CHAPTER 3 – RESEARCH METHODS

3.1 Venue and Time

The experiment was conducted in the LABTIAP BPPT Serpong for approximately 4 months starting from March 2017 until June 2017.

3.2 Materials and Equipment

3.2.1 Materials

Unripe " Kepok" banana were used as the raw material for this study. Kepok Banana were purchased from local supplier in BSD , Tangerang. The characteristic of the selected banana were green, hard and no mold present. The banana were then put into the laboratory and further process for extraction.

Material used were modification of resistant starch were pullulanase enzyme (Novozyme), 0.1 M Acetate Buffer solution (Ph 5.2), distilled water.

Materials used in analysis of product were glucose standard (Merck, Darmstadt, Germany), maltose standard (Sigma Aldrich, USA), amylose standard (Sigma Aldrich), phosphate buffer solution, acetic acid 1N, alpha amylase enzyme (Sigma Aldrich, USA), distilled water, Ethanol 95%,Soidum Hydroxide (NaOH) 1N (Sumber Kimia Abadi) , Iodine (I2), Pottasium Iodide (KI), For the formulation of the batter coating the material will consist of wheat flour (Segitiga Biru), banana starch, glutamate (Ajinomoto), salt and baking powder (Koepoe – Koepoe), Pure starch (BDH Laboratory Supplies, England), DNS Solution

3.2.2 Equipment

The equipment used during the experiment were digital balance (Matrix AJ3002B), Waterbath shaker (Daihan Scientific) , Vortex (Top Heidolph , Germany), Oven (Memmert , Germany), hotplate, glassware, micropipette (Eppendorf), Incubator (Memmert, Germany), centrifuge (Beckman Coulter), microplate (Iwaki), microplate reader, , muslin cloth, 20 µnylon ,Centrifuge, and 100 – mesh sieve.

3.3 Design of Experiment

3.3.1 Stage 1 : Selection of RS 3 Modification method

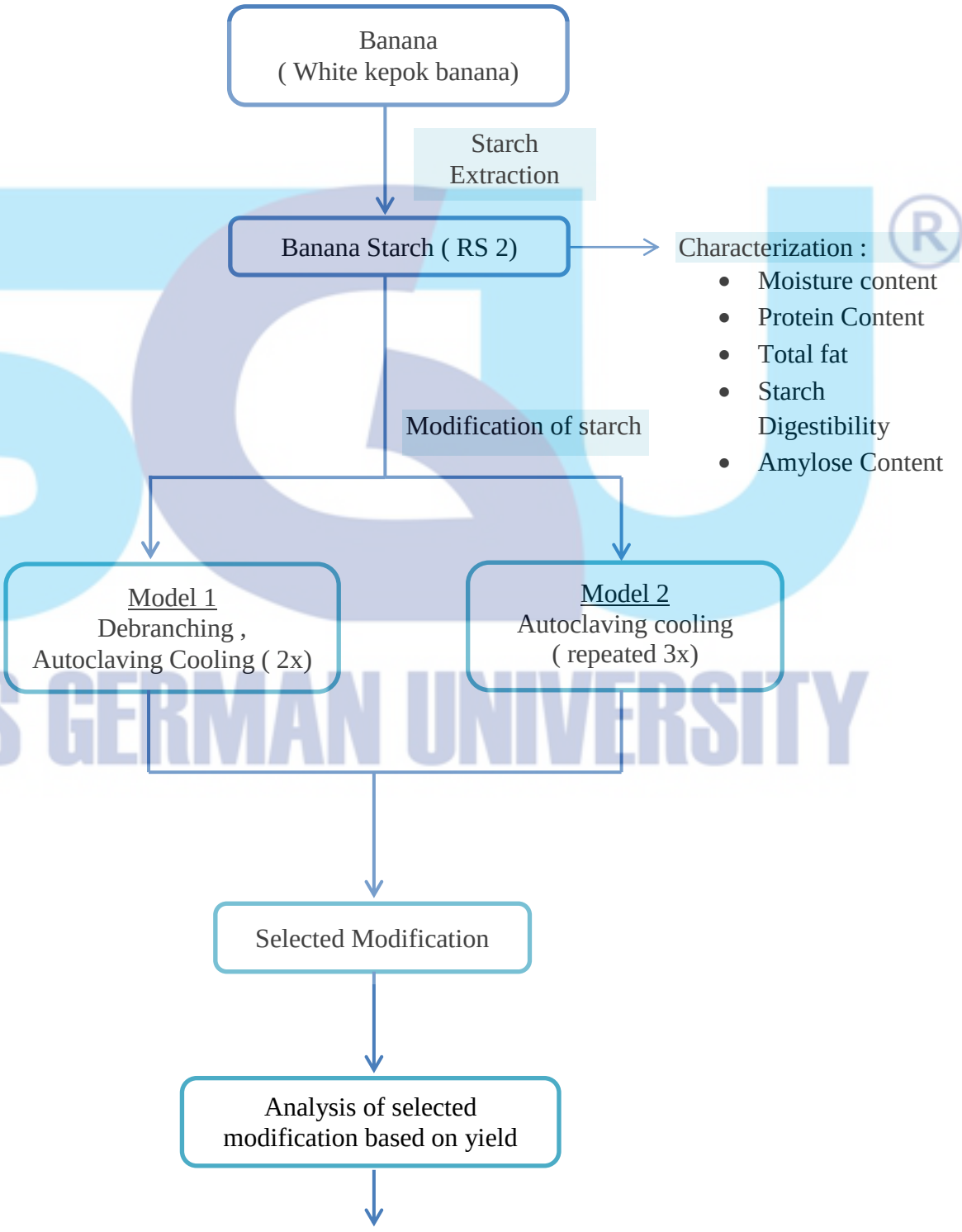
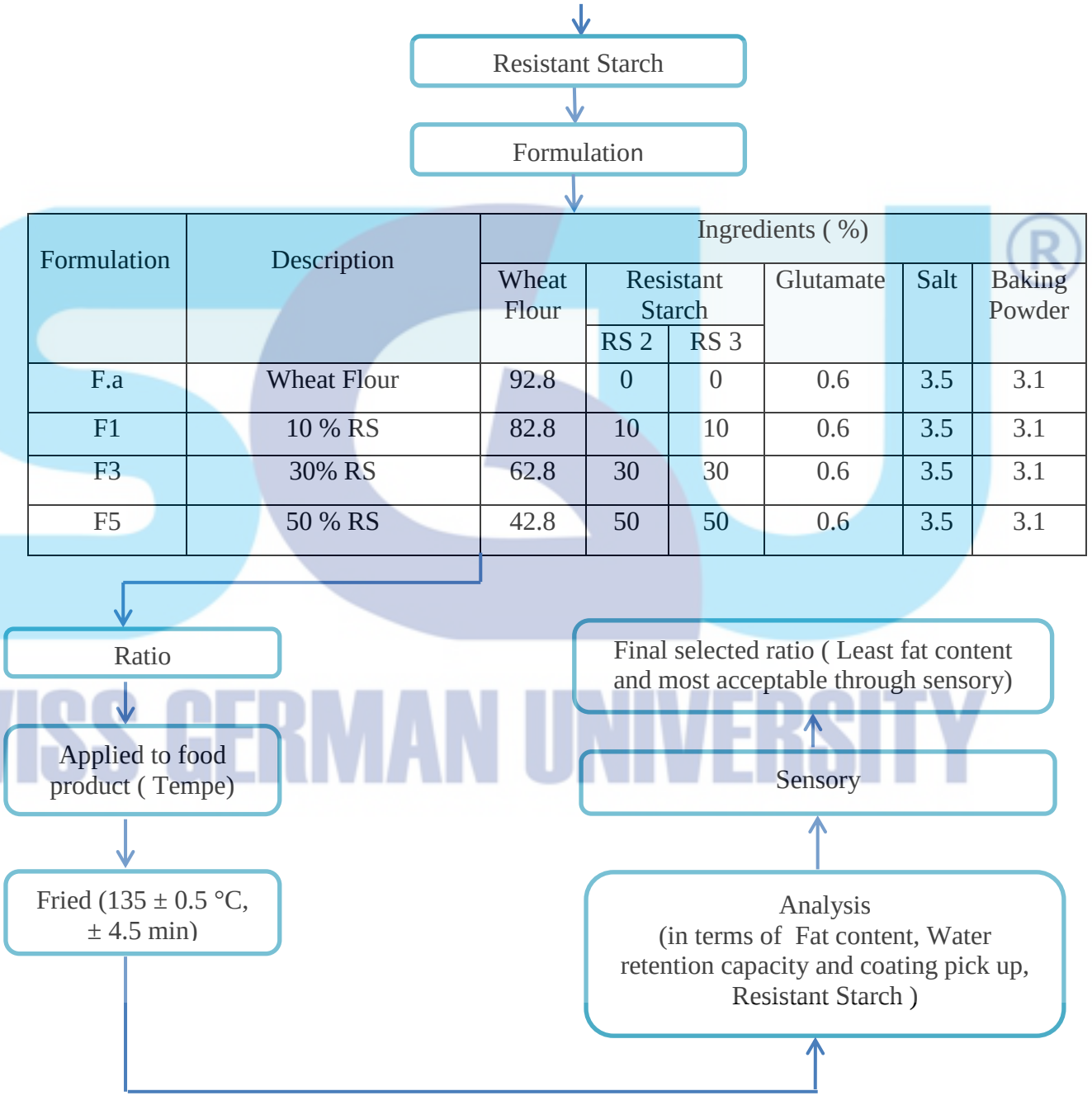


Figure 3.1 Process of selecting the RS 3 modification method

3.3.2 Stage 2 : Substitutions of wheat flour with RS from banana in batter coating



Formulation	Description	Ingredients (%)					
		Wheat Flour	Resistant Starch		Glutamate	Salt	Baking Powder
			RS 2	RS 3			
F.a	Wheat Flour	92.8	0	0	0.6	3.5	3.1
F1	10 % RS	82.8	10	10	0.6	3.5	3.1
F3	30% RS	62.8	30	30	0.6	3.5	3.1
F5	50 % RS	42.8	50	50	0.6	3.5	3.1

Figure 3.2 Process of Substitution of wheat flour to RS 3

3.4 Experimental Procedure

Methods of getting the data are explained in this subchapter as the following :

3.4.1 Extraction of RS2

Unripe white kepok banana (Musa paradisiaca formatypica) are used as the raw material and source of resistant starch . The selection of the banana will have the standard parameter of age ranging from 90 to 20 days, green and no diseases such as molds and rotten areas.. After the banana have been gathered, extraction of the banana starch will be conducted. The method of extraction will follow and adopted by the previous research done by Zhang et al. 2005 and Vatanasuchart et al., 2012 using the water alkaline extraction process with some modification and represented in figure 3.3

Banana is cut into thin sliced and then put in the blender with NaOH 0.1 N for approximately 10 minutes. The mixture of banana and NaOH are then pun into plastic container to be mix for 3 hours. The mixture is then filtered with muslin cloth to separate the supernatant and residue. Once separated, supernatant are let stand for 2 hours and the residue are dispersed with NaOH .Residue will follow be treated like supernantant but one step behind. After 2 hours, supernatant that have been let to stand for 2 hours will have 2 phase which consist of water at the top and starch portion at the bottom. The water is removed and 8L of distilled water is added to the starch portion and let stand for another 2 hours and then continued with removing the water , adding 8 L of distilled water to the starch portion and letting stand overnight. The process is then continued with removing the water and adding 1 L of distilled water to the starch portion at the bottom part and let stand for another 2 hour. The water is then removed and the starch gathered at the bottom is placed in aluminum plate to be heated in the oven at 40°C for 12 hours to remove the excess moisture. Once the starch is dried, the starch is sieved with sieve of 100 mesh and then stored in sealed condition.

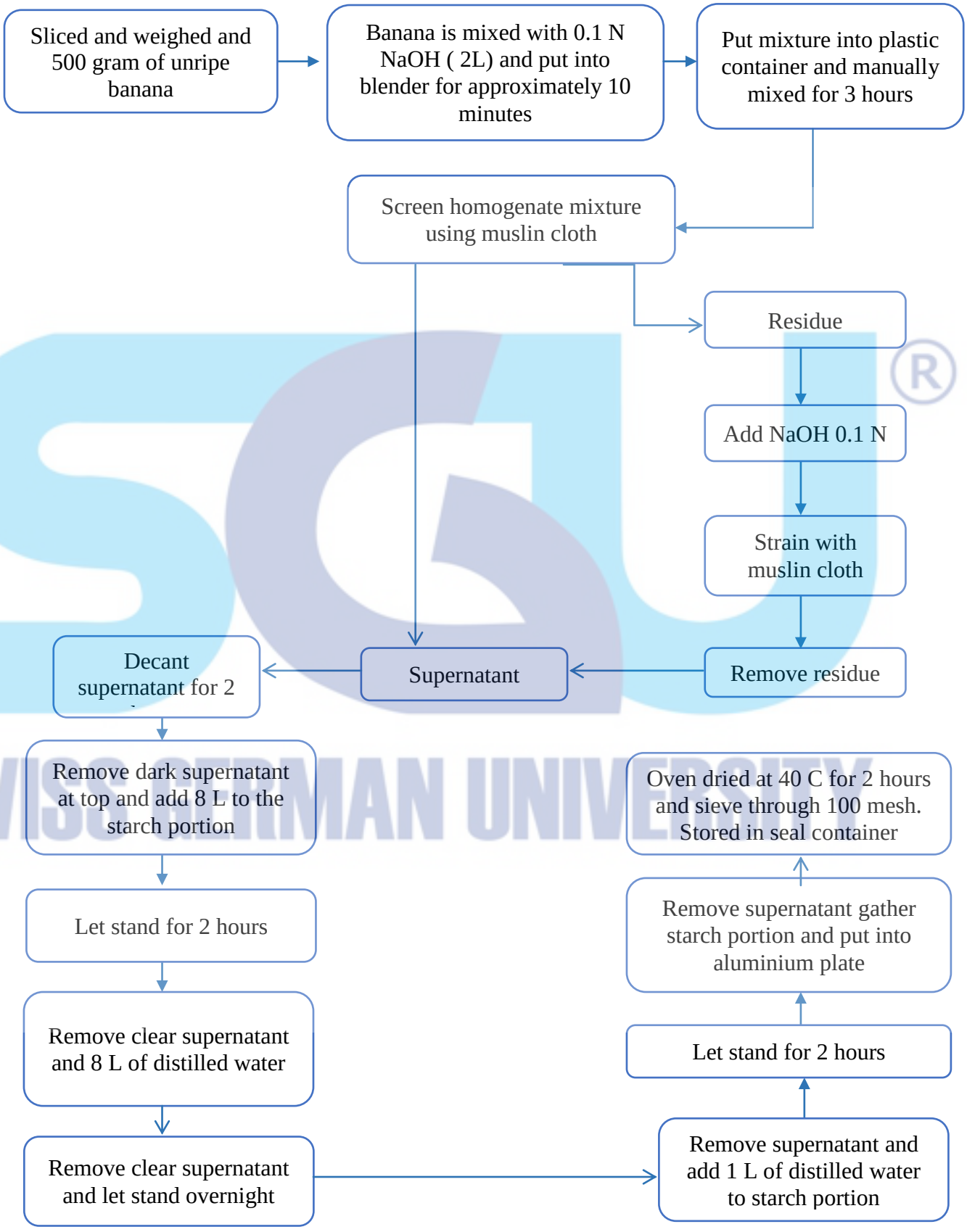


Figure 3.3 Extraction of Banana Starch

3.4.2 Modification of Resistant Starch 2 to Resistant starch 3

As mentioned by Musita .2009 , resistant starch type 2 that is found in many raw food such as the unripe white "kepok" banana (*Musa paradisiaca formatypica*) have the characteristic of low gelatinization ability and unstable in heat treatment modification of resistant starch is needed to generate resistant starch type 3. The optimization of the banana starch will follow the method done previously by Lehmann et al., 2002 with some modification.

The modification of RS 2 to RS 3 consist of 2 methods which consist of debranching – autoclaving cooling (2x) and repeated cycles of only autoclaving cooling. Each process will be explained in the following subchapter.

3.4.2.1 Debranching – Autoclaving Cooling Methods

The debranching methods are as followed, 50 grams of native starch are mixed with 250 ml distilled water and mixed in 500 ml Erlenmeyer Flask. The solution will then undergo gelling through heating at 80C for 5 minutes. After undergo gelling, the suspension is then autoclaved at 121 C for 15 minutes. The suspension is then added with 125 ml buffer acetate and then cooled at the incubator until the temperature reach 50 C. After the temperature have reached 50 C, 125 ml buffer enzyme is then added into the suspension and placed in the water bath shaker at 160 rpm with 50C for 24 hours. After 24 hours, the suspension is then put into autoclave for 15 minutes at 121 C, then cooled in the chiller for 24 hours for 2 times. After it had undergone debranching and autoclaving cooling for 2 cycles it is then sprayed dried to reach a fine particle.

3.4.2.2 Autoclaving – Cooling Methods

The autoclaving method are as followed, Starch are mix with distilled water with a ratio of 1 :5. The solution is starch mixture is then put into heat at 80°C for 5 minutes with constant stirring. The suspension is then put into autoclaved at 121° C for 15 minutes and let rest at room temperature until it reach approximately 40°C. It is then

put into the chiller and let rest for 24 hours. The cycle of autoclaving – cooling were repeated for another 2 more times before then the gel- like suspension is put into the oven at 40°C to dry. It is then grind to get fine particles of starch.

3.4.3 Formulation of batter coating

The amount of ingredient used in the batter coating formulation will refers to the study of Sanz et al., 2008. The ingredients that are going to be used consist of wheat flour, resistant starch, salt and baking powder ($NaHCO_3$). There will be a total of 4 batter formulation prepared where it consist of control and 3 samples by replacing the 10%, 30% and 50% of wheat flour in the batter coating formula with resistant starch represented in Table 3.4.4.1. The control will consist of batter formulation without RS (fully used wheat flour). The formulation will be labeled with F.a (Control A : Only wheat flour), F1 (10% RS), F3 (30% RS), and F5 (50% RS).

Although the propose method will include the ratio of formulation from 10 – 50% resistant starch, a preliminary study should be done to the ratio of batter formulation that will continue to be analyzed especially the sensory evaluation. 10% is the considered as the bottom ratio limit since the bottom limit will contribute result significance . To little amount of wheat flour substitute might result in not be able to see the result that want to be analyzed. 50% are put in the formulation but will be first observed. If using the ratio 50% shows inaccepbility through sensory such as undesired texture after fried then the ratio be considered to be ignored. In contrary, if up to 50% still shows good result, higher ratio will also might be observed.

The ingredients in the batter formula will be mixed with tap water with a ratio of 1.0 : 1.5. Then the batter is applied to Tempe. The tempe will be cut into the size of 4 cm x 3 cm x 1 cm. Then battered with the different batter formulation and then fried in cooking oil at approximately 130 ± 5° C for 3.5 minutes. The coated tempe will be taken out after giving a golden brown color.

Table 3.1 Composition of different batter formulation

Formulation	Description	Ingredients (%)					
		Wheat Flour	Resistant Starch		Glutamate	Salt	Baking Powder
			RS 2	RS 3			
F.a	Wheat Flour	92.8	0	0	0.6	3.5	3.1
F1	10 % RS	82.8	10	10	0.6	3.5	3.1
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F5	50 % RS	42.8	50	50	0.6	3.5	3.1

3.5 Analytical Procedure

3.5.1 Characterization of banana starch

After gaining the banana starch through the water alkaline extraction method, the starch will then be characterized using the proximate analysis to know the initial characteristic of the starch before any modification are done. Furthermore, with the characterization will also tell the author whether the extraction method that follow previous studies do produce the banana starch that is similar compare to the reference. In the proximate analysis parameters of moisture content, ash, fat , protein and carbohydrate are measured. Amylose and starch digestibility will also be measured.

3.5.1.1 Moisture Content (SNI 2891 1992 . 5.1)

One until two gram of sample were weighed using crucible with known initial weigh. The samples were oven dried at 105 °C for 3 hours. It is then cooled down in desiccator and the weighed is measured. The process were repeated until constant and stable weight of obtained.

Water content (% wet basis) = {[a – (b – c)] / a } x 100

Where,a = initial weight of sample (g); b = weight of crucible and sample after being dried (g); c = weight of empty crucible (g).

3.5.1.2 Ash content (SNI 2891 1992 . 6.1)

Weighed five to ten gram of sample inside porcelain crucible which known initial weighed. Heat the sample inside the furnace at 550 C. Samples were then cooled at dessicator. Weighed the sample and repeat the process until constant weighed were obtained.

Ash Content = (W1 – W2) / W x 100%

W = initial weight of sample (gram); W1 = weight of sample and porcelain crucible after being furnace (gram) ; W2 = weight of empty porcelain crucible.

3.5.1.3 Total Carbohydrate

Total carbohydrate was calculated by difference using the formula

Carbohydrate (%) = 100% - (% ash + % moisture content + % protein + % fat)

3.5.1.4 Crude Protein (960.52 AOAC 1998)

The crude protein will be analyze using the Kjeldahl method. Sample of around 100 – 250 gram of sample is put into the Kjeldahl flask and then add with 1.9 ± 0.1 gram of K_2SO_4,10 mg HgO, 2.0 ± 0.1 mL concentrated H_2SO_4, and 2-3 boiling stones. Sample is then heated with constant increase temperature until reaching its boiling point for 1 until 1.5 hours until a clear liquid is obtained. Once the solution is cool enough, the content is then poured into the distillation flask. The left over residue is then rinse with 1 – 2 mL of distilled water and poured into the distillation flask. The total solution is then added with 8 – 10 mL of 60% NaOH and 5% $Na_2S_2O_3$. In a separate Erlenmeyer, 5.0 mL H_3BO_3 solution and 2-4 drops of indicator methyl red-methyl blue is prepared. The Erlenmeyer is then place under condensor with the end of the condensor is soaked under the H3BO3 solution. Distillation process is carried out until about 15.0 mL of distillate is obtained. The obtained distillate is diluted with distilled water until 50.0 mL, then it is titrated by using HCl 0.02 N. The same procedure is

carried out for the blank solution. Protein content is then calculated in the wet basis (wb) and dry basis (db) by using the correction factor of 6.25 as the following:

$$N(\%) = (v1 - v2) \times NHCl \times 14.007 \times 100 / w$$

with : v1= volume of HCl spent for sample (mL); v2=volume of HCl spent for blank (mL); NHCL= concentration of HCl (0.02N), w=weight of sample (mg)

Protein content (%wb) = % N x convertion factor (6.25)

Protein content (%db) = Protein content (%w.b.) x 100 / (100 – water content)

3.5.1.5 Total Fat (SNI 01-2891-1992)

The total fat content is measured using the soxhlet methods. The Flat-bottom flask is oven dried at 105 °C for 15 min and then cooled in desiccator. It is then weighed before used. As much as 1-2.0 g sample is wrapped in dried filter paper. The filter paper containing the sample is dried at temperature not more than 80 ^{o}C for ± 1 h. It is then placed in the extraction chamber, which is suspended above the solvent flask and below a condenser. The fat is extracted by using hexane for ± 6 hours. The fat extract inside the flask is oven-dried at 105 °C for 12 hours, let cooled in the desiccator and then weighed. The drying process is repeated until the constant weight is obtained. Total fat content is calculated in wet basis (wb) and dry basis (db) with the formula:

Total Fat (%wb) = (a-b) x 100 / c

Total Fat (%db) = Total fat (%wb) x 100 / (100 – water content)

where : a = weight of solvent flask after extraction (g); b = weight of empty solvent flask before extraction (g); dan c = weight of sample (g)

3.5.1.6 Starch Digestibility (Anderson et al., 2002)

In vitro digestibility was determined by using spectrophotometric method. Preperation of standard solution are as followed. Standard used were Maltose was used as the standard and used in the standard solution. 0.25 ml of maltose solution were prepared in test tube (0.25 mg maltose/ ml distilled water). The maltose solution were put into different concentration of 0, 0.05, 0.1, 0.15, 0.20 and 0.25. The solution is then added with 0.75 ml dinitrosalicylic acid solution (DNSA) and was heated in the water bath at 95 C for 12 minutes. The process is then continued with adding 4ml of distilled water and homogenized using vortex. Then 260 µL of each solution was pippeted and put into microplate. The microplate is then read using microplate reader at 520 nm. The regression linear equation were made as a relation between concentration (x – axis) and absorbance (y – axis) .

For the preparation of sample, 1 gram of sample were prepared in Erlenmeyer flask (250 ml) and added with 100 ml of distilled water. Erlenmeyer flask is then covered with aluminum foil and heat in the waterbath at 90° C with constant stirring and then cooled at room temperature. 2 ml of the sample were pippeted into the test tube and add with 3 ml of distilled water and 5 ml of phosphate buffer solution at pH 7. Each sample were made in duplo where one of them is considered as blank. The sample solution is added with 5 ml of enzyme α – amylase solution (1 mg/ ml of phosphate buffer solution pH 7) and for the blank solution, 5 ml of phosphate buffer were added with 5 ml of phosphate buffer solution. Both blank and sample were incubated for 30 minutes. Then 2 ml of DNSA is added and heat in water bath for 12 minutes at 95 C. 4 ml of distilled water is then added and homogenized using vortex. Then 260 µL of each solution is pippeted into the microplate. Then the micro plate is read with miccroplate reader at wavelength 520 nm and done to each micro plate. The starch digestibility were calculated with the formula.

(A –a) /(B –b)

Where, A = maltose in sample (mg) ; a = maltose in blank (mg) ; B = maltose in pure starch (mg) b = maltose in pure starch blank (mg)

3.5.1.7 Amylose Content (IRRI 1978)

The Amylose content will be determined by using spectrophotometric method. Pure amylose will be used a standard solution. For the preparation of the sample, as much as 40.0 mg pure amylose is poured into 100 mL volumetric flask. As much as 1.0 mL ethanol 95% and 9.0 mL NaOH 1 N solution is added into the flask. The solution is heated in waterbath at 95 °C for 10 min. After allowed for a while, the amylose gel is added with distilled water until the tare sign. The amylose solution is used as a stock solution. From the stock solution, pipetted 1.0, 2.0, 3.0, 4.0, and 5.0 mL, respectively, into 100 mL volumetric flask. Acetic acid solution 1 N is added into each test tube 0.2, 0.4, 0.6, 0.8, and 1.0 mL. Then, 2.0 mL iodine solution (0.2 g I2 and 2.0 g KI dissolved in 100.0 mL distilled water) is added and distilled water is added until the tare sign. The solution is allowed for 20 min and the absorbance is measured by using micro plate reader at wavenumber 625 nm. The regression linear equation and standard curve are made as relationship between amylose content in the x axis and absorbance in the y - axis

For the preparation of the sample, starch sample of about 100.0 mg is placed in 100 mL volumetric flask,then added with 1.0 mL ethanol 95% and 9.0 mL NaOH 1 N solution. The solution is heated in waterbath at 95 °C for 10 min. After cooling, the gel solution is added with distilled water until the tare sign and homogenized. As much as 5.0 mL of the gel solution is poured into 100 mL volumetric flask, added with1.0 mL acetic acid 1 N,and 2.0 mL iodine solution, and distilled water until the tare sign. The solution is allowed for 20 min at room temperature before measured by using spectrophotometer UV-Vis at wavenumber 625 nm. Amylose content were calculated with the following formula :

Amylose content (%) = (A / S) * (FP / W) * 100 %

A = Absorbance of sample at 625 nm ; S =slope of standard curve; FP = dilution factor 0.05 ; W = mass of sample

3.5.2 Chemical Analysis

3.5.2.1 Total Fat content

The total fat content present in the fried product that have been fried and coated with different ratio of resistant starh will be analyzed. With knowing the total fat content, determination of which ratio give the significant value of reduced oil content could be done. The method that will be used is according to the (SNI 01-2891-1992) which have been explained in section 3.5.1.3.

3.5.3 Physical Analysis

3.5.3.1 Coating Pick Up

Coating pick up is the total of batter coating that still stick to the product (tempe) after frying. The coating pick up will be calculated according to the following formula

Coating pick-up (%) = B / (B + C) x 100

where B is the mass of the batter coating of one tempe after final frying, and C is the mass of the tempe excluding batter coating after final frying.

3.5.3.2 Water retention Capacity

The water retention capacity will be determined based on the procedure by Sanz et al., (2007) in the previous study. Thirty gram of the batter will be weighed and place in 50 mL centrifuged at 16,300 g for 10 min at 15 ^{o}C in a refrigerated highspeed centrifuge. After centrifugation, the supernatant is removed and weighed. Water retention

capacity is expressed as 100% minus the percentage of water released. The data will be done in triplo.

3.5.4 Sensory Analysis

For the sensory analysis, hedonic scoring test will be used. The purpose of the sensory evaluation is to know the preference and the acceptability of the product compare to control. The given test will be in form of Tempeh that have been coated with different formulation of resistant starch and fried . The sensory attributes that will be observed is “appearance”, “color”, “crispness”, “flavor”, “overall acceptability” and “oiliness”. The test will be conducted to 35 untrained panelists from SGU students.

The samples are presented monadically in plastic tray in random order. Samples will be scored for “appearance”, “color”, “crispness”, “flavor”, and “overall acceptability”. The sensory data will then be analyzed statistically using Friedman’s Test and if there is significant different , the result will be further analyzed using Wilcoxon’s Test