CHAPTER 3

Research Design and Methods

3.1 Materials and Instruments

3.1.1 Raw materials

Coffee (*Coffea arabica*) pulps were obtained from Highland Research and Training Center (Chiang Mai University, Chiang Mai, Thailand) during December 2013.

3.1.2 Other materials

1) Shrimp chitosan polymer type; 94.34% degree of deacetylation (Ta Ming Enterprise Co., Ltd., Samutsakon, Thailand)

2) 99.5% Glycerol; AR grade (QRec®, New Zealand)

3.1.3 Chemicals

1) 37% Hydrochloric acid ; AR grade (RCI Labscan Ltd., Bangkok, Thailand)

2) 97% Sodium hydroxide ; AR grade (RCI Labscan Ltd., Bangkok, Thailand)

3) 30% Hydrogen peroxide (stabilized) for synthesis (Merck, Darmstat, Germany)

4) Sodium silicate neutral solution QP (Panreac™, Barcelona, Spain)

5) 95% Ethanol; food grade (Alcoh-AH, Bangkok, Thailand)

6) Ethanol absolute for analysis (Merck, Darmstat, Germany)

7) Methanol; CG grade (RCI Labscan Ltd., Bangkok, Thailand)

8) Primary reference standard of chlorogenic acid; 5-caffeoylquinic acid (Sigma-Aldrich, MO, USA)

9) Analytical grade isopropanol (RCI Labscan Ltd., Bangkok, Thailand)

10) 99% Monochloroacetic acid (Sigma-Aldrich, MO, USA)

11) Acetic acid glacial; AR grade (RCI Labscan Ltd., Bangkok, Thailand)

12) 99% Sodium chloride; AR grade (RCI Labscan Ltd., Bangkok, Thailand)

13) Magnesium nitrate hexahydrate (Merck, Darmstat, Germany)

14) 98% Sulfuric acid; AR grade (Merck, Darmstat, Germany)

15) Lead (II) oxide (Sigma-Aldrich, MO, USA)

16) Lead (II) acetate trihydrate (Sigma-Aldrich, MO, USA)

17) Potassium acetate (Merck, Darmstat, Germany)

18) Sodium carbonate (Merck, Darmstat, Germany)

19) Gallic acid certified reference material (Sigma-Aldrich, MO, USA)

20) m-Cresol Purple indicator grade, Dye content 90 % (Sigma-Aldrich, MO, USA)

21) 2,2-Diphenyl-1-picrylhydrazyl (Sigma-Aldrich, MO, USA)

22) Folin-Ciocalteu's phenol reagent (Merck, Darmstat, Germany)

3.1.4 Instruments

Process

1) Tray dryer Model 160 x 180 x 200 (Navaloy Co., Ltd., Bangkok Thailand)

2) Two-digits weighing machine Model HR202 (A&D Engineering Inc., CA, USA)

3) Four-digits weighing machine Model Vibra (Shinko Denshi, Kalideres, Indonesia)

4) Drying oven with forced air and timer Model FED (Binder, NY, USA)

5) Milling machine (Hitachi, Tokyo, Japan)

6) Hot plate equipped with temperature control probe (IKA®, Selangor, Malaysia)

7) Polypropylene filter back with 10 microns pore sized (Union Science, Chiang Mai Thailand)

8) Ultra centrifugal mill Model ZM 200 (Retsch®, Haan, Germany)

9) Buchner funnel (Union Science, Chiang Mai Thailand)

10) Vacuum pump (Gardner Denver Thomas, Inc., Wisconsin, USA)

11) Whatman No.1 filter paper (GE Medical Systems, Bangkok, Thailand)

12) Whatman No.4 filter paper (GE Medical Systems, Bangkok, Thailand)

13) Batch disperser Model PT 10/35 (Kinematica, Luzern Schweiz, Switzerland)

14) Sonicator Model Elma S 100 H (iLabEquipment, New Jersey, USA)

15) 15.5 x 23.5 cm^2 polypropylene tray

Analysis

1) pH meter Model Orion 520A (Thermo Fisher Scientific, MA USA)

2) Furnace Model CWF 1100 (Scientific Promotion Co. Ltd., Bangkok, Thailand)

3) UV spectrophotometer Model UV 1800 (Shimadzu, Kyoto, Japan)

4) Vortex Model Genee-2 (Scientific Industry Inc., NY, USA)

5) WVTR aluminum cup

6) Desiccator

7) Silica gel (Union Science, Chiang Mai Thailand)

8) Paraffin wax (Union Science, Chiang Mai, Thailand)

9) Micrometer Model GT-313-A (Gotech testing machine Inc., Taichung City, Taiwan)

10) Universal Testing Machine Model 1000; H1K-S (Instron®, MA, USA)

11) Drop shape analyzer Model DSA30 (KRUSS, Hamburg, Germany)

12) Scanning electron microscope Model JEOL JSM-5910LV (JEOL UAS Inc., MA, USA)

13) Differential scanning calorimetry Model Pyris Diamond (PerkinElmer, MA, USA)

14) FT-IR spectrometer (Thermo Fisher Scientific, MA USA)

3.1.5 Microbiological testing

Microorganisms

1) *Staphylococcus aureus* (Department of Biotechnology, Faculty of Agro-Industry, Chiang Mai University, Chiang Mai, Thailand)

2) *Escherichia coli* (Department of Biotechnology, Faculty of Agro-Industry, Chiang Mai University, Chiang Mai, Thailand)

3) *Aspergillus niger* (Department of Biotechnology, Faculty of Agro-Industry, Chiang Mai University, Chiang Mai, Thailand)

Chemicals

1) Plate count agar (Difco™, MD, USA)

2) Potato dextrose agar (Difco™, MD, USA)

3) L (+) Tartaric acid (Carlo Erba, Val de Reuil, France)

4) Nutrient broth (Merck, Darmstat, Germany)

5) Peptone (Difco™, MD, USA)

Instruments

1) Autoclave Model Hiclave™ HVN-85 (Hirayama, Saitama, Thailand)
2) Stomacher 400 lab blender (Seward Medical, London, UK)
3) Vortex Mixer Uzusio VTX-3000 L (LMS®, Kampala, Uganda)
4) Laminar flow hood (Nuaire, MN, USA)
5) Incubator Model MIR-553 (Sanyo, Osaka, Japan)
6) Stomacher bag Steriblend™ (Sterilin, Staffs, UK)
7) Plastic petri dish Hycon Plastics (Union Science, Chiang Mai, Thailand)

3.1.6 Biodegradability testing

Materials

1) Disposable cellulose plate (Gracz™, Bangkok, Thailand)
2) Saw dust
3) Rabbit feed Smart Hearth (Charoen Pokphand, Bangkok, Thailand)
4) Live compost (Royal Project, Chiang Mai, Thailand)
5) Corn starch Knorr (Unilever, Bangkok, Thailand)
6) Sugar (Mitr Phol, Bangkok, Thailand)
7) Corn seed oil Mazola (ACH Food Companies Inc., TN, USA)
8) Urea; AR grade (RCI Labscan Ltd., Bangkok, Thailand)

Instruments

1) 15 x 10 x 10 cm^3 polypropylene box
2) Incubator (Memmert, D-91107 Schwabach, Germany)
3) 4 mm sieve (Mesh No.5)
4) 2 mm sieve (Mesh No.10)

5) Two-digits weighing machine Model HR202 (A&D Engineering Inc., CA, USA)

6) Four-digits weighing machine Model Vibra (Shinko Denshi, Kalideres, Indonesia)

3.1.7 Shelf life testing

Material

1) Carrot (*Daucus carota* ssp. *sativus*) was purchased from Waroros market (Chiang Mai, Thailand).

2) Polyvinyl chloride cling film (11 microns thickness)

3) 8.5 x 8.5 x 7 cm^3 HDPE tray

Chemicals

1) Sodium hypochlorite solution (6-14% active chlorine) (Merck, Darmstat, Germany)

Instruments

1) Incubator (Memmert, D-91107 Schwabach, Germany)

2) Four-digits weighing machine Model Vibra (Shinko Denshi, Kalideres, Indonesia)

3) Minolta chromameter Model CR-400 (Konika Minolta Sensing Inc., Osaka, Japan)

3.1.8 Statistical software

1) Design-Expert software version 7.1 (Statease Inc., Minneapolis, USA)

2) Microsoft Excel 2010 (Microsoft, WA, USA)

3.2 Process of Making Coffee Pulp Bioplastic

Fresh coffee pulps, free from impurity and defect, were dried in a tray dryer at 60°C until a constant weight (moisture content 7.4 ± 0.5% in dry basis). Then dried pulp

was ground to powder and sieved through 1-millimeter sieve using a milling machine. The powder was kept under -18°C until being used.

Process of making bioplastic from coffee pulp is summarized in Figure 3.1. First step was to produce crude extract of coffee pulp containing pectin and chlorogenic acids. The step was achieved by extracting coffee pulp powder at a solid to liquid ratio of 1:16 with distilled water containing specific amount of HCl. The extraction was performed on a hot plate equipped with continuous stirring using magnetic stirrer and temperature control probe. The powder was added to the extracting solution when the solution temperature reached a designed point and kept at that temperature for a specific time. The resulting extract was filtered through polypropylene filter bag with 10 microns pore size separating solid residue from coffee pulp crude extract. The crude extract was partially used in the film solution; while, the solid residue was made into CMC in the next step.

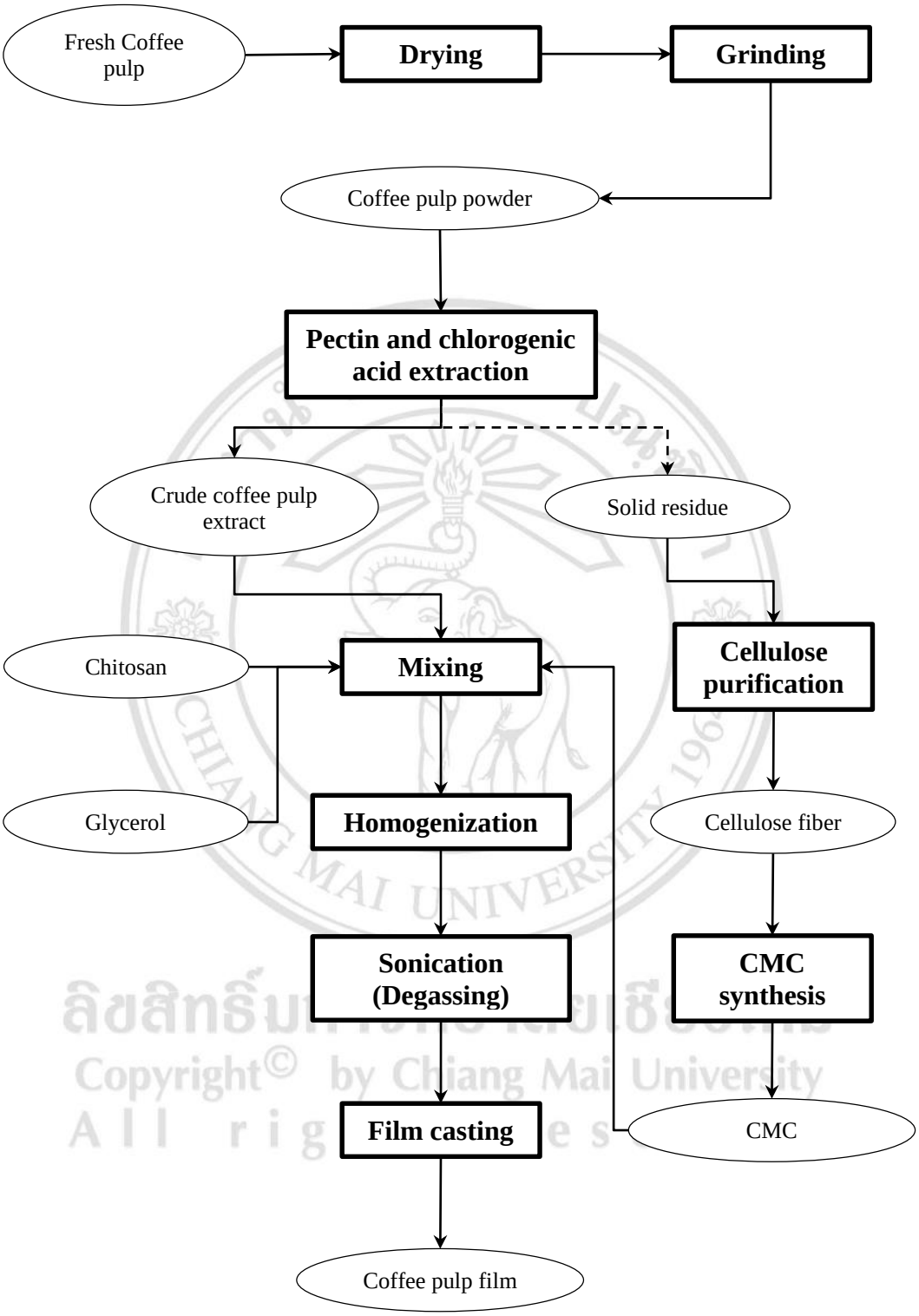

Figure 3.1 Process of making coffee pulp bioplastic

On a hot plate equipped with magnetic stirrer and temperature control probe, the solid residue left from the extraction process was bleached 2 times to extract for cellulose fiber using the solution containing 2% hydrogen peroxide, 0.2% sodium silicate and 0.2% sodium hydroxide (with solid to liquid of 1:20). Firstly, it was bleached at 80°C for 3 hours and washed with distilled water for 5 times. The remaining solid residue was bleached again at 50°C for 14 hours and washed with distilled water 5 times, as well. The resulting cellulose fiber was dried in a drying oven with forced air at 50°C for overnight and ground into powder with particle size less than 500 microns using ultra centrifugal mill. 15 grams of cellulose powder was dispersed in 450 mL isopropanol on a hot plate equipped with continuous stirring using magnetic stirrer and temperature control probe. Then, 50 mL of NaOH at a specific concentration was slowly added into the suspension and let it stand at room temperature for 30 minutes. After that, the temperature was raised to 55°C and 18 grams of monochloroacetic acid dissolved in 30 mL of 50% isopropanol were added into the suspension and let it stand for 3.5 hours. During this time the suspension was separated into 2 phases. The liquid phase was discarded, while the solid phase was transferred to 250 mL of 70% methanol, neutralized using glacial acetic acid and filtered using Buchner funnel with Whatman No.4 filter paper. Then, it was washed and filtered 4 times using 250 mL of 70% ethanol, 80% ethanol, absolute ethanol and absolute methanol, respectively. The solid residue was dried at 60°C for overnight in the drying oven with forced air. Thus, CMC from coffee pulp was obtained in a powder form.

CMC and crude coffee pulp extract were used to formulate coffee pulp bioplastic along with chitosan and glycerol as a thin film. CMC, chitosan and glycerol were separately weighed according to film formulation with a combined concentration of the three compounds equal to 3 grams per 100 mL of the film solution. To produce 1000 mL film solution, chitosan was prior dissolved in 400 mL solution of 1% acetic acid and 1% sodium chloride with constant stirring at room temperature for overnight. Meanwhile, on a hot plate equipped with temperature control probe, CMC was dissolved in 600 mL of crude coffee pulp extract at 50°C for 2 hours with constant stirring. Then, the solution of chitosan and glycerol were added into the mixture, adjusted pH to 4 and continued stirring for 30 minutes at 50°C. The mixture was

homogenized using a batch disperser with a speed of 6000 rpm for 10 minutes and degassed by sonication for 45 minutes. Finally, a portion (150 mL) of the film solution was casted on a 15.5 x 23.5 cm^2 polypropylene tray and dried at 25°C for exactly 3 days. The dried film was removed and conditioned at 25°C and 53% relative humidity (in a desiccator containing saturated solution of magnesium nitrate) for at least 48 hours before further analysis.

3.3 Research Design

This research aimed to produce bioplastic based on coffee pulp and characterizing the resulting bioplastic as a film. To achieve that goal, the study started from optimizing pectin and chlorogenic acid extraction followed by the optimization of process condition for CMC synthesis. Then, the optimal film formulation was found; thus, the final product was achieved. The next step of the study involved characterization of the film in two steps: 1) the film was characterized for its basic properties and structure and 2) the film was tested for its active properties, such as antioxidant property, antimicrobial property, biodegradability. Finally, the resulted bioplastic from coffee pulp was tested for its possible application as active food packaging by using fresh cut carrot as a model for studying.

The research was divided into 2 parts; production and characterization.

Production

3.3.1 Process optimization for pectin and chlorogenic acids extraction

Coffee pulp contains significant amount of pectin and chlorogenic acids. Both compounds can be extracted by water extraction, but with different conditions. Pectin is extracted under high temperature and strong acid condition, while chlorogenic acids are extracted by moderate temperature and neutral solvent. Thus, in order to achieve an extract containing suitable amount of both compounds, the process parameters must be studied. With the use of response surface methodology, the effects of acid concentration, extraction temperature and extraction time were investigated against pectin yield, pectin degree of esterification (DE), chlorogenic acid content and total phenolic compounds.

The experimental design and result analysis were established using the Design-Expert software version 7.1 (Statease Inc., Minneapolis, USA). To obtain interaction between process variables and responses, a 2^3 factorial design with center points was employed (Table 3.1). The studied process variables included: concentration of hydrochloric acid (x_1) ranging from 0 M (pH~7) to 0.1 M (pH~1); extracting temperature (x_2) from 60°C to 90°C and extraction time (x_3) from 30 minutes to 120 minutes. Eleven treatments including 3 replications of center points were measured for:

1) Pectin yield (Y_1)
2) Degree of esterification of pectin; DE (Y_2)
3) Chlorogenic acids content (Y_3)
4) Total phenolic compounds (Y_4)

Table 3.1 Experimental design for pectin and chlorogenic acid extraction

Treatment	Label	Acid concentration (M)	Temperature (°C)	Time (minute)
1	(1)	0	60	30
2	a	0.1	60	30
3	b	0	90	30
4	ab	0.1	90	30
5	c	0	60	120
6	ac	0.1	60	120
7	bc	0	90	120
8	abc	0.1	90	120
9	CP1	0.05	75	75
10	CP2	0.05	75	75
11	CP3	0.05	75	75

The relationship between each response and process variables were estimated by a second order polynomial (Equation [4]). Analysis of variance (ANOVA) was used to examine the statistical significance of the models.

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3$$
$$+\beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{123} x_1 x_2 x_3 \quad [4]$$

where Y is a response, x is a process variable and β is a coefficient of the term.

The optimum extracting condition was achieved using the following criteria:

1) Maximum pectin yield (Y_1)

2) DE less than 50% (Y_2)

3) Maximum chlorogenic acid content (Y_3)

4) Maximum total phenolic compounds (Y_4)

3.3.2 Process optimization for CMC synthesis

After bleaching the coffee pulp cellulose fiber was used to synthesize CMC by etherification using monochloroacetic acid as reactant with monochloroacetic acid to cellulose fiber ratio of 6 to 5 in isopropanol media for 3.5 hours at 55°C. Prior the reaction, addition of NaOH solution was used to expand the fiber structure. With different plant structure, the amount of NaOH required to expand the cellulose would be varied. While, other process parameters such as the amount of reactant, temperature and time are factors for chemical reaction between the reactant and cellulose; thus, they would be slightly deterred by the change of raw material.

Therefore, to find the optimum condition for CMC synthesis, the NaOH concentration was varied from 20% to 60%. With the use of response surface methodology, the effects of NaOH concentration (x) were studied against:

1) CMC yield (Y_1)

2) Degree of substitution; DS (Y_2)

One factor analysis was established using Design-Expert software and summarized in Table 3.2.

Table 3.2 Experimental design for CMC synthesis

Treatment	NaOH concentration (%)
1	20
2	30
3	40
4	50
5	60
6	30
7	50

The relationship between responses and variables was tested using cubic (Equation [5]). Analysis of variance (ANOVA) was performed using Design-Expert software to evaluate the significance of the models.

$$Y = \beta_0 + \beta_1 x + \beta_{11} x^2 + \beta_{111} x^3 \quad [5]$$

where Y is a response, x is a NaOH concentration and β is a coefficient of the term.

The optimal NaOH concentration was achieved by using criteria as followed:

1) Maximum CMC yield (Y_1)

2) Maximum DS (Y_2)

3.3.3 Optimization of film formulation

Polymer blend with polyelectrolyte complex structure was used to improve the mechanical and barrier properties of bioplastic from coffee pulp. The structure utilizes an ionic interaction between polymers with opposite charges (anionic pectin and CMC with cationic chitosan) to make a complex that can enhance properties of the plastic. In this case, CMC acted a major film component for polyelectrolyte complex structure while chitosan acted as a minor component. Appropriate ratio of both compounds need to be determined for fully utilization of polyelectrolyte complex structure. Therefore, in this study, CMC and crude coffee

pulp extract containing pectin and chlorogenic acid were used as a base to produce bioplastic. Chitosan was used as reinforcing agent to produce polyelectrolyte complex structure; while, glycerol acted as a plasticizer. To find optimum formulation for the coffee pulp film, mixture design (Table 3.3) established by Design-Expert software was used to investigate the effects of film composition, including CMC (x_1) from 60 to 80 percent, chitosan (x_2) from 0 to 20 percent and glycerol (x_3) from 20 to 40 percent, against:

1) Tensile strength (Y_1)
2) Elongation at break (Y_2)
3) WVT (Y_3)

Table 3.3 Product formulations in mixture design

Formulation	CMC (%)	Chitosan (%)	Glycerol (%)
1	80	0	20
2	60	20	20
3	60	0	40
4	70	10	20
5	70	0	30
6	60	10	30
7	66.67	6.67	26.67
8	66.67	6.67	26.67
9	66.67	6.67	26.67

The mathematical model used in the study followed an Equation [6]. Design-Expert software was used to verify significance of the models by performing the analysis of variance (ANOVA).

$$Y = \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \beta_{123} x_1 x_2 x_3 \quad [6]$$

Where Y is a response, x is percent of composition and β is a coefficient of the term.

The optimum film formulation was achieved by following criteria:

1) Maximum tensile strength (Y_1)
2) Elongation at break (Y_2) more than 50%
3) Minimum WVT (Y_3)

Characterization

3.3.4 Film studies

The final film formulation; called CC, obtained from previous part was compared with basic film formulation without chitosan (Formulation 1 in Table 3.3); called CM. The purpose of this study was to confirm the presence of polyelectrolyte complex and its effects in improving the film properties of coffee pulp bioplastic. Rfreesults were statistically analyzed with Microsoft Excel 2010 using student t-test. Both film formulations were compared for:

1) Tensile strength
2) Elongation at break
3) WVT
4) Moisture content
5) Contact angle
6) Film morphology using Scanning Electron Microscope (SEM)
7) Thermal property using Differential Scanning Calorimetry (DSC)
8) Chemical functional groups using Fourier Transform Infrared Spectrometry (FT-IR)

3.3.5 Coffee pulp bioplastic active properties

The final film formulation obtained from section 3.3.3 is evaluated for it active properties including antioxidant, antimicrobial and biodegradability. Then, the film was tested for its possible application as active food packaging by using fresh cut carrot as a model.

1) Antioxidant activity

The film was tested for its antioxidant activity using DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay; method proposed by Siripatrawan and Harte (2010). The result was expressed in term of milligram gallic acid equivalent and EC_{50}.

2) Antimicrobial activity

The film antimicrobial activity was investigated against *Staphylococcus aureus* (gram positive bacteria), *Escherichia coli* (gram negative bacteria) and *Aspergillus niger* (filamentous fungi) as model microorganisms. The testing method was disk diffusion method according to Manual of Clinical Microbiology (American Society of Microbiology, 2011). Effectiveness of antimicrobial activity was measured in term of inhibition zone.

3) Biodegradability

This experiment applied a method from ISO 20200: 2004, "Plastics - Determination of the degree of disintegration of plastic materials under simulated composting conditions in a laboratory-scale test" (International Standard Organization, 2004), to measure biodegradability of coffee pulp film in comparison to a commercial biodegradable plastic as a reference (Gracz™). The biodegradability was measured in term of degree of disintegration. The test was performed three times for each sample and the results were statistically analyzed with Microsoft Excel 2010 using student t-test.

4) Application testing: shelf life extension of fresh cut carrot

Fresh cut carrot is deteriorated by three main mechanisms; moisture loss, microbial spoilage and oxidative browning, which can be decreased by packaging system that contain antioxidant and antimicrobial compounds. Therefore, fresh cut carrot is an appropriate model for testing coffee pulp bioplastic application as active food packaging that contains antimicrobial and antioxidant activity of chlorogenic acids. To assess effectiveness of coffee pulp active packaging system, fresh cut carrot wrapped by coffee pulp film was compared with the commercial packaging system that utilized plastic container covered with hygienically food wrapping film (polyvinyl chloride cling film).

Carrots free from defects were cut to remove their ends and divided into 4-cm portions using a kitchen knife. Each portion was peeled and grated into 0.3 x 0.3 x 4 cm^3 strips using kitchen grater. Carrot shreds were submerged in 100 ppm chlorine solution (Sodium hypochlorite) for 60 seconds. Excess water was manually removed and the carrots were dried at room temperature for 10 minutes. The resulting fresh cut carrots were put into two types of packaging: 1) carrots were wrapped with 22 x 14 cm^2 coffee pulp film and put in 8.5 x 8.5 x 7 cm^3 HDPE tray; 2) carrots were put in the HDPE tray that wrapped with polyvinylchloride cling film (control). 8 samples were prepared for each treatment with each sample containing 50 g of the fresh cut carrot samples. The packed carrot samples were then stored at 10°C in incubator for 8 days. Initial carrot sample and the samples with 2, 4, 6 and 8 days of storage were analyzed for:

1) Weight loss

2) Microbial load (Total plate count)

3) Color (L*a*b*)

End of carrot shelf life was determined using microbial criteria. The value of total plate count beyond 8 log CFU/g indicated the end of the

product shelf life (Gómez-López *et al.*, 2007). Parameters were statistically compared using student t-test using Microsoft Excel. All measurements were performed in duplicates.

3.4 Methods of Analysis

3.4.1 Coffee pulp extract analysis

1) Pectin yield

Pectin yield was measured using alcohol recovery method (Prakash Maran *et al.*, 2013). The pectin was precipitated by adding 95% ethanol three times the volume of the extract and kept at room temperature for 2 hours. The precipitate was recovered by filtration through a Whatman No.4 filter paper using Buchner funnel. Then, the recovered pectin was washed with 70% ethanol, 80% ethanol and 95% ethanol, respectively, to remove salts and soluble impurities. Finally, wet pectin was dried at 50°C in the hot air oven until constant weight was reached and weighed. The yield (%) was calculated from the following equation:

$$Pectin\ yield\ (\%) = \frac{m}{m_0} \times 100 \qquad [7]$$

where m is a weight (g) of dried pectin and m_0 is the weight (g) of dried coffee pulp powder.

2) Pectin degree of esterification (DE)

The degree of esterification (DE) is determined by acid-base titration method (Wai *et al.*, 2010). Pectin (500 mg) were moistened by isopropanol and dissolved in 100 mL of distilled water. The completely dissolved solution of pectin was titrated to pH 7.5 with 0.1 M NaOH. The volume of titrant (mL) representing amount of free carboxyl group (a) was recorded. Then, 30 mL of 0.1 M NaOH were added to the titrated pectin solution and left with constant stirring for 30 minutes. After saponification, 30 mL of 0.1 M HCl were added to the mixture. The pectin solution was again titrated with 0.1 M NaOH to pH 7.5. The volume of titrant (mL) responsible for an

amount of esterified pectin (b) was recorded. The measurement was performed in triplicate and the degree of esterification (DE) was calculated as follows:

$$DE\ (\%) = \frac{100b}{a+b} \quad [8]$$

3) Chlorogenic acid content

Chlorogenic acid was determined by spectrophotometric method (AOAC, 2002). The method started by preparing basic lead acetate solution. Firstly, lead (II) oxide was activated by heating at 650°C in furnace for 3 hours (until lemon in color). Then, 40 grams of activated lead (II) oxide and 80 grams of lead (II) acetate were dissolved in 250 mL of boiling water for 45 minutes. The solution was cooled, filtered through Whatman No.1 filter paper and diluted to density of 1.25 at 20°C.

To determine chlorogenic acid content in the coffee pulp extract, 10 mL of the extract (or dilution with known ratio) were diluted to 100 mL with distilled water. Absorbance of the diluted solution was measured at 324 nm and apparent concentration before lead acetate treatment (C_0) was determined by comparing the absorbance to the standard curve (Figure 3.2). After that, 2 mL of saturated potassium acetate solution and 10 mL of the lead acetate solution were added to the sample. Sample was put in boiling water bath for 5 min with occasionally swirling. The sample was cooled and put in ice bath for 1 hour with mechanically stirring. The solution was warmed to room temperature, diluted to 100 mL with water. The resulting solution was filtered through Whatman No.1 filter paper. First 50 mL of the filtrate were discarded, but the remaining solution were immediately measured for absorbance at 324 nm and apparent concentration after lead acetate treatment (C_1) was determined from the standard curve. The corrected concentration of chlorogenic acid was calculated by an Equation [9]. Measurement was performed in duplicate.

$$Chlorogenic\ acid\ content\ (\frac{mg}{mL}) = C_0 - [\frac{C_1 - 0.00045}{5}] \quad [9]$$

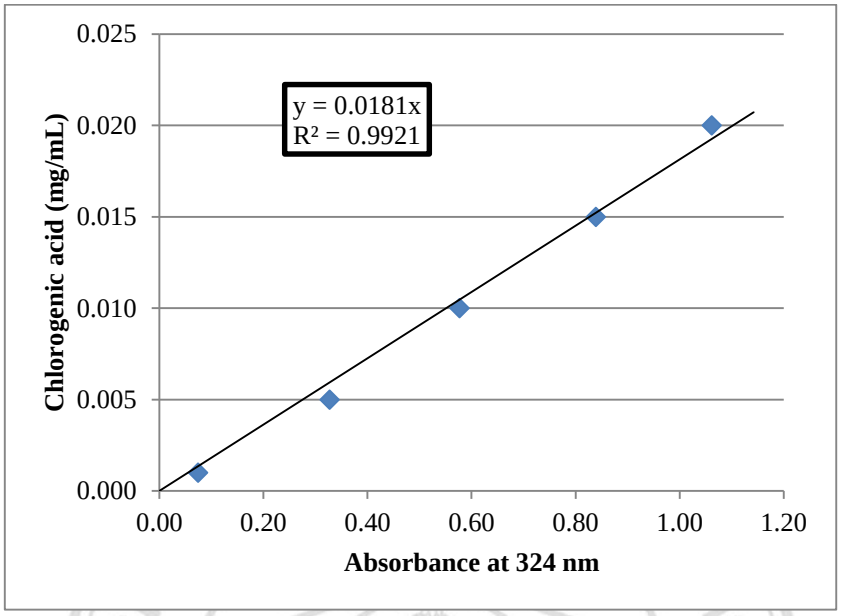

Figure 3.2 Standard curve of chlorogenic acid

Standard curve was prepared by measuring absorbance at 324 nm of 5-caffeoylquinic acid in a series of concentration: 0.001, 0.005, 0.10, 0.015 and 0.025 mg/mL. The curve was established by plotting the concentrations against absorbance.

4) Total phenolic compounds

Method of measuring total phenolic compounds was adapted from Upadhyay *et al.* (2012). Sample (or dilution with known ratio) in the volume of 1 mL was added with 7.5 mL of saturated sodium carbonate solution and 0.5 mL of Folin-Ciocalteu's phenol reagent. The mixture was adjusted to 10 mL by distilled water and vortexed for 30 seconds. Absorbance was measured at 760 nm and total phenolic compounds were determined by standard curve (Figure 3.3) in a unit of mg gallic acid equivalent per mL. The test was performed in triplicate.

Standard curve was established by plotting known concentrations of gallic acid (0.03, 0.06, 0.09, 0.12 and 0.15 mg/mL) against their absorbance after Folin-Ciocalteu's phenol reagent treatment.

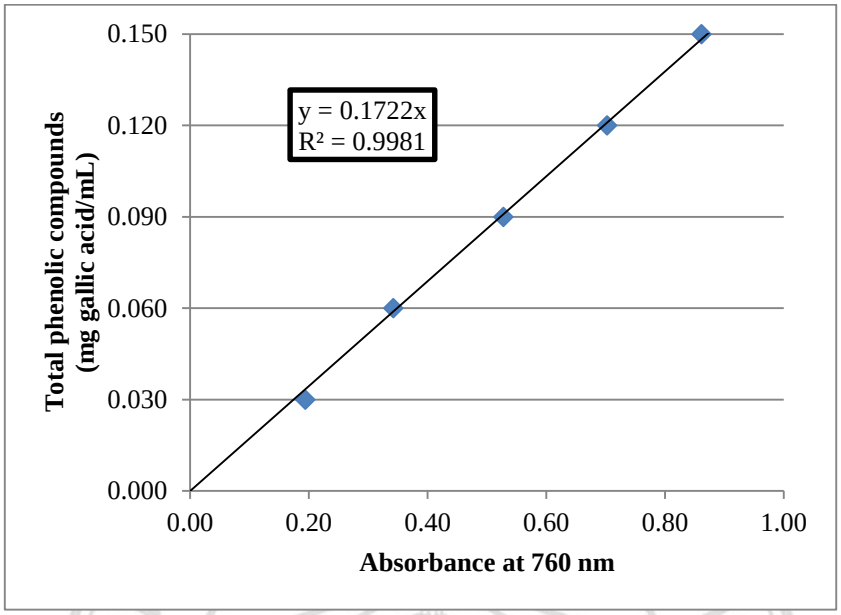

Figure 3.3 Standard curve of total phenolic compounds in term of gallic acid equivalent

3.4.2 CMC analysis

1) Yield

To calculate the yield of coffee pulp CMC, the dry weight of cellulose fiber (m_0) was recorded along with the dry weight of CMC (m) after washing and drying process. Then, CMC yield was calculated by the following formula:

$$CMC\ yield\ (\%) = \frac{m}{m_0} \times 100 \qquad [10]$$

2) Degree of substitution (DS)

Degree of substitution (DS) was calculated according to the USP XXIII method as described by Kittipongpatana *et al.* (2006). DS was measured by two processes; titration and residue on ignition.

In titration process, 1 gram of CMC was dissolved in 300 mL of 10% sodium chloride solution for 5 minutes. 15 mL of 0.1 M hydrochloric acid and 5 drop of m-cresol purple were added to the CMC solution. If the solution remained purple, 0.1 M hydrochloric acid was added until the color became yellow. Then, the sample was back titrated using 0.1 M sodium

hydroxide until the solution color had turned purple. The volume of base was recorded and calculated to milliequivalent (M), mmole of base required to neutralize 1 gram of CMC multiplied by valence (valence of sodium hydroxide is 1). The titration test was performed 2 times for each sample.

Residue on ignition process started from adding 1 gram of CMC (m_0) into a dried crucible. The crucible with CMC was put in a furnace at 400°C for 1 hour to obtain black residue. Then, on a hot plate, the black residue was moistened by adding sulfuric acid (approximately 1 mL) and heat at 350°C until all white fumes were vaporized. The remaining residue was put in the furnace at 850°C for 3.5 hours resulting in a white residue. Finally, the white residue was cooled in desiccator for 30 minutes and weighed (m). The percentage of residue (C) on ignition was calculated as in Equation [11]. Residue ignition test was performed in triplicate for each sample.

$$C = \frac{m}{m_0} \times 100 \qquad [11]$$

DS of CMC was calculated using the following equation.

$$DS = A + S \qquad [12]$$

where A is a degree of acid carboxymethyl substitution, which was calculated as:

$$A = \frac{1150M}{7120 - 412M - 80C} \qquad [13]$$

and S is a degree of sodium carboxymethyl substitution, which was calculated as:

$$S = \frac{(162 + 58A)C}{7120 - 80C} \qquad [14]$$

3.4.3 Coffee pulp film analysis

1) Film thickness

Thickness of film was measured using a micrometer Model GT-313-A (Gotech testing machine Inc., Taichung City, Taiwan). Each film sample was measured 5 times at random areas throughout the film.

2) Mechanical properties

Mechanical properties were determined according to ASTM D882 (ASTM, 1994a). Sample was cut into 15 x 120 mm^2 rectangular pieces. Film strips were conditioned at 25 ± 2°C and 53% RH (desiccator containing saturated magnesium nitrate solution) for 48 hours before testing. The test was performed using Instron universal testing machine (Model 1000; H1K-S). The testing condition was at grip separation of 50 mm, strain rate of 10 mm per minute, 25 ± 2°C and 53% RH. At least 6 measurements were performed for each film sample. Measured mechanical properties were tensile strength (MPa) and elongation at break (%).

3) Water vapor transmission rate (WVT)

Water vapor transmission rate was determined according to ASTM E96 (ASTM, 1994b). The film sample was cut into circles with 70 mm diameter and conditioned at 25 ± 2°C and 53% RH (desiccator containing saturated magnesium nitrate solution) for 48 hours before testing. The circular film was placed on an aluminum cup with mount diameter of 60 mm containing 10 grams of dried silica gel. The edge of the film was sealed to the cup using paraffin wax, so that the 60 mm in film diameter were exposed (film test area; A (m^2)). The cup was kept at 25 ± 2°C and 53% RH. The cup weight (G) was recorded daily for 14 days. After that, the cup weight was plotted against time (t) and the linear slope ($\frac{G}{t}$) was calculated. WVT of the sample was determined according to an Equation [15]. All measurements were performed in triplicate.

$$WVT\ \left(\frac{g}{h.m^2}\right) = \left(\frac{G}{t}\right) \times \frac{1}{A} \quad [15]$$

4) Moisture content

Film sample was cut into 2 x 2 cm^2 square pieces and put in hot air oven at 105°C for 24 hours. Then, the sample was cooled in desiccator for 30 minutes and weighed (m_0). After that, the dried film was kept at 25 ± 2°C and 53% RH (desiccator containing saturated magnesium nitrate solution) for 48 hours and weighed (m). The test was performed in triplicate and moisture content of the film was calculated as followed.

$$Moisture\ content\ (\%) = \frac{m-m_0}{m_0} \times 100 \quad [16]$$

5) Film water solubility

Triplicate measurement of film solubility was performed according to the method from Rotta *et al.* (2009) with some modification. Sample with known moisture content ($M;\ \%$) was cut into 2 x 2 cm^2 square pieces. Each piece was weighed (m_0) and immersed in 100 mL distilled water at 25°C for 24 hour with periodically swirling. After that, the film was recovered and dried at 105°C for 24 hours. The weight of the dried film was recorded (m). Then, the film solubility was calculated according to the following equation.

$$Film\ water\ solubility\ (\%) = \frac{m}{m_0 \times (1-\frac{M}{100})} \times 100 \quad [17]$$

6) Contact angle

Film contact angle was measured using drop shape analyzer Model DSA30 (KRUSS, Hamburg, Germany). The film preconditioned at 25 ± 2°C and 53% RH (desiccator containing saturated magnesium nitrate solution) for 48 hours, was used as a base for water droplet to form. The picture of water droplets was taken and contact angel was automatically calculated according to the following formula.

$$Contact\ angle\ (°) = 2\tan^{-1}(\frac{b}{2h}) \quad [18]$$

where b is the length of interface between water droplet and film, and h is the height of the water droplet.

7) Scanning electron microscope (SEM)

Film sample was cut to 0.5 X 10 cm^2 stripes and conditioned at ambient temperature in desiccator containing a silica gel for 48 hours. Then, the film stripes were immersed in liquid nitrogen for 10 second and snapped using tweezers. The fracture pieces were thawed at ambient temperature and taped to a stub using double sided conductive carbon tape (3M) exposing both film surface and cross-sectioned area. The samples were gold-sputtered. The SEM micrographs were taken using Scanning electron microscope Model JEOL JSM-5910LV (JEOL UAS Inc., MA, USA) under secondary electron mode.

8) Differential scanning calorimetry (DSC)

Thermal property of the film was determined using differential scanning calorimetry Model Pyris Diamond (PerkinElmer, MA, USA). The film sample was cut into circular disks (5 mm in diameter) and conditioned at 25 ± 2°C and 53% RH (desiccator containing saturated magnesium nitrate solution) for 48 hours. The 10 ± 0.1 mg film disks was place into the 40 μL aluminum pan. The test was performed using a heating rate of 10°C/min from 25 to 250°C with empty aluminum pan as a reference. Each sample was determined for melting point and corresponding enthalpy (ΔE) in duplicate measurement.

9) Fourier transform infrared spectrometry (FT-IR)

A 1 x 1 cm^2 film sample was determined for its functional groups using FT-IR spectrometer (Thermo Fisher Scientific, MA USA). The test was performed using attenuated total reflectance mode (ATR). Absorbance of the sample was measured from 400 cm^{-1} to 4,000 cm^{-1} wavenumber.

10) DPPH free radicals scavenging assay

Antioxidant activity of the film sample was determined using DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay. The method was modified from Siripatrawan and Harte (2010). Firstly, 1 gram of the film was extracted for antioxidants using 100 mL of absolute methanol for 2 hours. Then, 3 mL of extracted solution was mixed with 1 mL of 1 mM DPPH solution in methanol. The solution was vortexed for 30 seconds and kept in the dark for 30 minute at room temperature. After that the mixture was immediately evaluated for its absorbance at 517 nm. Percent inhibition was calculated using the following equation.

$$Inhibition\ (\%) = \frac{Abs_{DPPH} - Abs_{sample}}{Abs_{DPPH}} \times 100 \qquad [19]$$

where Abs_{DPPH} is an absorbance at 517 nm of 1 mM methanolic solution of DPPH and $Abs_{sample}$is an absorbance at 517 nm of the sample after the reaction with DPPH.

Antioxidant activity in terms of mg gallic acid equivalent was calculated by comparing the percent inhibition of the sample with standard curve (Figure 3.4). The measurement was performed 5 times for each sample and expressed in a unit of mg gallic acid per gram of the film sample (mg gallic acid/g). The standard curve was established by plotting concentrations of gallic acid (0.1, 0.2, 0.3 and 0.35 mg/mL) against their corresponding percent inhibition.

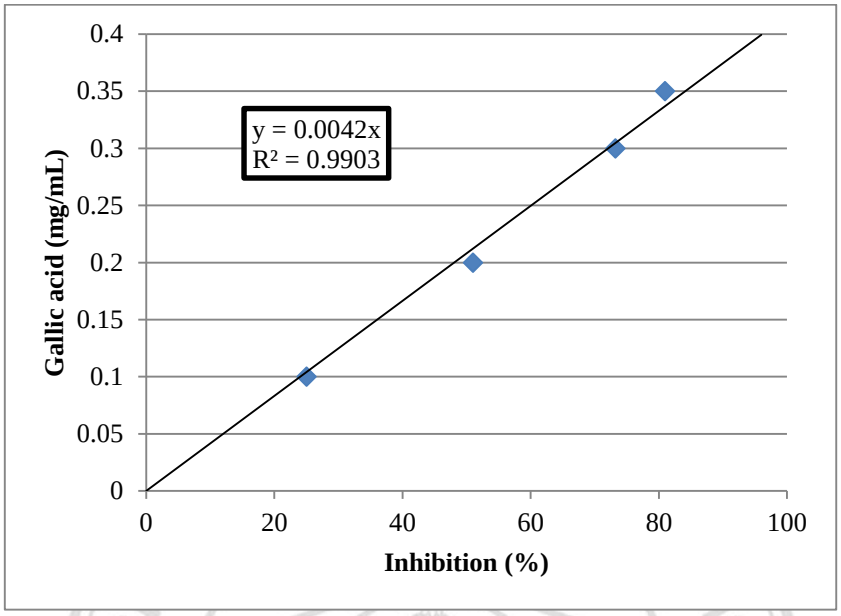

Figure 3.4 Standard curve antioxidant activity in term of gallic acid equivalent

EC_{50} measures the amount of film required to reduce DPPH free radical by half. The value was calculated by a formula (Equation [20]).

$$EC_{50}\left(\frac{\mu g}{mL}\right) = \frac{Antioxidant\ activity_{\ sample}}{EC_{50}^{gallic\ acid}} \times 10^4 \qquad [20]$$

where $Antioxidant\ activity_{sample}$ is an antioxidant activity of the sample in the unit of mg gallic acid/g and EC_{50}^{gallic} is a concentration of gallic acid required to inhibit 50% of free radicals (0.2043 mg/mL).

11) Disk diffusion method for antimicrobial testing

Film antimicrobial testing was adapted from disk diffusion method from Manual of Clinical Microbiology (American Society of Microbiology, 2011).

For antimicrobial testing, selected strain of bacteria from overnight grown culture in nutrient broth was adjusted to match turbidity of 0.5 McFarland standard (absorbance at 625 nm in between 0.08-0.13). The resulting culture would have approximately 10^8 CFU/mL. Then, the inoculum was diluted 3 times to have bacteria concentration of 10^5 CFU/mL by transferring 1 mL of prior dilution to test tube containing 9 mL of 1%

peptone solution and vortexed using Mixer Uzusio VTX-3000 L (LMS®, Kampala, Uganda) for 30 seconds. After that, one milliliter of bacteria suspension was used to inoculate a freshly prepared plate containing solidified Plate Count Agar (with uniform depth of approximately 4 mm) using spread plate method. Within 15 minutes after inoculation, 4 disks of coffee pulp film (5 mm in diameter) were evenly placed on to the inoculated agar surface. The plate was inverted and incubated at 37°C for 24 hours in an incubator (Memmert, D-91107 Schwabach, Germany). Effectiveness of antimicrobial activity was measured by inhibition zone; diameter (mm) of the area where no obvious growth visible to the naked eye subtracted by the film diameter (mm).

For antifungal testing, selected strain of filamentous fungi from one week old culture on Potato Dextrose Agar slants was used to prepared conidial suspension by flooding 1 mL of 1% peptone solution onto the slants. The mixture was recovered and heavy particles were allowed to settle for 5 minutes. The upper homogenous suspension was adjusted to a volume where the absorbance at 530 nm was in between 0.09 and 0.13. The resulting inoculum would have approximately 1.6×10^6 CFU/mL. Then, the inoculum was diluted 1:50 by transferring 1 mL of the suspension to 49 mL of 1% peptone solution and vortexed using Mixer Uzusio VTX-3000 L (LMS®, Kampala, Uganda) for 30 seconds. After that, one milliliter of conidia suspension was used to inoculate a freshly prepared plate containing solidified Potato Dextrose Agar (with uniform depth of 4 mm) using spread plate method. Within 15 minutes after inoculation, 4 disks of coffee pulp film (5 mm in diameter) were evenly placed on to the inoculated agar surface. The plate was inverted and incubated at 37°C for 24 hours in an incubator (Memmert, D-91107 Schwabach, Germany). Effectiveness of antimicrobial activity was measured by inhibition zone; diameter (mm) of the area where no obvious growth visible to the naked eye subtracted by the film diameter (mm).

12) Biodegradability

This experiment applied a method from ISO 20200: 2004, "Plastics - Determination of the degree of disintegration of plastic materials under simulated composting conditions in a laboratory-scale test" (International Standard Organization, 2004), to measure biodegradability of coffee pulp film in comparison to a commercial biodegradable plastic as a reference (Gracz™, Bangkok, Thailand). The biodegradability was measured in term of degree of disintegration. The test was performed three times for each sample and the result was statistically analyzed with Microsoft Excel using student t-test.

In each replication, the test sample was cut into square pieces with a dimension of 2.5 x 2.5 cm^2 and dried in a drying oven with forced air and timer Model FED (Binder, NY, USA) at 50°C until constant weight. Five grams (m_i) of film sample were submerged in distilled water for 30 seconds and put into a polypropylene box (15 cm × 10 cm × 10 cm) with 2 holes (5 mm in diameter) in the middle of the two 10 cm wide sides and 3 cm height from the bottom of the box. The box contained 500 grams of synthetic waste produced by mixing the components in Table 3.4 with distilled water (distilled water was added until reaching 55% total water content) and sieving the mixture through 4 mm sieve (Mesh No.5). The film samples and synthetic waste were gently mixed to avoid any mechanical degradation to the test pieces. The lid was closed and the test was started by putting the box in an incubator (Memmert, D-91107 Schwabach, Germany) at 60°C. During the 30 days of incubation period following procedures were followed to ensure good composting process. On the 1^{st}, 2^{nd}, 3^{rd}, 4^{th}, 7^{th}, 9^{th}, 11^{th} and 14^{th} days of incubation, the mixture initial weight was restored using distilled water and the composting system was mixed. On the 8^{th}, 10^{th}, 16^{th}, 18^{th}, 21^{th}, 23^{th}, 25^{th} and 28^{th} days of incubation, the initial weight was restored without mixing the composting mixture.

Table 3.4 Composition of synthetic waste used for biodegradability testing

Material	%
Saw dust	40
Rabbit feed	30
Live compost	10
Corn starch	10
Saccharose	5
Corn seed oil	4
Urea	1
Total	100

Source:International Standard Organization (2004)

After 30 days of composting, the test was terminated by opening the lid and drying the composting matter until constant weight at 50°C in a drying oven with forced air. The dried mixture was sieved through 2 mm sieve (Mesh No.10) to recover the remaining test sample (break any lump of composts). The sample was carefully cleaned from compost (if appropriate the sample was dipped in distilled water), and dried in a drying oven with forced air at 50°C until constant weight. The final weight (m_f) of the sample was recorded and biodegradability of the plastic was determined in degree of disintegration (Equation [21]).

$$Degree\ of\ disintegration\ (\%) = \frac{m_f - m_i}{m_i} \times 100 \qquad [21]$$

3.4.4 Fresh cut carrot shelf life

1) Weight loss

The weight loss of carrot sample was calculated by a following formula.

$$Weight\ loss\ (\%) = \frac{m}{m_0} \times 100 \qquad [22]$$

where m is a weight of fresh carrot sample and m_0 is an initial weight of the fresh cut carrot measured right before shelf life testing.

2) Color

Hunter color (L* a* b*) values were measured using a Minolta chromameter Model CR-400 (Konika Minolta Sensing Inc., Osaka, Japan). Three portions of 10 grams fresh cut carrot were collected from each sample to perform the measurement. The chromameter was standardized with white standard tile (L* = 97.67, a* = -0.18 and b* = 1.84). L* (brightness), a* (redness) and b* (yellowness) color values were determined as an average value of the three measurements.

3) Microbial load (Total plate count)

Total plate count was measured according to the method from AOAC international (AOAC, 2003). Ten grams of fresh cut carrot were aseptically collected from multiple points of the sample and transferred to a stomacher bag containing 90 mL of 1% peptone solution. The bag was mixed using Stomacher 400 Lab Blender (Seward Medical, DE1-1PP, UK) for 2 minutes resulted in 10^{-1} dilution. Series of dilution (10^{-2} to 10^{-8}) were prepared by transferring 1 mL of prior dilution to test tube containing 9 mL of 1% peptone solution and vortexed using Mixer Uzusio VTX-3000 L (LMS®, Kampala, Uganda) for 30 seconds. In each dilution starting from the smallest dilution, 1 mL sample was transfered to 2 plastic petri dishes. Then, approximately 15 mL of liquid Plate Count Agar (45-55°C) were poured into inoculated petri dishes. The dishes were mixed and left until solidified. After solidified, all petri dishes were turned upside down and incubated at 37°C for 24 hours in an incubator (Memmert, D-91107 Schwabach, Germany). Finally, the plates containing microbes between 30-300 colonies were counted and total plate count was determined by their average value. The measurement was performed in duplicate for each sample and total plate count was expressed in log CFU/g.