CHAPTER 3

Materials and Methods

3.1 Materials

3.1.1 Equipment

Name of Equipment	Suppliers
Analytical Balance (PA214)	Ohaus, USA
Autoclave (ACV-3167)	Hirayama, Japan
Auto Fat Extraction System (Soxtec™ 2050)	Foss, Denmark
Automated Distillation Units for Protein Analysis (Kjeltec™ 8100)	Foss, Denmark
Automate DNA Sequencer (3100-Avant Genetic Analyzer)	ABI Hitachi, USA
Autosystem Gas Chromatography (GC-2010)	Shimadzu, Japan
Autosystem Gas Chromatography (GC-7890A)	Agilent Technology, USA
Blender (HR2001)	Philips, China
Centrifuge (Z326K)	Hermle, Germany
Erlenmeyer Flask (250 mL)	Duran, Germany
Freezer (SF-C997 (GYN))	Sanyo, Thailand
Glass Beads (size 3 mm)	Superior, Germany
High Performance Liquid Chromatography (HPLC) (LC-10AT vp)	Shimadzu, Japan
Hot Air Oven (EN 400)	Nüve, Turkey
HP-INNOWax Column (0.25 µm, 30 m × 0.25 mm)	Agilent Technology, USA
HPLC Column C18 (5 µm, 250 m × 4.6 mm)	Restek, France
Incubator Shaker (Lab-Therm)	Kühner, Switzerland
Kjeldahl Digestion Units (DK6)	Velp Scientifica, Thailand
Mass Spectrometer (MSD 5975C)	Agilent Technology, USA
Micropipette (10 µL)	Rainin, USA

Micropipette (200 µL)	Rainin, USA
Micropipette (1,000 µL)	Rainin, USA
Micropipette (5,000 µL)	Rainin, USA
Nylon Membrane Filter (13mm, 0.2 µm)	FiltrEX, USA
pH Meter (C830)	Consort, Belgium
Rotary Evaporator (Rotavapor® R-3)	Büchi, Switzerland
Screw Cap Tube (25×150 mm)	Pyrex, Germamy
Spectrophotometer (UV-VIS) (Genesys 10-s)	Thermo Fisher Scientific, UK
Stirred Tank Bioreactor	BEM Marubishi, Japan
Tabletop Electron Microscope (TM300)	Hitachi, Japan
Ultrasonic Bath (Elmasonic S 60H)	Elma Schmidbauer, Germany
Vortex Mixer (G560E)	Scientific industries Inc., USA
Water Bath (NB 20)	Nüve, Turkey

3.1.2 Chemicals

Name of chemicals	**Suppliers**
Acetic acid (CH_3COOH)	RCI Labscan, Thailand
Acetone (C_3H_6O)	Merck, Germany
Acetonitrile (C_2H_3N) (HPLC grade)	RCI Labscan, Thailand
Ammonium sulphate ($(NH_4)_2SO_4$)	RCI Labscan, Thailand
α-Amylase (SPEZYME FRED)	Genencor, USA
Amyloglucosidase or AMG (DISTILLASE VPH)	Genencor, USA
Ascorbic acid ($C_6H_8O_6$)	Fluka, Switzerland
Boric acid (H_3BO_3)	Merck, Germany
Bromocresol green ($C_{21}H_{14}Br_4O_5S$)	Sigma, USA
β-Carotene ($C_{40}H_{56}$)	Sigma, USA
Chloramphenicol ($C_{11}H_{12}Cl_2N_2O_5$)	Sigma, USA
Chloroform ($CHCl_3$)	RCI Labscan, Thailand
Citric acid monohydrate ($C_6H_8O_7.H_2O$)	RCI Labscan, Thailand
Deionized water	RCI Labscan, Thailand
Dichloromethane (CH_2Cl_2) (HPLC grade)	Thermo Fisher Scientific, UK
3,5-Dinitrosalicylic acid ($C_7H_4N_2O_7$)	Sigma, USA

Ethanol 95% (CH_3CH_2OH)	Apex Alco Co., LTD, Thailand
Ethyl acetate ($C_4H_8O_2$)	RCI Labscan, Thailand
Glucose ($C_6H_{12}O_6$)	Ajax Firechem, Australia
Glycerol ($C_3H_8O_3$)	Merck, Germany
Hexane (C_6H_{14})	RCI Labscan, Thailand
Hydrochloric acid (HCl)	RCI Labscan, Thailand
Lauric acid (C12:0)	Nacalai Tesque, Japan
Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$)	Ajax Finechem, Australia
Malt extract	Difco, France
Manganese sulphate monohydrate ($MnSO_4.H_2O$)	Ajax Finechem, Australia
Methanol (CH_3OH)	RCI Labscan, Thailand
Methyl hexanoate ($C_7H_{14}O_2$)	Sigma, USA
Methyl red ($C_{15}H_{15}N_3O_2$)	Sigma, USA
Myristic acid (C14:0)	Nacalai Tesque, Japan
p-Nitrophenyl-α-D-glucopyranoside ($C_{12}H_{15}NO_8$)	Sigma, USA
Oleic acid (C18:1)	QRëC, New Zealand
Olive oil	Srichand United Dispensary, Thailand
Palmitic acid (C14:0)	Nacalai Tesque, Japan
Phenol (C_6H_6O)	Polskie Odczynniki Chemiczne S.A., Poland
Petroleum ether (40–60°C) (C_6H_{14})	RCI Labscan, Thailand
Phosphoric acid (H_3PO_4)	Merck, Germany
Potassium di-hydrogen phosphate (KH_2PO_4)	Loba Chemie, India
di-Potassium hydrogen phosphate (K_2HPO_4)	RCI Labscan, Thailand
Potassium hydroxide (KOH)	RCI Labscan, Thailand
Potassium sodium tartrate ($C_4H_4KNaO_6$)	RCI Labscan, Thailand
Kjeldahl catalyst	Oskon, Thailand
Sodium chloride (NaCl)	RCI Labscan, Thailand
tri-Sodium citrate ($Na_3C_6H_5O_7$)	RCI Labscan, Thailand
Sodium hydroxide (NaOH)	RCI Labscan, Thailand

Sodium sulphate anhydrous (Na_2SO_4)	RCI Labscan, Thailand
Soluble starch $(C_6H_{10}O_5)_n$	SRL, India
Sudan black B powder ($C_{29}H_{24}N_6$)	Sigma, USA
Sulphuric acid 98% (H_2SO_4)	RCI Labscan, Thailand
TLC Silica gel 60, F_{254}	Merck, Germany
Yeast extract	Himedia, India

3.2 Raw Material

Rice residue from food waste was daily collected from canteen of the Faculty of Agro-Industry, Chiang Mai University, Chiang Mai 50100, Thailand, during September to October of the first semester of academic year 2015. Rice residue was manually separated from other contaminated solid waste residues e.g. bone, meat, egg and vegetable residues. Then, it was crushed using a blender and kept at -20°C in a freezer. The crushed rice residue was thawed and dried at 60°C for 2 days. After completely drying, it was fine ground using a blender, pooled and kept at 4°C until used.

3.3 Experimental Methods

3.3.1 Screening and isolation of oleaginous red yeast for lipids and carotenoids productions

Oleaginous red yeasts were screened from flowers, fruits and leave samples obtained from Doi-Inthanon National Park and Faculty of Agro-Industry, Chiang Mai University as well as the culture collection of Thailand Institute of Scientific and Technological Research (TISTR), Thailand and the Division of Biotechnology, Faculty of Agro-Industry, Chiang Mai University. They were enriched in yeast-malt extract medium (YM) which supplemented with 100 ppm chloramphenicol to minimize bacterial growth. The initial pH of the medium was adjusted to 6.0 with H_3PO_4 or 0.1 M KOH and then, autoclaved at 121°C for 15 min. All of samples were cultivated on incubator shaker at 28°C with shaking speed of 200 rpm for 3 days (Manowattana et al., 2012). After that, the culture broth was diluted by 10–folds serial dilution technique and spread on YM medium agar plate. The yeasts colonies were selected and re-streaked on YM medium agar plate. The red to yellow color intensity of colonies were used as

criteria for primary screening the carotenoid-producing yeasts. The lipids accumulated in yeast cell was selected by Sudan black B technique (Kitcha and Cheirsilp, 2011) (Appendix C–1). Moreover, the pure yeasts isolates were kept on YM slant at 4°C as well as maintained in glycerol stock (60% w/w) at -20°C until used (Manowattana et al., 2012).

3.3.2 Inoculum preparation

One mL of the glycerol stock of yeasts isolate was transferred into 250 mL Erlenmeyer flasks containing 50 mL of YM medium on incubator shaker at 28°C with a shaking speed of 200 rpm for 3 days until the optical density (OD_{600}) reached to 8.0. The starter culture was 10.0% (v/v) inoculated by batch fermentation.

3.3.3 Identification of oleaginous red yeast strain

The selected oleaginous red yeasts strains were identified based on their 26S rDNA gene sequence which were analyzed by the Mahidol University and Osaka University Collaborative Research Center for Bioscience and Biotechnology (MU-OU: CRC). The 26S rDNA sequence was compared with the sequences 26S rDNA accessible in the GenBank by BLAST search of the National Center Biotechnology Information (NCBI) databases. The polymerase chain reaction (PCR) amplification of 26S rDNA D1/D2 regions was initiated by using the F63-Forward primer (5'-GCATATCAATAAGCGGAGGAAAAG-3') and LR3-Reverse primer (5'-GGTCCGTGTTTCAAGACGG-3'). PCR sequencing for 26S rDNA was programmed with an initial denaturing at 95°C for 5 min followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 50°C for 10 sec and extension at 60°C for 4 min and final extension at 60°C for 4 min with Automate DNA Sequencer. The sequences chromatograms were assembled into one complete sequence using BioEdit Sequence Alignment Editor version 7.0.9.0 (Hall, 1999) and compared to all known sequences in the GenBank by use of BLASTN 2.6.1+ program (Zhang et al., 2000). The phylogenetic tree was made based on comparison of 26S rDNA sequences of their strain with other strain of same species which were obtained from GeneBank database (http://www.ncbi.nlm.nih.gov). The obtained 26S rDNA sequence has been deposited in NCBI GeneBank to get accession number.

3.3.4 Production of rice residue hydrolysate from food waste

3.3.4.1 Production of enzymatic-rice residue hydrolysate

Rice residue from food waste was used as a substrate for fermentable sugars production. The substrate was subjected to enzymatic hydrolysis by mashing with distilled water and pH was adjusted to be 4.5 by adding 10% H_2SO_4. Then, 180 U of α-amylase per gram of rice residue was added, and the reaction was carried out at 80ºC. After 2 h of reaction time, 120 U of amyloglucosidase (AMG) per gram rice residue was added and incubated at 60°C for 72 h (Manysoat, 2013). The original activity of α-amylase and AMG was 18,000 and 7,800 U/mL, respectively. The reducing sugar content of hydrolysate was measured by the DNS method according to Miller (1959) (Appendix C–5).

3.3.4.2 Production of acid-rice residue hydrolysate

For the acid hydrolysis, the rice residue was mixed with 3.0 M HCl at the solid to liquid ratio of 7:3 before autoclaving at 121°C for 15 min. After that, the pH of hydrolysate was adjusted to neutral pH (6.5–7.5) by adding 2.5 M NaOH (Woiciechowski et al., 2002). The reducing sugar content of hydrolysate was measured by the DNS method according to Miller (1959) (Appendix C–5).

3.3.5 Screening of oleaginous red yeast for lipids and carotenoids productions using rice residue hydrolysate from food waste as a carbon source

The glycerol stocks of red yeast isolates (from selection 3.3.1) were transferred into 250 mL Erlenmeyer flasks containing 50 mL of YM medium and cultivated in an incubator shaker at 28°C with a shaking speed of 200 rpm for 3 days until the OD_{600} reached 8.0. The starter culture was then 10.0% (v/v) inoculated into the basal medium by batch fermentation. The basal medium was supplemented with either glucose (10 g) or enzymatic-rice residue hydrolysate from food waste (equivalent to 10 g glucose) or acid-rice residue hydrolysate from food waste (equivalent to 10 g glucose). The compositions of basal medium (per liter) were 1.0 g yeast extract, 5.5 g KH_2PO_4, 5.3 g $(NH_4)_2SO_4$, 3.7 g K_2HPO_4, 0.5 g $MgSO_4.7H_2O$, 0.2 g $MnSO_4.H_2O$ and 0.5 g NaCl (Manowattana et al., 2012). The initial pH of the medium was adjusted to 6.0. The culture was incubated on an incubator shaker at 28°C with a shaking speed of 200 rpm

for 5 days. The oleaginous red yeast isolate which showed high ability to use the rice residue hydrolysate from food waste as a carbon source for the growth and high lipids and carotenoids productions was selected for further study.

3.3.6 Screening of oleaginous red yeast for direct using of rice residue from food waste as a carbon source

The red yeast isolates (from selection 3.3.5) were transferred into 250 mL Erlenmeyer flasks containing 50 mL of YM on incubator shaker at 28°C with a shaking speed of 200 rpm for 3 days until the OD_{600} reached 8.0. Then, the starter culture was 10.0% (v/v) inoculated into the basal medium which supplemented with either 10 g soluble starch (equivalent to 10 g glucose) or 11.1 g rice residue from food waste (equivalent to 10 g glucose). The culture medium composition and the culture condition were previously described as the section 3.3.5. The oleaginous red yeast isolate which showed high ability to directly convert rice residue from food waste to biomass, lipids and carotenoids was chosen for further study.

3.3.7 Optimization of lipids and carotenoids productions by the selected oleaginous red yeast *Sporidiobolus pararoseus* KX709872

3.3.7.1 Effect of medium compositions on biomass, lipids and carotenoids productions using the Plackett-Burman statistical design

The Plackett-Burman statistical design was used to screen the effect of the culture medium compositions on the volumetric productions of biomass, lipids, lipids content, total carotenoids, total carotenoids yield, β-carotene and β-carotene yield by *Sporidiobolus pararoseus* KX709872. A total of 8 variables, including C:N ratio (rice residue (g/L)) (X_1), K_2HPO_4 (X_2), KH_2PO_4 (X_3), NaCl (X_4), $MgSO_4.7H_2O$ (X_5), $MnSO_4.H_2O$ (X_6), $(NH_4)_2SO_4$ (X_7) and yeast extract (X_8) that were studied in 12 experiments. Each variable was investigated at two level, high (+) and low (-) (Chodok et al., 2010). The statistical software package Design Expert 6.0.10 (Stat-Ease, Minneapolis, MN) was used to design and analysis of the each experiment data. Table 3.1 shows the Plackett-Burman design with eight variables at various levels used for biomass, lipids and carotenoids productions by strain KX709872, based on the first-order polynomial model as follows:

$$Y = \beta_0 \sum \beta_i X_i \quad (3.1)$$

Where Y was the response (the volumetric production of biomass (g/L) or lipids (g/L) or total carotenoids (mg/L) or β-carotene (mg/L) or lipids content (% w/w) or total carotenoids yield (μg/g) or β-carotene yield (μg/g)), β_0 was model intercept, β_i was the linear coefficient, and X_i was the level of the independent variable.

Table 3.1 Experiment variables at various levels used for biomass, lipids and carotenoids productions using the Plackett-Burman design

Variables	**Unit**	**Symbol codes**	**Experimental values**	
			Low (-1)	High (+1)
C:N ratio* (rice residue (g/L))	-	X_1	C:N (25)	C:N (50)
K_2HPO_4	g/L	X_2	0.5	7.0
KH_2PO_4	g/L	X_3	1.0	10.0
NaCl	g/L	X_4	0.1	1.0
$MgSO_4.7H_2O$	g/L	X_5	0.2	2.0
$MnSO_4.H_2O$	g/L	X_6	0.01	0.1
$(NH_4)_2SO_4$	g/L	X_7	1.0	10.0
Yeast extract	g/L	X_8	0.5	5.0

*C:N ratio was calculated from the carbon source in a relation to the nitrogen source (Appendix A–7).

This model did not describe interaction among the factors. It was used to screen and evaluate the important factors that influenced the response. The magnitude of the coefficient of positive or negative indicated the corresponding impact on the titer. The coefficient value approached to zero, which implied small or no effect. The *p*-value was the probability that described the magnitude of a contrast coefficient resulting from random process variability. A low *p*-value indicated a significant effect (Chaiyaso et al., 2011). The volumetric productions of biomass (g/L) and lipids (g/L), lipids content (% w/w), total carotenoids (mg/L), total carotenoids yield (μg/g), β-carotene (mg/L) and β-carotene yield (μg/g) were carried out in triplicate and the average values of observed values and predicted values by the equation model were shown as response Y_1, Y_2, Y_3, Y_4, Y_5, Y_6 and Y_7, respectively. Based on a regression analysis of the variables, a confidential

level of 90% ($p<0.1$) for each factor was considered to have a significant effect on each response Y. The significantly factor was chosen for further study.

3.3.7.2 Optimization of cultivation condition using response surface methodology (RSM) via a central composite design (CCD)

A central composite design (CCD) was used to evaluate the coefficients in a mathematical model, predict the response, and check the fitting of the model (Chaiyaso et al., 2011). The factor of pH (X_9), temperature (X_{10}) and agitation rate (X_{11}) were examined with one variables of C:N ratio (X_1), which obtained from Plackett-Burman design study (Table 3.2). The CCD contained an imbedded factorial or fractional factorial matrix with center points and star point around the center point that allowed estimation of the curvature. The distance from the center of the design space to a factorial point was ±1 unit for each factor, and the distance from the center of the design space to a star point was $\pm\alpha$, where $|\alpha| > 1$. The precise value of α depended on certain properties needed for the design and on the number of factors used (in this case α =1.68). Similarly, the number of center point runs that the design must contain also depended on certain properties required for the design. The CCD always contained twice as many star points as factors in the design. The star points represented new extreme values (low and high) for each factor in the design. To maintain notability, the value of α depended on the number of experimental runs in the factorial portion of the CCD. The statistical software package Design Expert 6.0.10 (Stat-Ease, Minneapolis, MN) was used to design and analysis of the each experiment data. The experimental results of RSM were fitted via the response surface regression procedure, using the following second-order polynomial equation:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \qquad (3.2)$$

where Y was the predicted response, β_0 was the interception coefficient, β_i the coefficient for the linear effect, β_{ii} the coefficient for the quadratic effect, β_{ij} the ij^{th} coefficient of the interaction effect, and $X_i X_j$ was independent factors. The response variable in each trial was the average of three replicates (Chaiyaso et al., 2011).

pH temperature and agitation are the important factor for the synthesis and accumulation of biomass, lipids and carotenoids of oleaginous red yeast (Donot et al.,

2014). A range of pH value between 5–7 with a boundary of 4–8 for ±α, temperature between 20–30°C with the boundary of 15–35°C for ±α and agitation rate between 100–200 rpm with the boundary 50–250 rpm for ±α were selected in this experimental design. It included a total of 27 experiments with 3 trials of center points.

To confirm the applicability of CCD optimization model, production of biomass, lipids and carotenoids by *Sporidiobolus pararoseus* KX709872 would be carried out by cultivation this strain in the optimized medium under the optimal cultivation condition for the maximum of biomass, lipids and carotenoids productions.

Table 3.2 Experimental codes, ranges and levels of independent variables in the response-surface methodology experiment

Variables	**Units**	**Symbol codes**	**Levels**				
			-α	**Low (-1)**	**Center (0)**	**High (+1)**	**+α**
C:N ratio	-	X_1	0:1	18:1	23:1	25:1	27:1
pH	-	X_9	4	5	6	7	8
Temperature	°C	X_{10}	15	20	25	30	35
Agitation rate	rpm	X_{11}	50	100	150	200	250

3.3.8 Scale-up production in 5.0–L stirred tank bioreactor

Scale-up of lipids and carotenoids productions by *Sporidiobolus pararoseus* KX709872 was carried out in 5.0–L stirred tank bioreactor with 2.5–L working volume of optimized medium under the optimal cultivation conditions. The aeration rate was set at 3.0 vvm to maintain the dissolved oxygen (DO) level above 10%. The samples were withdrawn at interval of every 24 h for analysis of volumetric productions of biomass and lipids, lipids content, lipids productivity, total carotenoids, total carotenoids yield, β-carotene, β-carotene yield, α-amylase and amyloglucosidase (AMG) activities.

3.4 Analytical Method

3.4.1. Biomass measurement

Biomass (g/L) was collected from 5 day–olds of cultivation broth, which was taken from each flask and centrifuged at 6,000 rpm (4,830 *g*), 4°C for 15 min by a

refrigerated centrifuge. Then, the remaining rice residue was removed from cell pellet by hydrolysis using a mixture of commercial enzyme of α-amylase and AMG and the reaction was carried out at 60°C overnight to completely hydrolyzed remaining rice residue (Manysoat, 2013). After that, cell pellet solution was centrifuged at 6,000 rpm (4,830 *g*), 4°C for 5 min by a refrigerated centrifuge. The cell pellet was washed twice with distilled water and drying at 80°C overnight in the hot air oven and transferred to desiccators until constant weight. Meanwhile, the clear supernatant was subjected to determine the concentration of reducing sugar by the DNS method (Miller, 1959) (Appendix C–5). Then, the concentration of reducing sugar was expressed as the remaining of rice residue concentration which was calculated according to carbohydrate content (90%) in rice residue.

3.4.2 Lipids extraction

The lipids of cell pellet was extracted by chloroform-methanol method (Bligh and Dyer, 1959), which broke the yeast cell, carried out in screw cap tube with a mixture of chloroform : methanol (2:1, v/v) and 4.0 g glass beads. The mixture was vigorously shaken in a vortex mixer for 30 min, and then sonicated for 30 min using an ultrasonic bath. The ruptured cell and extracted lipids were centrifuged at 6,000 rpm (4,830 *g*), 4°C for 5 min by a refrigerated centrifuge. The clear supernatant was collected and removed by rotary evaporator. After that, crude lipids were transferred to desiccator until constant weight (g/L). The lipids content was expressed in the percentage of the volumetric production of lipids in relation to the dry biomass (% w/w) and the lipids productivity was expressed in term of volumetric production of lipids per day (g/L/day).

3.4.3 Carotenoids analysis

Carotenoids content of cell pellet was extracted following the procedure described by Manowattana et al. (2012), which broke the yeast cell, carried out in screw cap tube with 10.0 mL of acetone, 100 ppm of ascorbic acid and 4.0 g glass beads. The mixture was vigorously shaken in a vortex mixer for 15 min. The broken cell was centrifuged at 6,000 rpm (4,830 *g*), 4°C for 5 min by a refrigerated centrifuge. The clear supernatant was collected and removed by rotary evaporator, then re-dissolved in 1.0 mL hexane. The quantity analysis of carotenoids was investigated by the high performance liquid

chromatography (HPLC) equipped with C-18 column according the method of Manowattana et al. (2012) as explained in Appendix C–2. The carotenoids yield was expressed in the volumetric production of carotenoids (mg/L) in relation to the dry biomass (μg/g).

3.4.4 Fatty acid composition analysis

3.4.4.1 Gas Chromatography with Flame-Ionization Detector (GC-FID)

Fatty acid composition of lipids from *Sporidiobolus pararoseus* KX709872 was determined by converting all fatty acid to the corresponding fatty acid methyl esters (FAME) according to the method of Chaiyaso et al. (2012). Briefly, 50 mg of lipids or oil was methanolyzed with 2.5 mL of methoxide solution (0.5% KOH in methanol). The reaction was carried out at 60°C for 15 min. The FAME was extracted by hexane (2.0 mL). The hexane layer was washed with 1.0 mL deionized water and dried over sodium sulphate anhydrous. The FAME was filtered through a nylon membrane filter (0.2 μm) and subjected to GC analysis. The fatty acid composition in FAME was analyzed by using a GC-2010 Autosystem Gas Chromatography equipped with a HP-INNOWax column and flame ionization detector as explained in Appendix C–3. Hexanoic acid (C6:0) methyl ester was used as the internal standard.

3.4.4.2 Gas Chromatograph Mass Spectrometer (GC-MS)

The fatty acid profile of the lipids was also confirmed by GC-MS analysis which was analyzed by the Science and Technology Service Center, Chiang Mai University (STSC-CMU), Thailand. GC-MS was performed with GC-7890A Autosystem Gas Chromatography connected to 5975C Mass Spectrometer as explained in Appendix C–4.

3.4.4.3 Thin layer chromatography (TLC)

The qualitative analysis of fatty acid methyl esters (FAME) or biodiesel products were analyzed using thin-layer chromatography (TLC, silica gel 60 F_{254}) as described by Chaiyaso et al. (2012). The mobile phase of hexane : ethyl acetate : acetic acid (98:1.5:0.5 v/v/v) was used the mobile phase. The sample of 1.0 μL was applied to the TLC plate, dried at 80°C for 5 min and then developed in mobile phase for 30 min.

The plate was dried at 80°C and visualized by iodine vapor (Peramuna and Summers, 2014).

3.4.5 Composition of rice residue from food waste (Appendix A)

3.4.5.1 Proximate analysis of rice residue form food waste according method of AOAC 1990 (Helrich, 1990)

3.4.5.2 Determination of carbohydrate content (Dubois et al., 1956)

3.4.5.3 Determination of total nitrogen (Helrich, 1990)

3.4.5.4 Determination of total carbon (Watanabe et al., 2011)

3.4.5.5 Calculation of C:N ratio (Braunwald et al., 2013)

3.5 Enzyme Activity Assay

3.5.1 α-Amylase activity assay (Xie et al., 2014) (Appendix C–6)

3.5.2 Amyloglucosidase (AMG) activity assay (Teste et al., 2010) (Appendix C–7)