing products were collected and determined by HPLC as described in section 2. The main product peak was collected for further characterization.

2.15 Charaterization of OS products

2.15.1 Mass Spectrometry

Electrospray Ionization-Time of Flight Mass Spectrometry (ESI-TOF MS) profile was recorded on a micrOTOF at the Service Unit of the National Center for Genetic Engineering and Biotechnology. The mixture of methanol : H_2O was used as solvent.

2.15.2 Nuclear Magnetic Resonance

For structural elucidation, ^{1}H and ^{13}C NMR spectrum were determined using Varian Gemini 400 MHz spectrometer at the department of Chemistry, Chulalongkorn University. The operation was at 400 MHz for protons and 100 MHz for carbons. The chemical shifts were expressed in ppm downfield from the signal of tetramethylsilane (TMS), which was used as internal standard.

2.16 Determination of properties of OS product

2.16.1 Monitoring of bioactivity of OS product in supporting growth of *Lactobacillus acidophilus*

2.16.1.1 Starter inoculum

Lactobacillus acidophilus was streaked on solid MRS (MRS medium ; consisted of 1 % beef extract, 1 % peptone, 1 % yeast extract, 1 % tri- ammonium citrate, 1 % K_2HPO_4, 1 % $MgSO_4.7H_2O$, 1 % $MnSO_4$, 1 % Tween 80 and 2 % glucose) (de Man, Rogosa and Sharpe, 1960). Medium and anaerobically incubated at 37 °C for 36 hours. A single colony was then inoculated into 5 ml of MRS broth and incubated anaerobically at 37 °C overnight.

2.16.1.2 Various carbon source

Starter inoculum of *Lactobacillus acidophilus* was 1 % transfer red into 5 ml of MRS broth consisted of components as described but with 2 % of variable carbon sources (glucose, raffinose, melibiose, FOS (commercial) and the main product: product A). The cell culture was anaerobically cultivated at 37 °C for 30 hours and growth was monitored by measuring the absorbance at 600 nm at different time intervals.