CHAPTER 3

METHODOLOGY

3.1 Materials

3.1.1 Microorganisms

Eight probiotic LAB strains were used in this study; namely *Lb. pentosus* (DM068, JM0812, JM085, UM054, UM055, VM095, VM096, and YM122) (Kansandee, 2010) were obtained from the laboratory of Asst. Prof. Dr. Pariyaporn Itsaranuwat, Department of Biotechnology, Mahasarakham University, Thailand. The strains were maintained in MRS broth (Criterion, USA) with 20% glycerol at -20 °C. They were anaerobically activated twice in MRS broth using 1% (v/v) inoculums for 24 h, at 37 °C, 5% CO_2 incubator prior to use for the experiments.

3.1.2 Materials and media

3.1.2.1 De Man Rogosa Sharpe (MRS) (Citerion, USA)

3.1.2 2 Modified-MRS medium

3.1.2 3 Agar (Merck Darmstadt, Germany)

3.1.2 4 Soybean seed (Rai Thip brand)

3.1.2.5 100% Honey syrup (Suan Jit-radda brand)

3.1.3 Chemicals

3.1.3.1 Chemical reagents

(1) Oxgall (Sigma-Aldrich, USA)

(2) Absolute ethanol (Merck, Germany)

(3) 95 % Ethyl alcohol (Solvent solution Grade A; Commercial grade)

(4) n-Hexadecane; Cetane (Sigma-Aldrich, USA)

(5) Taurodeoxycholic acid; TDCA (Sigma-Aldrich, USA)

(6) *o*-Phthalaldehyde reagent (Sigma-Aldrich, USA)

(7) Sodium chloride (Univar, Ajak Finechem, NSW, Australia)

(8) 0.05 M Sodium acetate buffer (pH 5.0)

(9) 0.85 % NaCl

(10) Acetonitrile HPLC grade (Lab-Scan, Ireland)

(11) Methanol HPLC grade (Lab-Scan, Ireland)

(12) Reverse Osmosis Water for HPLC

3.1.3.2 Standard reagents

(1) Water soluble cholesterol (polyoxyethanyl cholesteryl sebaccate; PCS) (Sigma-Aldrich, USA)

(2) Zeralenone (Sigma-Aldrich, USA)

(3) Propionic acid (Merck, Germany)

(4) Butyric acid (Merck, Germany)

(5) Iso- butyric acid (Merck, Germany)

(6) n-Valeric acid (Merck, Germany)

(7) Lactic acid (Merck, Germany)

(8) Acetic acid (Merck, Germany)

(9) Fructooligosaccharide (FOS) (Sigma-Aldrich, USA)

(10) Maltotetraose (Sigma-Aldrich, USA)

(11) Raffinose (Sigma-Aldrich, USA)

(12) Stachyose (Sigma-Aldrich, USA)

(13) D (-) Maltose (Merck, Germany)

(14) Treharose (Merck, Germany)

(15) Lactose (Sigma-Aldrich, USA)

(16) D (+) glucose (Merck, Germany)

(17) Myo-innositol (Merck, Germany)

(18) Mannose (Merck, Germany)

(19) D (+) galactose (Univar, Ajax Finechem, NSW, Australia)

(20) D (-) fructose (Sigma-Aldrich, USA)

(21) D (+) xylose (Sigma-Aldrich, USA)

(22) Mannitol (Merck, Germany)

(23) Sorbitol (Merck, Germany)

(24) L (-) Rhamnose (Merck, Germany)

(25) L (+) arabinose (Merck, Germany)

(26) D (-) arabinose (Sigma-Aldrich, USA)

3.1.4 Apparatus and Instruments

3.1.4.1 Centrifuge (Beckman Coulter™, Allegra™ X-22R, Germany)

3.1.4.2 Water bath (Memmert, Germany)

3.1.4.3 Membrane filter (Whatman, England)

3.1.4.4 Microscopy (Olympus, BX60, USA)

3.1.4.5 Densimat (bioMérieux, Marcy l'Etoile, France)

3.1.4.6 Micropipette (Biohit, France)

3.1.4.7 Microwave (Turbora, TRX-2021, Thailand)

3.1.4.8 Refrigerator (Hitachi, Thailand)

3.1.4.9 Freezer (Sanyo, Thailand)

3.1.4.10 Hot air oven (Memmert, Germany)

3.1.4.11 Biosafety Cabinet Class II (Telstar Industrial, Biostar plus4, Spain)

3.1.4.12 CO_2 incubator (Contherm, MITRE 4000 Series, Australia)

3.1.4.13 Vortex mixer (Vortex-Genie2, Scientific Industries, USA)

Industries,

G-560E)

3.1.4.14 Autoclave (Hirayama, HICLAVETM, AllegraTM X-22R Cen)

3.1.4.15 UV-Vis spectrophotometer microplate reader (Shimadsu, UV-160A)

3.1.4.16 UV-Vis spectrophotometer microplate reader (SPECTROstar Nano)

3.1.4.17 pH meter (Mettler Toledo, SevenMulti, Switzerland)

3.1.4.18 Ultra sonicator (Cavitator Metter Lectronics crop.,ME11)

3.1.4.19 Balance 2 digits (Mettler Toledo, PB1502-5, Switzerland)

3.1.4.20 Refrigerator microcentrifuge (Eppendorf Centrifuge 5415R, UK)

3.1.4.21 Refrigerator microcentrifuge (Refrigerated Microfuge Sigma 1-15k, UK)

3.1.4.22 Hot plate (Harmony, LMS Laboratory & Medical supplies, VA, USA)

3.1.4.23 Thermostatically controlled shaker (Bioblock Scientific Ping-Pong 74582)

3.1.4.24 HPLC-PDA system (Shimadzu, Japan)

3.1.4.25 HPLC-RID system (Shimadzu, Japan)

3.1.4.26 HPLC system (ICS, France)

3.1.4.27 Aminex HPX-87H column (Bio-Rad, Hercules, CA)

3.1.4.28 C-18 spherisorb column (Prontosil 120-3-C18, 25 cm× 0.4 cm)

3.1.4.29 Inertsil® ODS-3 C18 analytical column (4.6 x 250 mm inside diameter, 5µm)

3.1.4.30 Steriled petri dishes (HYCON, ligand)

3.1.4.31 STERIN 96 well plate steriled

3.1.4.32 Microcentrifuge tube 1.5 mL

3.1.4.33 Sample vial for HPLC

3.1.4.34 Test tube

3.1.4.35 Glass ware

3.1.4.36 Syringe holder

3.1.4.37 Nylon membrane filters, 0.22 (Whatman, Japan)

3.2 Methods

This study was divided into two parts; the first part was studied on health-promoting properties of probiotic *Lb. pentosus* strains and the other one was to examine the use of the probiotic *Lb. pentosus* strains which had good health-promoting properties as a starter culture for probiotic soya beverage production.

Part I Study on health-promoting properties of probiotic *Lb. pentosus* strains.

3.2.1 Growth profile, pH change, sugar utilisation and SCFAs production of probiotic *Lb. pentosus* strains.

3.2.1.1 Culture preparation

Eight strains of *Lb. pentosus* (DM068, JM0812, JM085, UM054, UM055, VM095, VM096, and YM122) were cultured in MRS medium (Citerion, USA) at 37 °C for 24 h, in 5% CO_2 incubator (Contherm, MITRE 4000 Series, Australia). All strains were weekly sub-cultured in MRS broth using 1 % inoculums followed by incubation at 37 °C for 24 h. They were stored at 4 °C before used. The stock cultures were monthly sub-cultured in MRS agar slant. Each strain was sub-cultured twice consequently in steriled MRS broth using 1 % inoculums for 24 h incubation at 37 °C prior to use.

3.2.1.2 FOS, raffinose, lactose (Sigma-Aldrich, USA) were used to replace glucose as a carbon-source in modified-MRS media, namely FOS-MRS medium, Raffinose-MRS medium, and Lactose-MRS medium respectively- (see Appendix A for details). FOS, raffinose, and lactose were added at the same concentration of glucose in modified-MRS media (2 % w/v used in basically formula), mixed together in 1000 mL distilled water, and gently boiled to dissolve the medium completely. One thousand mL of each medium was distributed into Duran bottle (size 250 mL) and sterilized by autoclaving at 121 ^{o}C for 15 min. Thereafter, it was cooled down to be ready for the inoculation with *Lb. pentosus* strains. MRS medium with 2 % (w/v) glucose was used as a control. Triplicate samples of each treatment were prepared.

3.2.1.3 Propagation of *Lb. pentosus* strains and preparation of inoculums Eight strains of *Lb. pentosus* grown in MRS slant agar about 18-24 h were prepared as cell suspensions in steriled normal saline solution (0.85% NaCl). Cell concentrations were adjusted to obtain the final concentration at 3×10^{8} CFU/mL by adjusting turbidity at 1.0 McFarland Units using Densimat (bioMérieux, Marcy l'Etoile, France- (see Appendix C for details) designed to measure the bacteria density and gives values in McFarland Units, proportional to the average value of bacterial concentrations (Itsaranuwat, 2003) (Table 3.1). After that, 2 mL of the cell suspension was transferred to 200 mL the modified-MRS media which prepared as mentioned above. The initial cell concentration in modified-MRS media was approximately 10^{6} CFU/mL. The samples were incubated at 37 °C for 24 h in 5% CO_2 incubator. All experiments were replicated three times.

Table 3.1 Correspondences between McFarland scale/Bacteria concentration/Optical density

Standard McFarland scale	Bacterial concentration X 10^8 CFU/mL	Optical density At 550 nm
0.5	1.5	0.125
1	3	0.25
2	6	0.5
3	9	0.75
4	12	1.00
5	15	1.25
6	18	1.50
7	21	1.75

Source: Itsaranuwat (2003)

3.2.1.4 Growth profile and acid production

The growth and changes in pH were monitored as indicators of growth pattern. The growth profiles of eight strains were measured as optical density (OD) at 600 nm every hour over incubation period by using UV-Vis spectrophotometer microplate reader (SPECTROstar Nano) (see Appendix C) with 96 well plate steriled microplate (STERIN96) at 37 °C of 24 h. Aliquots from each batch were taken at 0, 3, 6, 9, 12, 15, 18, 21, and 24 h to monitor pH value by using a pH meter (model 8417, Hanna Instruments, Singapore). The machine was calibrated before use with buffer solutions of pH 7.0 and 4.0. All experiments were replicated three times.

3.2.1.5 Sugar utilisation

3.2.1.5.1 Samples preparation

To determine sugar contents, 1 mL of each sample was taken at 0, 6, 12, and 24 h of incubation period into 1.5 mL microcentrifuge tube. Samples were centrifuged at 10,000 rpm for 10 min by using the refrigerated microcentrifuge (Refrigerated Microfuge Sigma 1-15k, UK). Supernatant was then filtered though a 0.22 µm pore size nylon membrane HPLC filter (Whatman, Japan) before injecting to HPLC. The experiment was repeated twice.

3.2.1.5.2 Determination of sugars

Sugar contents were determined by modified method described previously by Eyéghé-Bickong et al. (2012) and Liu et al. (2012). Briefly, HPLC analysis was performed on a Shimadzu Prominence System (Shimadzu, Japan) (see Appendix C) equipped with LC-20AD series pumping system and SIL-20A series auto injector system equipped with refractive index detector (RID); RID-10A Series (A waters 410 Milfold, MA) , which was used to simultaneously separate and analyze sugars at wavelength of 210 nm. Aminex HPX-87H (Bio-Rad, Hercules, CA) ion exchange column (300 mm × 7.78 mm) maintained at 85 ^{o}C and protected with a Bio-Rad micro-guard cartridge (30 mm × 4.6 mm). Deionized H_2O with 5 mM H_2SO_4 was used as mobile phases and maintained at a flow rate of 0.5 mL/min. The injection volumn was 20 µL and run time was 55 min.

3.2.1.5.3 Preparation of calibration standard curve

Standard sugar was obtained from Sigma Chemical Co (Sigma-Aldrich, USA). The quantification of sugar in samples was carried out using external calibration standard curve method. The calibration standard curves of 18 sugars were prepared at five concentrations ranging from 0.1 to 10.0 mg/mL (see Appendix D for details). The reference samples were injected repeatedly three times. The calibration curves were constructed by plotting the relative peak area versus sugar concentrations. The concentration of sugar in samples were derived from external standard calibration based on peak are and was expressed as mg/mL.

3.2.1.6 SCFAs production of probiotic *Lb. pentosus* strains

3.2.1.6.1 Samples preparation

The samples for SCFAs determination prepared as described in Section 3.2.1.5. The experiment was repeated in duplicate.

3.2.1.6.2 Determination of SCFAs

SCFAs determination was applied according to the previous method by Itsaranuwat (2008). The SCFAs analyses were carried out by using HPLC. Briefly, 20 µL of samples were injected directly into HPLC RID system (Shimadzu, Japan) LC-20AC Series pumping system; SIL-10AD Series auto injector system with Shimadzu SPD-M20A diode array detector. SCFAs in samples were separated using a Inertsil® ODS-3 C18 analytical column (4.6 x 250 mm inside diameter, 5µm) at 38 °C. The

target compounds were detected using a UV-diode array detector set at wavelength of 210 nm. The mobile phase consisted of 10 mM $NaHPO_4$ buffer (pH 2.5), filtered through 0.22 µm nylon membrane and degassed by sonication for 10 min before use. Flow rate was 1.0 mL/min and injection volumn 20 µL. The SCFA compounds in the samples were identified by comparing their relative retention times and were concentrations were calculated by using peak areas of an external standard.

3.2.1.6.3 Preparation of calibration standard curve

Standard SCFAs such as acetic, propionic, butyric, isobutyric, and *N*-valeric acid were obtained from Merck, Germany. The calibration standard curves were prepared with concentration levels ranging from 0.1 to 1.0 mmol/mL, except lactic acid was used with concentrations ranging from 0.1 to 10.0 mg/mL. SCFAs concentration was derived from external standard calibration and was expressed as mmol/mL.

3.2.3 Screening of strains for Bile Salt Hydrolase (BSH) activity

3.2.3.1 Preparation of strains

Stationary phase growth of *Lb. pentosus* 8 strains were investigated for BSH activity. The strains grew in MRS slant agar for 24 h and cell suspension was prepared in steriled normal saline solution (0.85 % NaCl), the cell concentration was adjusted to have turbidity of 3.0 McFarland Units.

3.2.3.2 BSH activity of *Lb. pentosus* strains

The BSH activity assay of the cultures was evaluated by modified method of Du Toit et al. (1998) and Mahrous (2011). Agar plate assay was developed to detect the BSH activity in lactobacilli. Briefly, MRS agar plates were prepared with 0.5 % (w/v) sodium salt of taurodeoxycholic acid (TDCA; Sigma-Aldrich, USA) as a bile acid and 0.37 g/L of $CaCl_2$ ((Merck, Germany). After sterilization, the plates were poured into steriled plastic petri dishes. Once solidified, the plates were inverted and placed in the anaerobic condition for at least 72 h before use. Steriled filter discs (diameter 0.7 cm) were placed on MRS agar plates and were spotted with a 10-µL of the cell suspension of each tested strain which prepared as mentioned above. All plates were incubated anaerobically at 37 °C for 72 h in 5 % CO_2 incubator. After which, the precipitation zone was observed as an indicator for the BSH activity of the tested strain and diameters of the precipitation zones surrounding filter discs were measured. The

MRS agar plates without supplementation were used as negative controls. Each strain was performed in triplicates.

3.2.4 *In vitro* cholesterol binding activity.

In vitro cholesterol binding activity was performed according to the previous method by Mahrous (2011) and Mirlohi et al. (2012) with slight modifications. Eight strains of *Lb. pentosus* were investigated for cholesterol binding activity. Standard cholesterol (Water soluble cholesterol; polyoxyethanyl-cholesteryl sebacate (PCS), Sigma-Aldrich, USA) was prepared at the concentration of 10 mg/mL in steriled water and filtered with 0.22 µm sterilized membrane. For each culture to be tested, 70 µL of cholesterol solution was added to 10 mL of freshly prepared MRS broth (final cholesterol concentration of 70 µg/mL) supplemented with 0.3 % oxgall (w/v) (Sigma-Aldrich, USA) as a source of bile salt. To the MRS broth, 1 % (v/v) of freshly grown culture of each strain was inoculated and incubated anaerobically at 37 °C for 24 h. An un-inoculated MRS broth at the same condition was used as a negative control. After 24 h of growth, the cells were removed by refrigerator microcentrifuge (Refrigerated Microfuge Sigma 1-15k, UK) at 10,000 g for 10 min at 4 °C. Spent broth was then filtated by using 0.45 mm membrane sterilized. The cholesterol levels were determined in the supernatant using the method of Rudel and Morris (1973) and Liong and Shah (2005). Briefly, 1 mL of the supernatant with 1 mL of 33 % (w/v) KOH and 2 mL of absolute ethanol were placed in a capped test tube, vortexed for 1 min and incubated for 15 min at 37 °C. After incubation, the mixture was removed and cooled under tap water, and then 3 mL of hexane and 3 mL of distilled water were added and mixed for 15 min. One milliliter of the hexane layer was transferred into a dry clean test tube and evaporated under nitrogen gas. The dried material was dissolved in 2 mL of freshly o-phthaladehyde reagent (0.5 mg/mL of glacial acetic acid) (Sigma-Aldrich, USA). After that, 0.5 mL of 12 N H_2SO_4 was added and the mixture was mixed for 1 min. After 10 min, absorbance was measured at 550 nm with a UV-Vis Spectrophotometer microplate reader (Shimadsu, UV-160A). The experiment was repeated in three different runs. The activity of cholesterol binding was calculated as a percentage by the treatment compared with the control from the following equation:

$$\% \text{ cholesterol binding} = [1-(OD_A / OD_B)] \times 100$$

When; OD_A = OD of sample was inoculated the cells

OD_B = OD of un-inoculated MRS broth

3.2.5 ZEA binding ability of *Lb. pentosus* strains in buffer solution

3.2.5.1 Chemical and Reagents

All the organic solvents used in this study were HPLC grade (Fisher scientific, France), MRS media (Criterion, USA), and Standard ZEA was purchased from Sigma-Aldrich (St. Louis, MO, USA).

3.2.5.2 Preparation of the bacteria cells for toxin binding

Eight probiotic strains were cultivated by steak plate technique in MRS agar media and incubated at 37 °C for 24 h in 5 % CO_2 incubator. Cells were suspended in 0.85% normal saline solution and transferred to microcentrifuge tube (1.5 mL safe-lock). The cells were harvested by centrifugation at 10,000 rpm, 4 °C for 10 min (Eppendorf Centrifuge 5415R, UK). The cell pellets were washed twice with steriled water and suspended in 2 mL of 10% skim milk and the cells suspension was transferred into sterilized serum vials, freeze-dried (Heto power dry PL3000) and stored at -20°C for the next experiment.

3.2.5.3 Preparation of standard solutions ZEA

Standard stock solution of ZEA: stock solution was prepared at 0.1 mg/mL in methanol. The concentration of the ZEA stock solution was determined by measuring the UV absorbance at 236 nm and calculated by using the molar extinction coefficient of 29700 mol^{-1} cm^{-1} (IARC, 1993).

Standard working solutions of ZEA: initial standard solution (0.1 mg/mL) was diluted by 0.05 M sodium acetate buffer (pH 5.0) into 1, 5, 20, 50 and 75 µg/mL and kept at -20 °C for using in the experiment.

0.05 M sodium acetate buffer solution (pH 5.0) was prepared by dissolving 6.5 g of sodium acetate ($C_2H_3NaO_2$-$3H_2O$) in 450 mL of ultrapure water, the pH was adjusted to 5 by adding acetic acid, and then the final volumn was adjusted to 500 mL.

3.2.5.4 Binding step

The experiments were prepared according to the protocol of Joannis-Cassan et al. (2011). All assays were performed in centrifuge tube (1.5-mL, safe-lock). Each tube contained 5 mg of freeze-dried LAB cells and 990 µL of 0.05 M sodium acetate buffer (pH 5.0) was added into the tube. After that, the samples were vortexed for 20 s and rotated at 175 rpm on a thermostatically controlled shaker (Ping-Pong 74582, Fisher Bioblock Scientific, IIIkirch, France) (see Appendix C) at 37 °C for 5 min. Then, 10 µL of ZEA working standard solution (1, 5, 20, 50 and 75 µg/mL) was added to each tube. The final incubation volumn was 1 mL. The suspensions were mixed and incubated on a thermostatically controlled shaker at 37 °C for 15 min at 175 rpm. Subsequently, the suspensions were terminated by centrifugation (Refrigerated Microfuge SIGMA 1-15k, UK) at 9,200 × *g*, at 20 °C for 10 min (Hadjeba-Medjdoub et al, 2009). A control treatment without addition of working standard ZEA solution was included. All experiments were carried out in duplicates.

3.2.5.5 HPLC analysis to determine ZEA binding by *Lb. pentosus* strains

HPLC method was performed according to the protocol of Dall'Asta et al. (2004). HPLC-system (ICS, Bruges, France) consisted with a 20 µL injector loop, a high pressure pump (L-6200A) and an auto sampler (AS-2000A). A C-18 spherisorb column (Prontosil 120-3-C18, 25 cm × 0.4 cm), and a fluorescence detector (Shimadzu Fluorescence Detector RF-10AXK, Japan) was run in a temperature controlled room (25 °C). The mobile phases were gradient condition as described by Faucet-Marquis et al. (2006). Briefly, using the following gradient: solvent A; MeOH/Acetonitile (ACN)/6.5 mM ammonium formate (200/200/600) adjusted to pH 3.5 with formic acid. Solvent B; MeOH/ACN/6.5 mM ammonium formate (350/350/300) adjusted to pH 3.5 with formic acid. Program: T_0 100 % A; T_{25} 30 % A; T_{30} 30 % A; T_{45} 0 % A; T_{55} 0 % A; T_{58} 100 % A at a flow rate of 0.5 mL/min. The spectrofluorimetric detection was 275 nm for ZEA excitation and 450 nm for emission. The calibration curve was obtained by analyzing the peak area of ZEA standard solutions of concentrations in the range between 0.1 to 10 µg/mL. All experiments were carried out in duplicate, and the values are given as mean ± standard deviation.

3.2.6 Adhesion capacity

To study the adhesion capacity of *Lb. pentosus* strains to human intestine cells, *In vitro* cell surface hydrophobicity was tested in all bacteria by using bacterial adherence to hydrocarbon assay modified from the methods of Schillinger et al. (2005) and Klayraung et al. (2008). Briefly, 8 strains of *Lb. pentosus* were grown in MRS broth at 37 °C under anaerobic conditions (5 % CO_2 incubator) about 18-24 h. Cells were harvested by centrifugation at 9,000 rpm, 4 ^{o}C for 10 min, washed twice and resuspended in 0.85 % NaCl. Cell concentrations were adjusted to reach the turbidity at 4.0 McFarland Units (turbidity 1.0 at; OD550) as estimated by Densimat (bioMérieux, Marcy l'Etoile, France). The cell suspension was measured for OD at 600 nm (A_0) with UV-Vis spectrophotometer microplate reader (SPECTROstar Nano). A portion of 0.6 mL of n-hexadecane; Cetane (Sigma-Aldrich, USA) was added to 3 mL of bacterial suspension into a clean test tube with screw cap. The mixture was blended using a vortex mixer at 120 rounds per sec for 10 min. The aqueous and organic phases were allowed to separate at room temperature for 30 min. The aqueous phase in lower layer was carefully removed and OD of the aqueous phase (A_{30}) was measured. Hydrophobicity was calculated from three replicates as OD of the initial cells suspension decreasing due to cells partitioning into a hydrocarbon layer. Therefore, high percentage of cell surface hydrophobicity (% H) shows the strain has adhesion ability and the % H was calculated as follow;

$$\% H = [1-(A_{30} / A_0)] \times 100$$

When; A_0 = OD of cells suspension at 0 min

A_{30} = OD of the aqueous phase at 30 min

Part II Use of probiotic *Lb. pentosus* as starter culture for probiotic soya beverage production.

The selection of three *Lb. pentosus* VM095, VM096, and YM122 strains with health-promoting properties; such as utilisation of sugar as carbon-source in modified MRS media, SCFAs production, cell surface hydrophobicity, cholesterol

reduction and ZEA binding (from Part I) were prepared as starter culture for soybean milk fermentation in this study.

3.2.7 Soya milk fermentation

3.2.7.1 Preparation of soya milk

The soya milk preparation was modified from Carrão-Panizzi et al. (1999) and Sirilun (2012). Dehull soybean seeds (Rai Tip brand) purchased from Big C supermarket, Mahasarakham, Thailand, were washed twice and soaked in distilled water with a weight ratio of 1: 6 w/v for seeds and water, at room temperature overnight. After soaking, water was discarded and the soybean seeds were placed directly into boiling water containing 0.25% $NaHCO_3$, for 3 min. The water was discarded again and the soybean seeds were washed with tap water until final at pH of 6.8-7.2 was reached. The soybean seeds were ground in electric juice blender for 3 min in hot distilled water at 80 ºC using a weight ratio of 1:5 w/v for seeds and water. The blended solution of soybeans was filtered through double-layered cheese cloth and was then passed through the sieve 100 mesh. A filtrated solution was recognized as soya milk. The soya milk was boiled for 3 min and the volumn 50 mL of soya milk was then filled into Duran bottles size 150 mL (for inoculums preparation) and 200 mL filled into Duran bottles size 500 mL (for soya milk fermentation), and sterilized by autoclaving at 121ºC for 2 min (Itsaranuwat, 2003). The bottles were stored at 4 ºC before use.

3.2.7.2 Preparation of inoculum and fermentation

The strain cultures *Lb. pentosus* VM095, VM096, and YM122 were grown on MRS agar plate about 18-24 h. After that, one loop of cells of each strain was picked-off a plate and added to each bottle of 50 mL soybean milk prepared as mentioned above. The inoculated milks were incubated at 37ºC, 5% CO_2 incubator for 18 h. After first time inoculation, each culture was sub-cultured (5% v/v) into freshly 50 mL of soya milk twice. The activated *Lb. pentosus* strains after two successive transfers were used for the production of cultures for soya milk fermentation. The cultures were prepared by inoculating 5% (v/v) in 200 mL soybean milk and then incubated at 37ºC, 5% CO_2 incubator for 24 h. All fermentations were performed in triplicates. A control consisted of un-inoculated soya milk.

3.2.7.3 Determination of pH value and titratable acidity

Samples at 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24 h of

fermentation period were taken and determined for the pH value and titratable acidity. Changes in pH were recorded using the pH meter (Mettler Toledo, SevenMulti, Switzerland). The acid titration was determined according to the method adapted from Villaluenga and Gómez (2007). Briefly, 1 mL of the soybean milk was transferred in 10 mL of distilled water and titrated with 0.1 M NaOH using phenolphthalein as an indicator. The results were calculated as percentage of equivalent lactic acid (%LA) of fermented soya milk. All data were performed in triplicates and the values were averaged.

$$\%LA = (V_1(mL) \times 0.1(M) \times 90\ (g/mol)\ /V_2\ (mL))/100$$

When; V_1 = used volumn of 0.1 N NaOH for titration

V_2 = volumn of sample

Where the molecular weight of lactic acid is given as 90 g/ mol.

3.2.7.4 Enumeration of *Lb. pentosus* strains

Samples from each batch were taken at 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24 h to monitor cell growth. Enumerations of bacteria were determined by the pour plate technique with appropriate dilutions on selective MRS agar. The plates were anaerobically incubated at 37 °C for 48 h, after which all visible colonies were counted. Results were expressed as the number of log10 CFU/mL of sample. The experiment was done in triplicates.

3.2.7.5 Determination of sugar contents

The fermented soya milk from each batch was taken at 0, 6, 12, and 24 h fermentation period to determine the sugar contents. one mL of each sample in 1.5 mL microcentrifuge tube was centrifuged at 10,000 rpm for 10 min by using the refrigerator microcentrifuge and then filtered though a 0.22 μm pore size nylon membrane HPLC filter (Whatman, Japan). The experiment was repeated twice. The sugar determination was performed as described previously in the section 3.2.1.5.

3.2.7.6 Determination of SCFAs

The samples were prepared according to the procedure in the section 3.2.7.5. The SCFAs determination was carried out described previously in the section 3.2.1.6.

3.2.8 Preparation of soya beverage

Soya beverage were prepared from fermented soya milk as mentioned above in the section 3.2.6.1 and 3.2.7.2 by probiotic strains *Lb. pentosus* (VM095, VM096, YM122). Each batch, 300 mL of soya milk in Duran bottle (500 mL) was inoculated with a single strain 5 % (v/v) and incubated at the same condition as in the section 3.2.7.2. After incubation, each batch was transferred to steriled electric juice blender and 10% honey syrup (100% pure, Suan Jit-radda brand) was added. This was purchased at 7-eleven super store. The soya beverage was blended in electric juice blender for 5 min and was transferred into the sterilized Duran bottle (500 mL), kept at 4 ^{o}C in the refrigerator until sensory test. The sensory test results were compared between 3 batches of the soya beverage (SB095H, SB096H, and SB122H) with 3 batches of the fermented soya milk without honey syrup (SF095, SF096, and SF122) as controls. In all batches, 100 mL of culture in Duran bottle (250 mL) were studied for survival of probiotic strains during storage at 4 ^{o}C for 4 weeks.

3.2.8.1 Sensory evaluation

The sensory properties of probiotic soya beverage already prepared from the section 3.2.8 were evaluated by forty untrained panelists recruited from students of Department of Biotechnology, Mahasarakham University. The samples initially stored at 4 ^{o}C (refrigerator temperature) were removed from refrigeration approximately 1 h prior to evaluation and shaken well to distribute any sediments. Approximately 10 mL of each sample was served into 20 mL plastic cups and were coded with three-digit random numbers from a Random Table (Ott, 1988). Water was available for panelists to rinse their pallet between samples. The test comprising six sensory attributes namely appearance, color, odor, taste, mouth feel, and overall acceptance was given to each panelist. The final sensory evaluation was done by nine-point Hedonic scale to specify their preferences on the samples tested. The scale of satisfaction were as follows: like extremely = 9, like very much = 8, like moderately = 7, like slightly = 6, neither like nor dislike = 5, dislike slightly = 4, dislike moderately = 3, dislike very much = 2, dislike extremely = 1 (Lawless and Heymann, 1999 cited in Itsaranuwat, 2003)-(see Appendix B). The scores were analyzed statistically using Duncan's test for multi comparison of the means.

3.2.8.2 Survival of probiotic strains during storage periods

About 100 mL of each fermented soya milk (SF) and probiotic soya beverage (SB) were kept in 250 mL of steriled Duran bottles. All six batches were held at 4 ^{o}C in refrigerator for a period 4 weeks (28 days). During the storage time, samples were taken out at 7-day intervals (days 0, 7, 14, 21, and 28) to examine the survival of the probiotic strains and determine pH value. Total colony counts were performed using the pour plate technique and selective media (MRS agar) to determine pH changes and viable counts. Briefly, 1 mL of each sample was taken from each SF and SB. Serial tenfold dilution were prepared in a solution of 0.85 % NaCl (w/v) and suitable dilutions were placed on MRS agar which were subsequently plated (in duplicate) and incubated for 48 h at 37 °C, 5 % CO_2 incubator.

3.3 Statistical analysis

Collected data from the experiments were statistically analyzed by Analysis of Variance using a randomized completely designs with three replications. Experimental results are given as means plus the standard deviation of three parallel measurements. Analysis of variance were conducted to identify differences among means and Duncan's Multiple Range test (DMRT) was employed. All of the statistical analysis was conducted by the SPSS statistical. The level of significant difference was defined at $p<0.05$.