CHAPTER III

MATERIALS AND METHODS

MATERIALS

3.1 *Panicum maximum* cv TD 53 (purple guinea grass)

Pueple guinea grass (Figure 15) was collected from Department of livestock, Ministry of Agricultural, Pakchong district, Nakhon Ratchasima province, Thailand.

Figure 15 Purple guinea grass

3.2 Equipments

3.2.1 Analytical balance : Mettler Toledo, model AG 285, Switzerland.

3.2.2 Autoclaves : Tomy, model SS- 325 and Hirayama, model HV-28, Japan.

3.2.3 Biochemistry Analyzer : YSI 2700 SELECT, USA.

3.2.4 Fermentor : B.E. Marubishi, model 10L, Japan.

3.2.5 Gas chromatography : Shimadzu, model 7AG, Japan.

3.2.6 Hot plate : E.G.O., model RK18715, Poland.

3.2.7 High Performance Liquid Chromatography (HPLC) : Agilent Technology Ltd., model 1100 series, USA.

3.2.8 Laminar flow : Lab service Ltd., Clean model V6, Thailand.

3.2.9 Incubator shaker : New Brunswick Scientific, model Innova 2300, USA.

3.2.10 pH meter : Mettler Toledo, model SevenEasy, China.

3.2.11 Precision balance : Mettler Toledo, model PB 3002, Switzerland.

3.2.12 Refrigerated centrifugation : Sorvall, model Biofuge stratos, Germany. (Rotor #3334, Heraeus, USA).

3.2.13 Test seive : 20 mesh, Retesch, Germany

3.2.14 Test seive : 40 mesh, Retesch, Germany

3.2.15 Spectrophotometer : Spectronic Instruments, model Spectronic 20, USA.

3.2.16 Water bath : Tolabo, model TW20, Germany.

3.2.17 Water bath shaker : Amerex Instrument Inc., model Gyromax 939XL, USA. and GFL, model 1086, Germany.

3.3 Chemicals

Chemicals (Analytical grade)	Company
Agar	Becton
Ammonium heptamolybdate tetrahydrate ($(NH_4)_6Mo_7O_{24}.4H_2O$)	Merck
Ammonium phostphate, dibasic ($(NH_4)_2HPO_4$)	Merck
Ammonium Sulphate ($(NH_4)_2SO_4$)	Merck
Bacto-peptone	Becton
Calcium hydroxide ($Ca(OH)_2$)	Merck
Citric acid ($HOC(COOH)(CH_2COOH).2H_2O$)	Ajax Finechem
Copper (II) sulfate ($CuSO_4.5H_2O$)	Merck
Disodium hydrogen arsenate ($Na_2HAsO_4.7H_2O$)	Merck
di-Sodium hydrogen phosphate dodecahydrate ($Na_2HPO_4.12H_2O$)	Merck
Ethanol (C_2H_5OH)	Merck
Glucose ($C_6H_{12}O_6$)	Sigma
Hydrochloric acid (HCl)	Sigma
Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$)	Merck
Potassium dihydrogen phosphate (KH_2PO_4)	Merck

Potassium sodium tartate ($C_4H_4KNaO_6.4H_2O$)	Merck
Sodium hydroxide (NaOH)	Merck
Sodium sulfate (Na_2SO_4)	Merck
Sulfuric acid (H_2SO_4)	Merck
Trisodium citrate dihydrate ($Na_3C_6H_5O_7.2H_2O$)	Merck
Yeast extract	Difco

3.4 Microorganism

3.4.1 *Saccharomyces cerevisiae* TISTR 5596 was obtained from Thailand Institute of Scientific Technological Research (TISTR).

METHODS

3.5 Experiments

Flow diagram of experiments is shown in Figure 16

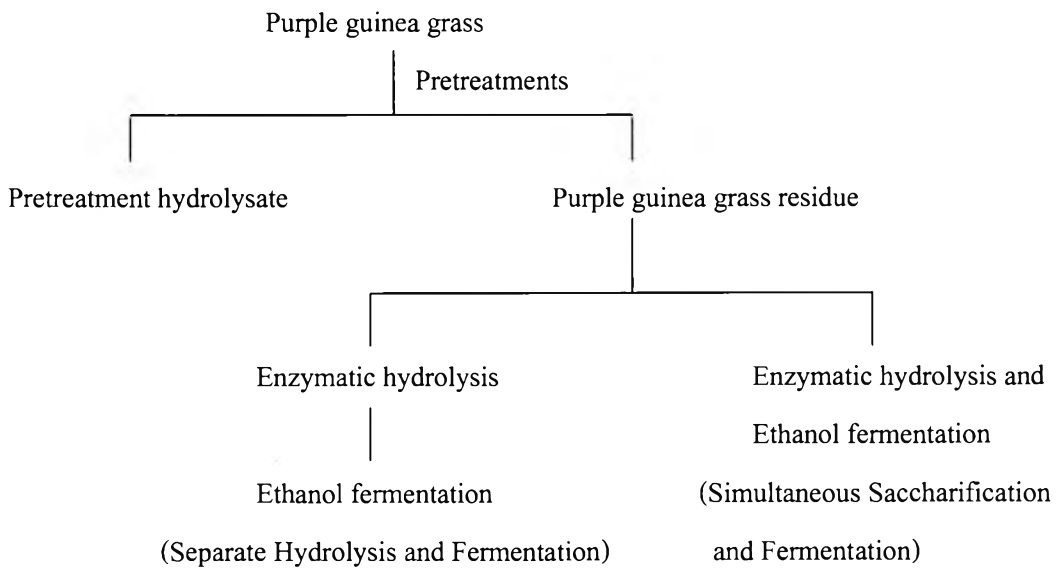

Figure 16 Flow diagrams of experiments

3.6 Microorganism

3.6.1 Maintenance of microorganism

Saccharomyces cerevisiae TISTR 5596 grown on yeast peptone dextrose (YPD) agar slant at 30 °C for 48 hours was kept at 4 °C and subcultured every week.

3.6.2 Cultivation of microorganism

A single colony of *S. cerevisiae* TISTR 5596 grown on yeast peptone dextrose agar at 30°C for 24 hours was inoculated to YPD broth and incubated at 30 °C (200 rpm) for 24 hours. The culture then transferred at 1% (v/v) to the same medium and incubated at the same condition was used as inoculum.

3.7 Raw material preparation

Purple guinea grass (*Panicum maximum* CV TD 53) collected from Department of livestock, Ministry of Agricultural, Pakchong district, Nakhon Ratchasima province,Thailand was oven-dried at 70-80 °C , cut and Hammer milled to 20-40 mesh particles. The dried purple guinea grass particle was kept in desiccator for preserving. A 500 g of the dried purple guinea grass particle was used for chemical composition analysis (Cellulose, Hemicellulose, Lignin) using method described by Technical Association of Pulp and Paper Industry at Department of Science Service, Ministry of Science and Technology.

3.8 Pretreatment of purple guinea grass

3.8.1 Effect of sulfuric acid concentration on cellulose susceptibility

Purple guinea grass particles (3% w/v , dry weight basis (DS)) was suspended in 1.0-3.5 %(w/v) sulfuric acid and autoclaved at 121 °C , 15 lb/inc^2 for 30 min. The pretreated purple guinea grass slurry was press-filtered through 20-40 mesh stainless steel sieves then the filtrate was recentrifuged at 4 °C, 11,292xg for 10 min. The pretreated purple guinea grass residual particle was resuspended back into pretreatment hydrolysate and pH adjusted to 6.0. This step was done to ascertain that purple guinea grass residual particle was treated with sulfuric acid by the same procedure as with calcium hydroxide. Cellulase GC 220 (6200 units/g DS of substrate or 23 units of β-glucosidase/g DS of substrate, Genencor International, Inc., USA) was added at a dose of 69 units/g DS of substrate or 0.26 units of β-glucosidase /g DS of substrate

and incubated with shaking (120 rpm) at 40 °C for 72 hours. Then, the slurry was press-filtered and the filtrate was recentrifuged at 4 °C, 11,292xg for 10 min. Reducing sugar liberated in the supernatant was analyzed by Somogyi- Nelson method (Somogyi, 1952).

3.8.2 Effect of calcium hydroxide or lime concentration on cellulose susceptibility

Purple guinea grass particle (3% w/v, dry weight basis (DS)) was suspended in 1.5-3.0 %(w/v) lime and autoclaved at 121 °C , 15 lb/inc^2 for 30 min. The pretreated purple guinea grass slurry was press-filtered through 20-40 mesh stainless steel sieves then filtrate was recentrifuged at 4 °C, 11,292xg for 10 min to separate lime powder. The pretreated purple guinea grass residual particle was resuspended back into pretreatment hydrolysate and pH adjusted to 6.0. Cellulase GC 220 (6200 units/g DS of substrate or 23 units of β-glucosidase /g DS of substrate, Genencor International, Inc., USA) was added at a dose of 69 units/g DS of substrate or 0.26 units of β-glucosidase /g DS of substrate and incubated with shaking (120 rpm) at 40 °C for 72 hours. Then, the slurry was press-filtered and the filtrate was recentrifuged at 4 °C, 11,292xg for 10 min. Reducing sugar liberated in the supernatant was analyzed by Somogyi-Nelson method.

3.8.3 Effect of substrate loading on cellulose susceptibility

An amount of purple guinea grass particle suspended in sulfuric acid or lime solution was varied (3, 6 and 8 %w/v, DS). Concentration of sulfuric acid and ratio of substrate/g lime used was results of 3.8.1 and 3.8.2, respectively. The slurry was autoclaved at 121 °C, 15 lb/inc^2 for 30 min, press-filtered, centrifuged. The pretreated purple guinea grass residual particle was then resuspended back into pretreatment hydrolysate, pH adjusted to 6.0 and hydrolyzed by Cellulase GC220 (see 3.8.1 and 3.8.2). After press-filtration and recentrifugation of the filtrate, reducing sugar liberated in the supernatant was analyzed. Optimal substrate loading was an amount of the purple guinea grass pretreated which released maximum reducing sugar in the cellulolytic hydrolysis.

3.8.4 Effect of autoclaving period on cellulose susceptibility

To maximize reducing sugar released, the purple guinea grass particle was pretreated at optimal substrate loading (result of 3.8.3) with sulfuric acid or lime solution as described in 3.8.3 but autoclaving period was varied; 0, 5, 10, 15, 30, 45 and 60 min. Reducing sugar liberated in the supernatant was analyzed.

3.9 Analysis of sugars and byproducts in pretreatment hydrolysate

The pretreated purple guinea grass slurry was press-filtered and centrifuged as described in 3.8.1 and 3.8.2. Supernatant obtained was pH adjusted to 7.0 and analyzed for reducing sugar by Somogyi-Nelson method; glucose, xylose and pretreatment byproducts by High Performance Liquid Chromatography (HPLC).

3.10 Cellulase hydrolysis

Cellulase hydrolysis of purple guinea grass pretreated at optimal condition (results of 3.8.1-3.8.4) was optimized by resuspending the pretreated purple guinea grass in 100 mM of sodium citrate buffer (pH 5.0). Accellerase^{TM}1000 (45 FPU/g DS of substrate or 400 units of β-glucosidase /g DS of substrate) at 53, 106, 159, 212 FPU/g DS of substrate (471, 942, 1413, 1884 units of β-glucosidase /g DS of substrate) was added and incubated with shaking at 120 rpm, 50 °C for 6, 12 or 18 hours. The reaction mixture was centrifuged at 4 °C, 11,292xg (10 min). Glucose in the supernatant was analyzed by glucose analyzer.

3.11 Ethanol production

3.11.1 Ethanol production by Separate Hydrolysis and Fermentation (SHF) method

Single colony of *S. cerevisiae* grown on YPD agar at 30 °C for 24 hours was inoculated into YPD broth (50ml) in 250 ml armed flask and incubated at 30 °C (200 rpm) for 24 hours. The culture transferred to the same medium at 1% (v/v) and incubated at the same condition was used as inoculum.

Hydrolysate obtained after cellulase hydrolysis which pH was adjusted to 4.5 supplemented with 0.2%(w/v)$(NH_4)_2SO_4$ and sterilized by autoclaving at 110 °C, 10 lb/inc^2 for 10 min was used as fermentation medium. *S. cerevisiae* inoculum was inoculated at 10%(v/v) into the fermentation medium, incubated at 30 °C, oxygen limit condition for 72 hours. Oxygen limit condition was performed by cultivating 40 ml culture in 50 ml flask without shaking, and the inoculated flask was covered by cotton plug, tightly wrapped with parafilm. After centrifugation, supernatant obtained was analyzed for ethanol by gas chromatography.

3.11.1.1 Effect of incubation period on ethanol production

S. cerevisiae inoculum was inoculated into the fermentation medium and incubated at 30 °C, oxygen limit condition for 72 hours. Every 12 hours, the culture was centrifuged at 4 °C, 7227xg (10 min). Supernatant obtained was analyzed for ethanol by gas chromatography.

3.11.1.2 Effect of $(NH_4)_2SO_4$ supplementation on ethanol production

Hydrolysate obtained after hydrolysis by Accellerase™1000 hydrolysate (result of 3.10) which pH was adjusted to 4.5 was supplemented by various concentration of $(NH_4)_2SO_4$ (0, 0.2, 0.4, 0.6 %w/v) and used as fermentation medium. *S. cerevisiae* inoculum was inoculated into the fermentation medium at 10% (v/v), incubated at 30 °C, oxygen limit condition. Incubation period was a result of 3.11.1.1. After centrifugation, supernatant obtained was analyzed for ethanol produced by gas chromatography.

3.11.1.3 Effect of nutrient supplementation on ethanol production

Hydrolysate obtained after hydrolysis by Accellerase™1000 hydrolysate (result of 3.10), which pH was adjusted to 4.5 and contained optimal concentration of $(NH_4)_2SO_4$ (result of 3.11.1.2) was supplemented with 3% (w/v) yeast extract and 0.025% (w/v) $(NH_4)_2HPO_4$ and used as fermentation medium. *S. cerevisiae* inoculum was inoculated into the fermentation medium at 10% (v/v) and incubated at 30 °C, oxygen limit condition for 72 hours. Ethanol produced in supernatant obtained after centrifugation of the culture at 4 °C, 7227xg (10 min) was analyzed by gas chromatography.

3.11.1.4 Effect of inoculum medium on ethanol production

The inoculum prepared as described in 3.6.2, was centrifuged at 4 °C, 7227xg for 10 min, cell precipitate was re-suspended in YPD with and without glucose at its original volumn and used as inoculum. Ethanol fermentation was performed as described in 3.11.1.3, nutrients supplementation was a result of 3.11.1.3.

3.11.2 Ethanol production by Simultaneous Saccharification and Fermentation (SSF) method

After pretreatment at optimal condition, the pretreated purple guinea grass residue was washed with distilled water and suspended in 100 mM sodium citrate buffer pH 5.0 at its original volume was simultaneous hydrolyzed by Accellerase™ 1000 (45 FPU/g DS of

substrate or 400 units of β-glucosidase /g DS of substrate at 50 °C for 6 hours) and fermented by *S. cerevisiae* inoculum (10% (v/v) at 30 °C, oxygen limited condition for 120 hours. After press-filtration and centrifugation, supernatant obtained was analyzed for ethanol by gas chromatography.

3.11.2.1 Effect of temperature on ethanol production

An effect of incubation temperature on the simultaneous saccharification and fermentation was performed by varying incubation temperature at 25, 30, 35, 40 and 50 °C. Ethanol produced in culture supernatant was analyzed by gas chromatography.

3.11.2.2 Effect of reaction pH on ethanol production

An effect of reaction pH on the simultaneous saccharification and fermentation was performed by resuspending the washed pretreated purple guinea grass residue in 100mM of sodium citrate buffer at various pH (4.5, 5.0 and 5.5) at its original volume. Then the pretreated purple guinea grass slurry was simultaneous saccharified and ethanol fermented as described above at optimal temperature (result of 3.11.2.1). Ethanol produced in culture supernatant was analyzed by gas chromatography.

3.11.2.3 Effect of incubation period on ethanol production

The pretreated purple guinea grass residue was simultaneous saccharified and ethanol fermented at optimal temperature and pH (result of 3.11.2) for 120 hours. Ethanol produced in culture supernatant was analyzed every 24 hours by gas chromatography.

3.11.2.4 Effect of nutrient supplementation on ethanol production

Yeast extract (3%w/v) and $(NH_4)_2HPO_4$ (0.025%w/v) were added into the suspension of washed pretreated purple guinea grass residue in sodium citrate buffer, and the pretreated purple guinea grass residue was simultaneous saccharified and ethanol fermented as described above (result of 3.11.2.3). Ethanol produced in culture supernatant was analyzed every 24 hours by gas chromatography.

3.11.2.5 Effect of inoculum medium on ethanol production

The inoculum prepared as described in 3.6.2 was centrifuged at 4 °C, 7227xg for 10 min, cell precipitate was re-suspended in YPD with and without glucose at its

original volumn and used as inoculum. Ethanol fermentation was performed as described in 3.11.2.4, nutrients supplementation was a result of 3.11.2.5.

3.12 Scale up for ethanol fermentation

The hydrolysate obtained after cellulase hydrolysis was fermented in an *in situ* sterilizable 5L fermentor (B.E. Marubishi, model 10L, Japan) (Figure 17). The cellulose hydrolysate (3.0 L) contained 12.0 g/l of glucose was used as fermented medium without any supplementation. Cell of *S. cerevisiae* inoculum (3.6.2) harvested by centrifugation, resuspended in YPD without glucose was used as an inoculum. The agitation of 100 rpm, temperature 30 °C, pH 4.5 ±0.4 and without aeration were maintained throughout the process. The pH was controlled at 4.5±0.4 by using 4 N HCL and 5 N NaOH, when required. A 10% (w/v) antifoaming agent solution was used for controlling foam when required. Samples were withdrawn and centrifuged at 4 °C, 7227xg for 10 min. Cell free supernatant obtained was analyzed for ethanol by gas chromatography.

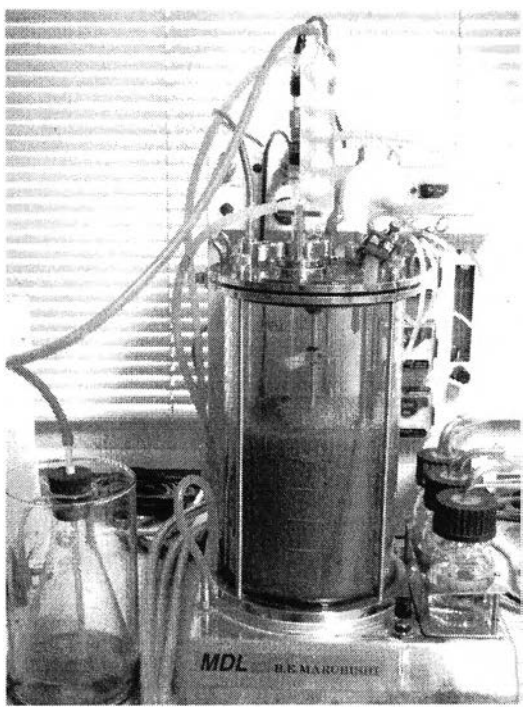

Figure 17 Ethanol fermentation by separate hydrolysis and fermentation method in 5L fermentor (B.E. Marubishi, model 10L, Japan).

3.13 Analytical procedure

3.13.1 Analysis of ethanol by gas chromatography

Sample (100 µl) was injected to Gas chromatography; GC (Shimadzu, model 7AG, Japan) using Porapak Q column, 3.3cm x 2m (Marubishi, Japan) equipped with flame ionization detector (Shimadzu, model 7AG, Japan) using nitrogen gas as carrier gas at flow rate of 50 ml/min .Injection and column temperatures were 240°C and 190°C, respectively. Retention time of ethanol was 2 min. Triplicate measurements were performed for each samples.

3.13.2 Analysis of sugar and pretreatment byproduct by High Performance Liquid Chromatography

Xylose and glucose were analyzed by High Performance Liquid Chromatography; HPLC (Agilent Technology Ltd., model 1100 series, USA) containing quaternary pump, online degassor, autoinjector, column thermostat, refractive index detector, and Chemstation software. HPLC condition was as followed.

Column	Aminex HPX-87P, 300mmx7.8mm (catalog#125-0098) (BioRad, USA)
Flow rate	0.6 ml/min
Mobile phase	Deionize water
Column temperature	80 °C
Sample	20 µl

Pretreatment byproduct was analyzed by High Performance Liquid Chromatography; HPLC (Agilent Technology Ltd., model 1100 series, USA) containing quaternary pump, online degassor, autoinjector, column thermostat, diode array detector and Chemstation software. HPLC condition was as followed.

Column	Pinacle II C18 5µm 250x4.6mm (BioRad, USA)
Flow rate	1 ml/min
Mobile phase	A: 1% acetic acid
	B: MeOH
Column temperature	40 °C
Sample	5 µl

3.13.3 Analysis of reducing sugar (Somogyi, 1952)

The proper dilution of sample (1 ml) in a test tube was mixed with alkaline copper solution (1 ml), placed in boiling water for 15 minutes, then immediately cool in ice water. Next, Nelson solution (1ml) was added, mixed and incubated at room temperature for 30 min. The reaction mixture was diluted with addition of 5 ml of distilled water, and then measured an absorbance at 520 nm (A520 nm). Various glucose concentration plotted VS their A520 nm were used as standard curve to determine glucose concentration of sample. Distilled water was used as a blank control.

3.13.4 Analysis of glucose by glucose analyzer

Glucose was analyzed by biochemistry glucose analyzer (GC YSI 2700 SELECT, USA). Sample (100 μl) was injected to the biochemistry glucose analyzer using glucose (dextrose) membrane (YSI 2365, USA). Triplicate measurements were performed for each sample.