CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Sugar depleted dried longan (SDDL)

SDDL used in this study was obtained from Thongpoon Food Limited Partnership, Lamphun province. It was ground by 5 l stainless steel blender. Dried longan powder was sieved to proper size (<40 mesh) and stored at -20°C until used.

3.1.2 Microorganisms

Aspergillus niger TISTR 3089 and 3063, *A. foetidus* TISTR 3461, *Trichoderma reesei* TISTR 3080 and 3081, as well as *Sacharomyces cerevisiae* TISTR 5606 were purchased from Thailand Institute of Scientific and Technological Research (TISTR) culture collection. The fungi were stocked as spore suspensions and yeast was stocked as cells suspension with 10% glycerol.

3.1.2.1 Spore suspension preparation

Fungi were cultured on potato dextrose agar (PDA) in flat bottle at 30°C for 7 days. Spores were collected by addition of 50 ml 0.1% (v/v) Tween 80 to the culture surface. Bottle was shaken vigorously and spore suspension was retrieved. The number of spores was counted by using a haemacytometer. If spore number was lower than 5×10^7 spores/ml, this spore suspension was added in to new fungal culture to collect more spore concentration. If spore number was higher than 5×10^7 spores/ml, it will be diluted to 5×10^7 spores/ml using sterilized

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reverse osmosis (RO) water. One ml of spore suspension was collected in a microcentrifuge tube and stored at -20°C with 10% (v/v) glycerol.

3.1.2.2 Yeast cells suspension preparation

S. cerevisiae TISTR 5606 were cultured in a yeast medium broth (YMB) for 48 h. Afterward, cells density was adjusted to an absorbance at 600 nm of 20. An aliquot of 1 ml in microcentrifuge tube with 10% glycerol was stored at -20ºC.

3.1.3 Culture medium

PDA (Harrigan, 1998) was used for fungi cultivation (see Appendix A). YMB was used for yeast cultivation (see Appendix A).

3.1.4 Chemical reagents

List of Chemical Reagents	**Company**
Acetic acid, Cat. No. A8401	LAB-Scan, Poland
Acetone, Cat. No. A3501	LAB-Scan, Poland
Agar, Cat. No. 214530	BD, France
Carboxymethycellulose, Cat. No. C5013	Sigma, Germany
3,5-Dinitrosalicylic acid, Cat. No. 42260	Fluka, Germany
Ethanol, Cat. No. 1.00983.2500	Merck, Germany
Fructose, Cat. No. L96-500	Fisher Scientific, UK
D-Galacturonic acid Cat. No. 48280	Fluka, Germany
D-Glucose, Cat. No. A783	Ajax FineChem, Australia
Glycerol, Cat. No. 242	Ajax FineChem, Australia
Hydrochloric acid, Cat. No. A8601	LAB-Scan, Poland
Locust bean gum Cat. No. G0753	Sigma, Germany
Malt extract, Cat. No. 218630	Criterion, USA
Mannitol, Cat. No. 310	Ajax FineChem, Australia

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List of Chemical Reagents	Company
D-Mannose	Nakara, japan
Meat peptone, Cat. No. RM635	HiMedia, India
Pectin, Cat. No. 76282	Sigma, Germany
Potassium sodium tartrate, Cat. No. 416	Ajax FineChem, Australia
Potassium hydroxide, Cat. No. A405	Ajax FineChem, Australia
Sodium acetate trihydtrate, Cat. No. 456	Ajax FineChem, Australia
Soluble starch, Cat. No. 527	Ajax FineChem, Australia
Sodium chloride, Cat. No. AR1227	LAB-Scan, Poland
Sodium hydroxide, Cat. No. AR1171	LAB-Scan, Poland
Sucrose, Cat. No. A530	Ajax FineChem, Australia
Sulfuric acid, Cat. No. A8302	LAB-Scan, Poland
Tween 80	Union Science, Thailand
Xylan, Cat. No. X0627	Sigma, Germany
D-Xylose, Cat. No. 1.08689.0025	Merck, Germany
Yeast extract, Cat. No. C7341	Criterion, USA

3.1.5 Equipments

List of Equipments	Company
Analytical balance ARB120	OHAUS, USA
Analytical balance AR2140	OHAUS, USA
Autoclave model ACV-3167	IWAKI, JAPAN
Blender	
Centrifuge MIKRO22R	HETTICH, GERMANY
Centrifuge Z296A	HERMILE, GERMANY
Electric pressure steam sterilizer model 25X	WISCONSIN, USA
Eppendorf centrifuge 5415C	EPPENDORF, GERMANY
Fume hood	TOPLAB, THAILAND
Evaporator	EYELAB, JAPAN
Hot air oven	MEMMERT, GERMANY
Hot plate stirrer RHB1	IKA, JAPAN

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List of Equipments	Company
HPLC AMINEX HPX-87H	BIO-RAD, USA
Laminar air flow NH3A	UNITECH, INDIA
Magnetic stirrer AGE	VELP, ITALY
Microprocessor control incubator i250	ACCUPLUS, THAILAND
Microscope CH20	OLYMPUS, JAPAN
Moisture analyzer MX-50	A&D, JAPAN
pH meter PH510	THERMOSCIENTIFIC, USA
Selectpette pipette SBP 100-200	SELECT BIOPRODUCTS, USA
Selectpette pipette SBP 100-1000	SELECT BIOPRODUCTS, USA
Shaking incubator NB205	N-BIOTEK, KOREA
Sorvall SUPER T21	KENDRO, USA
Spectrophotometer GENESIS 10-S	THERMOSCIENTIFIC, USA
Vortex mixer VM-300	GEMMY, TAIWAN
Water bath YCW-04M	GEMMY, TAIWAN

3.2 Methods

3.2.1 Determination of SDDL composition

3.2.1.1 Cellulose and hemicelluloses determination

Cellulose and hemicellulose were measured by a modified method from TAPPI T203 om-88. The holocellulose in SDDL was firstly determined with acid chlorite method. Three grams of dried SDDL in 250 ml Erlenmeyer flask were mixed with 160 ml distilled water, 0.5 ml acetic acid and 1.5 g sodium chloride in a fume hood cupboard. The flask was heated on a hotplate at 70-80℃ for 1 h with regular shaking. After that, 0.5 ml acetic acid and 1.5 g sodium chloride were added. The addition was repeated at 2 h and 3 h. Then, it was immersed in an ice bath until the temperature mixture was lower than 10℃. The mixture was filtered through a paper filter (Whatman No.1), washed by cold water and acetone. It was dried at 105℃ for 12 h. Holocellulose can be calculated by an equation below:

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$$\%\ \text{Holocellulose in sample} = \frac{\text{Dried weight of treated sample} \times 100}{\text{weight of sample}}$$

Holocellulose from SDDL about 1.5 g was placed in 500 ml beaker. It was added by 75 ml of 17.5% (w/v) sodium hydroxide. The mixture was kept at 25ºC. After that, the pulp was stirred with the apparatus until it was completely dispersed. The left over on a stirrer was rinsed with 25 ml of 17.5% (w/v) sodium hydroxide, stirred with a glass rod and kept on a water bath at 25ºC for 30 min. Then, the mixture was added with 100 ml of water, stirred with a glass rod and performed in a water bath at 25ºC for 30 min. After that, the mixture was filtered by a paper filter and washed by water and 40 ml of 10% acetic acid. It was dried at 105ºC for 12 h. α-Cellulose and hemicelluloses in the sample can be calculated by an equation below:

$$\%\ \alpha\text{-Cellulose in holocellulose} = \frac{\text{Dried weight of treated sample} \times 100}{\text{weight of holocellulose}}$$

$$\%\ \alpha\text{-Cellulose in sample} = \frac{\%\ \alpha\text{-Cellulose in holocellulose} \times \%\ \text{holocellulose in sample}}{100}$$

% Hemicellulose in sample = % Holocellulose - % α-Cellulose

3.2.1.2 Lignin determination

Lignin content in sugar deplete dried longan was determined by a modified method from TAPPI T222 om-88. One gram of dried sample was placed into 100 ml beaker and immersed in an ice-water bath. Then, 15 ml of cold 72% (v/v) sulfuric acid was added slowly and continuously stirred. The beaker was covered with a watch glass and kept in a 20ºC water bath for 2 h. The mixture was stirred every 15 min. After that, the mixture was transferred to 1000 ml of Erlenmeyer flask with 400 ml of distilled water. Rinsing was followed with water to total volume of 575 ml. The mixture was autoclaved at 120ºC for 1 h and left standing at room temperature for 12 h. The mixture was filtered through a filter paper (Whatman No.1) and the sediment was washed with hot water. The solid was dried at 105ºC for 12 h. The amount of lignin in the sample can be calculated by an equation below:

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$$\% \text{ Lignin in sample} = \frac{\text{Dried weight of treated sample} \times 100}{\text{Weight of sample}}$$

3.2.1.3 Pectin determination

Pectin content in SDDL was measured by a modified method according to Kertez (1951). One gram of dried SDDL powder was mixed with 2 ml of 95% (v/v) ethanol. The mixture was boiled at 70ºC for 10 min and filtered through a four fold cheesecloth. The sediment was dissolved and immersed in 20 ml of 30% (v/v) ethanol for 30 min. The mixture was filtered and extracted again with 20 ml of 30% (v/v) ethanol. After that, the mixture was extracted with 20 mM hydrochloric acid with the ratio of 1:30 (Sample w/ hydrochloric acid v). Then, the mixture was boiled for 1 h and filtered immediately through a cheesecloth. The supernatant was collected and the sediment was repeatly extracted. The total supernatant was left standing to cool down temperature and used for ethanol precipitation. The solution was mixed with cold 95% (v/v) ethanol with the ratio of 40:45 (v/v). The reaction was done in cold condition. The mixture was slowly stirred with a stirrer rod until pectin gel was appeared. Then, stirring was stopped and the mixture was kept at 4ºC for 12 h. The mixture was filtered by a filter paper with a suction pump and washed by 80% (v/v) ethanol with the ratio of pectin per ethanol at 1:15 (v/v) for 2 times. After that, it was washed with 95% (v/v) ethanol. The solid was dried at 105ºC for 12 h. The amount of pectin the sample can be calculated by an equation below:

$$\% \text{ Pectin in sample} = \frac{\text{Dried weight of treated sample} \times 100}{\text{Weight of sample}}$$

3.2.1.4 Starch determination

Starch content in SDDL was determined using a modified method from Chow and Landhausser (2004). One gram of SDDL was extracted in 50 ml centrifuge tube with 5 ml of 80% (v/v) ethanol. The mixture was performed by boiling in a water bath for 10 min and the addition was repeated three times.

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After that, the mixture was centrifuged at 2097 g for 10 min and the residue was collected for starch analysis. The residue was heated with 2 ml of 0.1 M sodium hydroxide at 50ºC for 30 min and neutralized with 2.5 ml of 0.1 M acetic acid. After neutralizing, 10 ml of the amylase mixture, 2000 U/ml of α-amylase (Spezyme AA, Genencor) and 10 U/ml of glucoamylase (Optidex® L-400, Genencor) were added into the mixture. The mixture was incubated in a water bath at 50ºC for 48 h. After incubation, it was centrifuged at 2,935 g for 10 min. The supernatant was used to measure reducing sugar by DNS method. The SDDL blank was carried out as described above except that the 10 ml of mixture amylase was replaced by 10 ml of 0.05 M acetate buffer pH 5.0. The enzyme blank was prepared similarly to the sample reaction but without SDDL. The standard curve was prepared by using 0.1, 0.2, 0.3, 0.4, 0.5 g of soluble starch as substrate. The amount of total starch in the sample can be calculated by an equation in Appendix C.

3.2.2 Substrate preparation

Ground SDDL was soaked in RO-water and squeezed through cheesecloth. Moisture content of substrate was analyzed by moisture analyzer and adjusted to approximate 70% (w/w) by RO-water. The substrate was sterilized by autoclaving at 120ºC at 15 psi for 15 min.

3.2.3 Solid state fermentation

Solid state fermentation was carried out in a 250 ml Erlenmeyer flask containing sterilized 50 g of ground SDDL with 70% (v/v) moisture content as substrate. Ten fermentations were carried out per fungal strain. Each flask was inoculated with 1 ml inoculum of 5×10^7 spore/ml and incubated at 30°C for 10 days. Samples were taken everyday by taking one flask to measure enzyme activity (cellulase, xylanase, mannanase, pectinase and amylase) and reducing sugar. The experiments were done in triplicate. The fungal strain and harvesting time which gave highest reducing sugar content was selected for further studies.

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3.2.4 Sample extraction for enzyme and reducing sugar analysis

Culture was extracted with 50 ml of 0.05 M acetate buffer pH 5.0. The extracts were performed on a rotary shaker at 200 rpm at 20°C for 1 hr. After that, they were squeezed through cheesecloth. The extract was centrifuged at 4,227 g for 10 min and filtered through Whatman No.1 filter paper. The clear filtrate were stored at -20°C until enzyme activities and reducing sugar were analyzed.

3.2.5 Sample extraction for ethanol production

A. niger TISTR 3089 and *A. foetidus* TISTR 3461 were selected to produce reducing sugar for ethanol production by *S. cerevisiae* TISTR 5606. Substrate preparation and fermentation treatment were similar to 3.2.2 and 3.2.3. except that fermentations were carried out for 7 days. Reducing sugar extraction was similar to 3.2.4 except that sterilized RO-water was used instead of 0.05 M acetate buffer pH 5.0. The extract from each fungal culture was analyzed for reducing sugar. It was then concentrated by an evaporator until reducing sugar was a100 g/l. Final reducing sugar content of extract was determined by DNS method.

Extract from non-microbial treatment SDDL was carried out as in above except in the evaporation step where the evaporation volume was similarly to *A. foetidus* TISTR 3461 extract. After that, glucose was added to adjust glucose concentration to approximate 100 g/l.

3.2.6 Yeast inoculum preparation

To prepare seed inoculum, 2 ml of glycerol stock of *S. cerevisiae* TISTR 5606 was added into 18 ml of YMB and cultured at 30°C on rotary shaker at 200 rpm for 18 hr.

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3.2.7 Ethanol production

Ethanol production was carried out to test the effect of microbial strain treatment, pH adjustment and sterilization on ethanol production. Fifteen ml of overnight culture of seed inocula was inoculated into 135 ml of substrate in a 250 ml Erlenmeyer flask. Fermentation was carried out at 30ºC in static condition for 5 days. Sample (4 ml) of each experiment was taken every 12 hr. One ml of sample was used for cells concentration determination by spectrophotometric method. Three ml of sample was centrifuged at 18,870 g for 10 min. The culture supernatant was retrieved and filtered through 0.45 µm nylon filter. They were kept at -20ºC and used for ethanol and sugar analysis. Experiments were done in triplicate.

3.2.7.1 Control experiment of ethanol production

Controls for ethanol production consist of YMB with 100 g/l glucose, 100 g/l glucose solution and extract of non-microbial treatment SDDL with 100 g/l glucose. YMB consisted of glucose (100 g/l) was prepared as described in Appendix A except that glucose was added to 100 g/l final concentration. pH of the controls were adjusted to 5.0 with 10 M potassium hydroxide. They were sterilized by autoclaving. Ethanol production was carried out as described in 3.2.7. Each experiment was carried out in triplicate.

3.2.7.3 Comparison of different fungal strains extracts on ethanol production

Fermentation of SDDL extracts from *A. niger* TISTR 3089 and *A. foetidus* TISTR 3461 treatment were carried out in 250 ml erlenmeyer flasks containing 135 ml of the extract with pH adjustment to 5.0 using 10 M potassium hydroxide and sterilized by autoclaving at 120ºC at 15 psi for 15 min. Fermentation was carried out as in 3.2.7. Experiments were done in triplicate.

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3.2.7.4 Comparison of pH adjustment of the extract on ethanol production

To study the effect of pH adjustment, the initial pH of the extracts from *A. niger* TISTR 3089 and *A. foetidus* TISTR 3461 cultured were adjusted to pH 5.0 with 10 M potassium hydroxide and sterilized by autoclaving at 120ºC at 15 psi for 15 min. Then, the fermentation was carried out as in 3.2.7. The ethanol yield was compared with fermentation of extract without pH adjustment. Experiment was done in triplicate.

3.2.7.5 Comparison of sterilization of the extract by autoclaving on ethanol production

To study the effect of sterilization of the extracts from *A. niger* TISTR 3089 and *A. foetidus* TISTR 3461 cultured were adjusted to pH 5.0 with 10 M potassium hydroxide and sterilized by autoclaving at 120ºC at 15 psi for 15 min. Then, the fermentation was carried out as in 3.2.7. The ethanol yield was compared with control in absence of sterilization. Experiment was done in triplicate.

3.2.8 Analysis

3.2.8.1 Reducing sugar analysis

Reducing sugar in the extract was measured by DNS Method (Miller, 1959) using glucose as a standard (see Appendix B). The reaction was carried out by mixing 0.5 ml of the extract with 0.5 ml of dinitrosalicylic acid solution (DNS). After that, the mixture was boiled for 15 min. The absorbance was read by spectrophotometer at 540 nm and used to calculate reducing sugar concentration by using standard curve constructed with known concentration of glucose.

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3.2.8.2 Enzyme activities analysis

Determination of cellulase activity was adapted from the method of Mendels *et al*., (1976). The reaction mixture contained 0.25 ml of the enzyme extract and 0.25 ml of 1.0% (w/v) carboxymethylcellulose in 0.05 M sodium acetate buffer (pH 5.0). Xylanase, mannanase and pectinase activity was assayed using the modified method of JECFA (2006a and b). The reaction mixture contained 0.25 ml of the enzyme extract and 0.25 ml of substrates containing 1.0% (w/v) xylan, 1.0% (w/v) locust bean gum and 0.5% pectin in 0.05 M sodium acetate buffer (pH 5.0), respectively. Amylase activity was measured by modified method described by Rick and Stegbauer (1974). The reaction mixture contained 0.25 ml of the enzyme extract and 0.25 ml of 1.0% (w/v) of soluble starch. The enzyme blank was prepared by mixing 0.25 ml of the enzyme extract with 0.25 ml of 0.05 M sodium acetate buffer (pH 5.0). The substrates blank for cellulase, xylanase, mannanase, pectinase and amylase determination were prepared by mixing 0.25 ml of the substrates solution (carboxymethycellulose, xylan, locust bean gum, pectin and soluble starch, respectively) with 0.25 ml of 0.05 M sodium acetate buffer (pH 5.0). All reaction mixtures were incubated at 40ºC for 10 min. Then, the reaction was stopped by adding 0.5 ml of dinitrosalicylic acid solution (DNS) and boiled for 15 min. Enzymes activities were determined by quantifying the release of reducing sugar by the dinitrosalicylic acid (DNS) method (Miller, 1959) using glucose, xylose, mannose and galacturonic acid as standards, respectively. One unit (U) of enzyme activity was defined as the amount of enzyme producing 1 μmol of product per minute under the assay conditions. Enzymatic activities in the extracts recovered from the solid-state cultures were determined in U/g dry substrate (U/gds) (see Appendix E).

3.2.8.3 Determination of cells density of *S. cerevisiae* and DCW

Cells density of *S. cerevisiae* TSTR 5606 was measured at 600 nm at appropriate dilution. Dried cell weight was obtained by comparing the OD with standard curve constructed with OD 600 nm and dried cell weight. Standard curve was prepared by diluting of culture in RO-water at ratio of 10/0, 8/2, 6/4, 4/6 and

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2/8 ml. One ml of each dilution was taken to measured dry cell weight. It was centrifuged at 14000 rpm for 10 min and supernatant was removed. The pellet was washed twice with RO-water. Finally, It was dried at 105ºC for 12 hr. This experiment was done in triplicate. The OD 600 nm of remaining sample was measured at appropriate dilution (see Appendix G).

3.2.8.4 Sugars and ethanol analyses

Ethanol and sugars (sucrose, maltose, glucose, xylose, mannose, fructose and galacturonic acid) concentration were analyzed by high performance liquid chromatography (HPLC) using Aminex HPX-87H column. The mobile phase was 0.05 mM sulfuric acid pH 2.0 and the flow rate was 0.75 ml/min. Sucrose, glucose, xylose, mannose, galacturonic acid and ethanol were used as standard at various concentration *i.e.* 500, 1,000, 2,000, 3,000, 5,000 and 10,000 ppm. Mannitol was used as internal standard and its concentration in the sample was 2,000 ppm. Samples and standards were injected using the HPLC auto-injector and was run for 20 min.

Area under the curves were integrated by Agilent ChemStation program. The area from various concentration of standards were used to construct standard curves (see Appendix H). The area of each peak was compared with standard curve and internal standard concentration. If the internal standard concentration was lower or higher than 2000 ppm, the sample concentration will be adjusted corresponding to internal standard.

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3.2.8.5 Formulas for estimation of kinetic parameters

Specific growth rate (μ) during exponential growth was measured by an equation below:

$$\mu = \frac{\text{In}X_2 - \text{In}X_1}{T_2 - T_1}$$

T = Time

X_1 = Biomass concentration at time T_1

X_2 = Biomass concentration at time T_2

Glucose consumption rate (r_s) of each cultivation time in exponential growth was determined by an equation below:

$$r_s = \frac{S_1 - S_2}{T_2 - T_1}$$

T = Time

S_1 = Glucose concentration at time T_1

S_2 = Glucose concentration at time T_2

Specific rate of glucose consumption (q_s) during exponential growth was determined by an equation below:

$$q_s = \frac{1}{X}\left(\frac{S_1 - S_2}{T_2 - T_1}\right)$$

T = Time

X = Biomass concentration at time T_2

S_1 = Glucose concentration at time T_1

S_2 = Glucose concentration at time T_2

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Ethanol production rate (r_p) of each cultivation time in exponential growth was calculated by an equation below:

$$r_p = \frac{P_2 - P_1}{T_2 - T_1}$$

T = Time

P_1 = Ethanol concentration at time T_1

P_2 = Ethanol concentration at time T_2

Specific rate of ethanol production (q_p) during exponential growth was determined by an equation below:

$$q_p = \frac{1}{X}\left(\frac{P_2 - P_1}{T_2 - T_1}\right)$$

T = Time

X = Biomass concentration at time T_2

P_1 = Ethanol concentration at time T_1

P_2 = Ethanol concentration at time T_2

The ethanol production yield ($Y_{p/s}$) of each experiment was determined by equation below:

$$Y_{p/s} = \frac{P_2 - P_1}{S_1 - S_2}$$

P_1 = Ethanol concentration at time T_1

P_2 = Ethanol concentration at time T_2

S_1 = Glucose concentration at time T_1

S_2 = Glucose concentration at time T_2

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