CHAPTER 3

Materials and Methodology

Research design

Study on the development of supplements food from soy germ (soy germ is separated from soybean seeds of tofu industry) using enzyme technology. The experiments were divided into 4 part: 1) The optimal isoflavone glucosides extraction for precursor to isoflavone aglycones production 2) Study of β-glucosidase production from *B. coagulans* PR03 (Isolated from Tua-nao in Chiang-Mai province) 3) The optimal condition for the production of isoflavone aglycones from soy germ 4) Development of health supplements beverage form isoflavone aglycones.

Experiment 1 The optimal isoflavone glucosides extraction for precursor to isoflavone aglycones production

1.1 Study on isoflavone glucosides extraction from soy germ

For this experiment, three isoflavone glucosides extraction methods were used: (1) Supercritical carbon dioxide (SCO_2) (Figure 3.1), (2) Supercritical fluid extraction with co-solvent (SFE- CS) (Figure 3.2) and (3) High-power ultra sonication (HPU) (Figure 3.3) using the same extraction temperature and time of 55 °C for 40 minutes with soy germ 100 g.

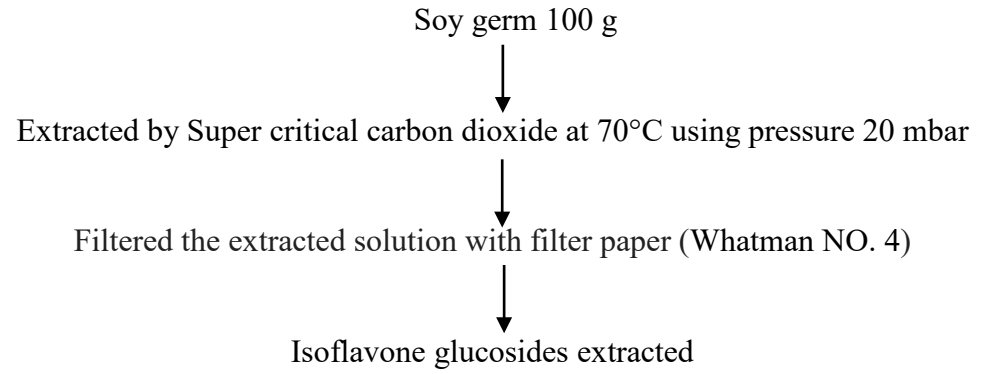

Figure 3.1 Isoflavone extraction with supercritical carbon dioxide

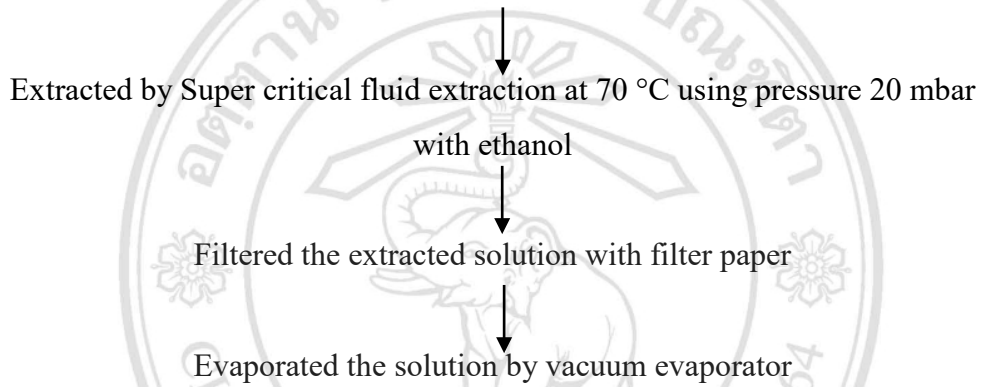

Figure 3.2 Isoflavone extraction with supercritical fluid extraction

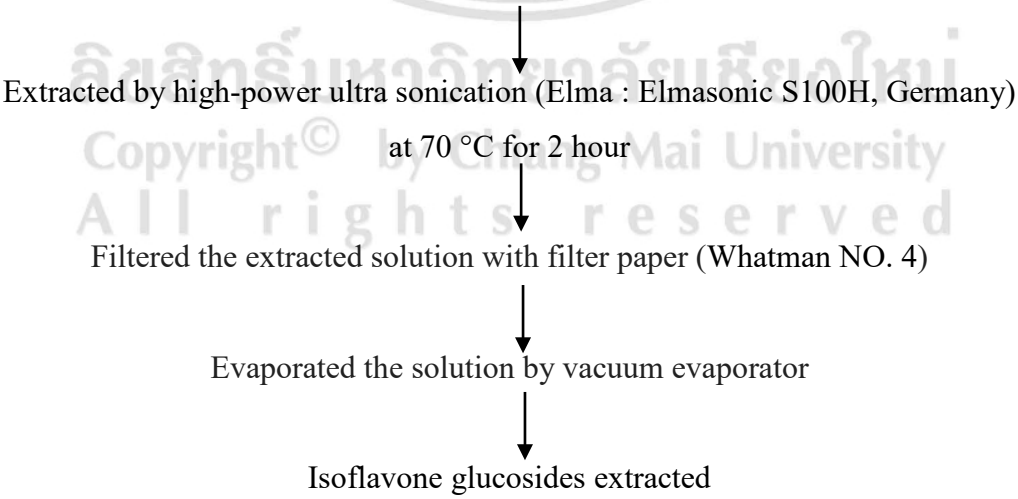

Figure 3.3 Isoflavone extraction with high-power ultra sonication

Then, isoflavone glucosides was analyzed by Klejdus method (2005) using high performance liquid chromatography (HPLC) at a wavelength of 200-350 nm.

1.2 The optimal concentration of ethanol on efficiency isoflavone glucosides extraction

From experiment 1.1, the optimal extraction method was used for extracting isoflavone glucosides was high-power ultra sonication. Then, this experiment studied ethanol concentration for isoflavone glucosides extraction at 4 levels that were 40, 60, 80 and 100%, respectively using completely randomized design (CRD) (Wiriyacharee, 2012).

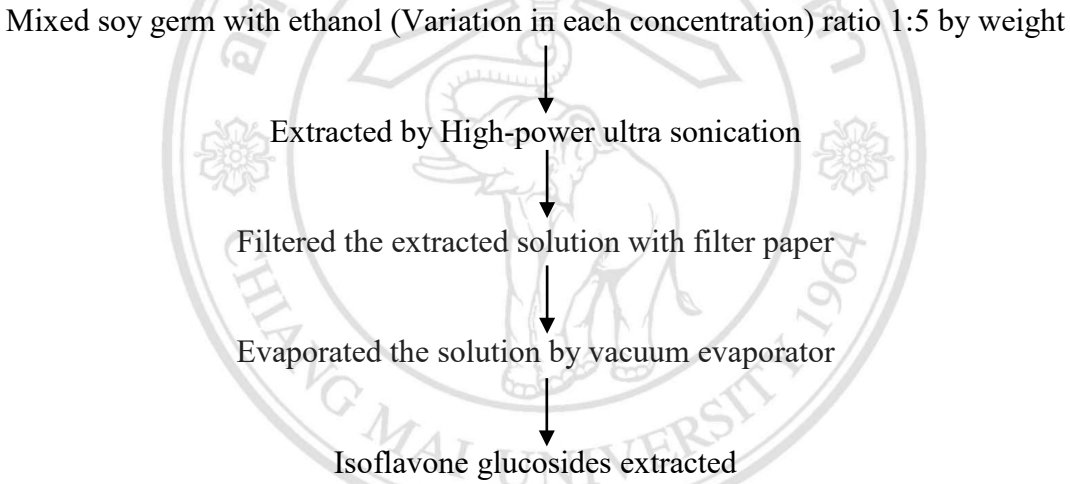

Figure 3.4 Isoflavone extractions from soy germ by variation ethanol concentration

Then, isoflavone glucosides was analyzed by Klejdus method (2005) using high performance liquid chromatography (HPLC) at a wavelength of 200-350 nm.

1.3 Effect of extraction time and temperature on efficiency of isoflavone glucosides from soy germ.

For this experiment, time and temperature of extraction were studied. To investigation the efficiency of isoflavone glucosides extraction as a precursor for isoflavone aglycones production, the condition was varied with extraction time (0-110 minutes) and temperature (30-80 °C) using 2^2 factorial experiment with central composite design (Wiriyacharee, 2012) are shown in Table 3.1.

Table 3.1 Experimental design of 2^2 factorial experiment in central composite design on extraction time and temperature for isoflavone glucosides extraction

Treatments	Code	Extraction temperature (ºC)	Extraction Time (minutes)
1	-1	-1	-1
2	a	1	-1
3	b	-1	1
4	ab	1	1
5	-α a	-1	0
6	+α a	1	0
7	-α b	0	-1
8	+α b	0	1
9	Cp1	0	0
10	Cp2	0	0
Level of code	-1	0	0
	0	55	55
	1	80	110

Then, isoflavone glucosides were analyzed by Klejdus method (2005) using high performance liquid chromatography (HPLC) at a wavelength of 200-350 nm.

1.4 Effect of extraction time investigation on efficiency of isoflavone glucosides from soy germ

From result of the experiment 1.3, it was found that the temperature and time of isoflavone glucosides extraction could be increased for increasing the extraction efficiency. But the temperature limited of high power ultra sonication was maximum at 80 º C. Therefore, in this experiment were studied extraction time at 80, 80, 100, 120, 140, 160 and 180 minutes, respectively at extraction temperature 80 º C. Then, isoflavone glucosides were analyzed by Klejdus method (2005) using high performance liquid chromatography (HPLC) at a wavelength of 200-350 nm.

Experiment 2 Study of β-glucosidase production from *B. coagulans* PR03

2.1 Study of suitable formulas *B. coagulans* PR03 for β-glucosidase production

The media and condition were optimized for the β-glucosidase activity by screening factors affecting β-glucosidase activity which was composed of beef extract, peptone, magnesium sulfate, glucose, pH and incubation temperature. Plackett and Burman design (n = 8) (Wiriyacharee, 2012) was used to determine the high (+) and low (-) level of each factor, as shown in table 3.2 and 3.3.

Table 3.2 The levels of factors for the composition of *B. coagulans* PR03 cultural media and incubation condition at low (-) and high (+) levels affecting β-glucosidase production

Factors	Lower (-)	Upper (+)
A = Beef extract (%)	2	10
B = Peptone (%)	2	10
C = Magnesium sulfate (%)	0.02	0.1
D = Glucose (%)	2	6
E = pH value	5	7
F = Incubation temperature (˚C)	30	50

Table 3.3 Plackett and Burman design for modifying condition factors affecting the β-glucosidase activity

Treatment	A	B	C	D	E	F	G
1	1	1	1	-1	1	-1	-1
2	1	1	-1	1	-1	-1	1
3	1	-1	1	-1	-1	1	1
4	-1	1	-1	-1	1	1	1
5	1	-1	-1	1	1	1	-1
6	-1	-1	1	1	1	-1	1
7	-1	1	1	1	-1	1	-1
8	-1	-1	-1	-1	-1	-1	-1

Note: A = Beef extract, B = Peptone, C = Magnesium sulfate, D = Glucose, E = pH value, F = Incubation temperature (˚C) and G = Dummy variable

B. coagulans PR03 at 10% and 24 hours of incubation were fixed for each experiment. The samples were analyzed for β-glucosidase activity (Appendix B).

2.2 Study on optimal conditions affecting the β-glucosidase production from screened factors.

The results from previous experiment showed that beef extract and pH affected on activity of β-glucosidase activity. Therefore, the levels of beef extract (8-15%) and pH (6-8) were studied using 2^2 factorial design with 2 center points (Wiriyacharee, 2012).

Table 3.4 2^2 factorial experiments with central composite design for find optimal β-glucosidase production

Treatments	Code	Beef extract (%)	pH
1	-1	-1	-1
2	a	1	-1
3	b	-1	1
4	ab	1	1
5	-α a	-1.414	0
6	+α a	1.414	0
7	-α b	0	-1.414
8	+α b	0	1.414
9	Cp1	0	0
10	Cp2	0	0
Level of code	-1.414	8	6
	-1	9.03	6.29
	0	11.5	7
	1	13.97	7.71
	1.414	15	8

B. coagulans PR03 at 10% and 24 hours of incubation were fixed for each experiment. Then, β-glucosidase activity was analyzed.

2.3 Kinetics of β-glucosidase production from *B. coagulans* PR03

Study of kinetics of β-glucosidase production using the optimal conditions for the previous experiment was investigated. The β-glucosidase activity using optical

absorption in optical form (Optical Density ;OD) from spectrophotometer at 620 nm wavelengths and counts of microbial colonies were used to determine the growth rate of microorganisms every 3 hours. Then, the coefficient of β-glucosidase production (q β) was calculated to predict the optimal harvesting time of β-glucosidase.

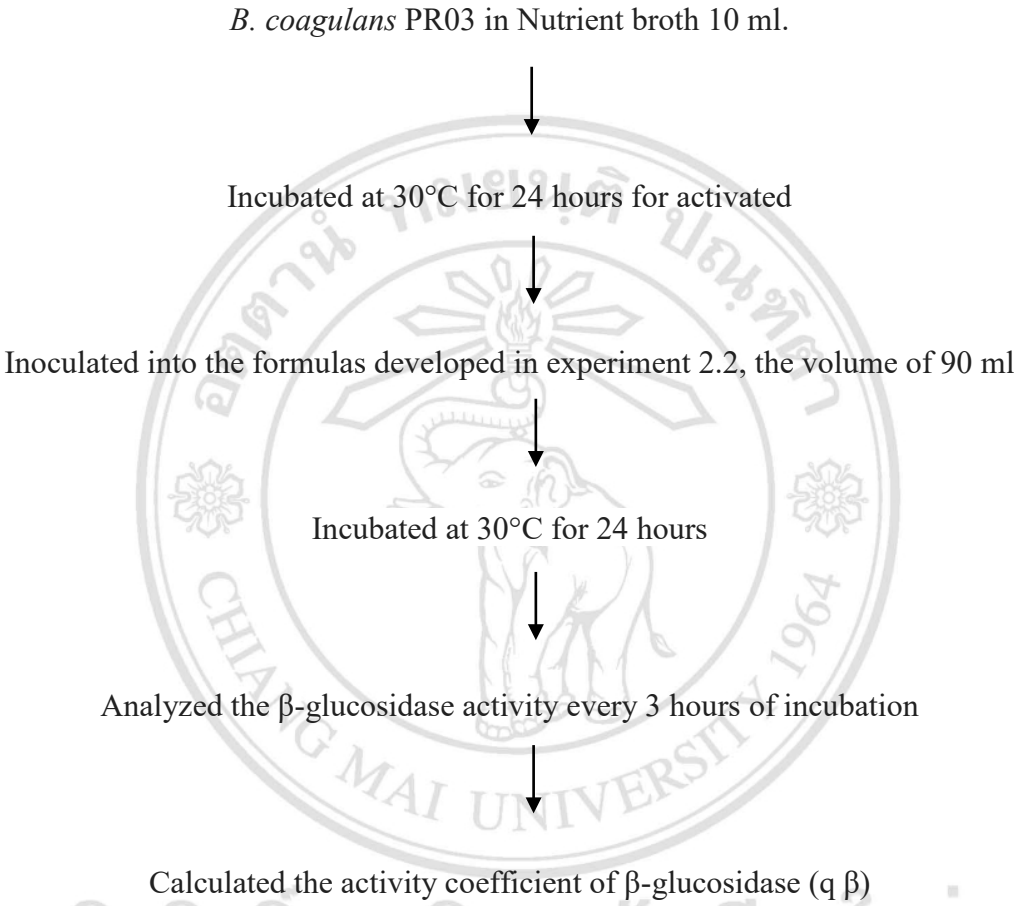

Figure 3.5 The study of the kinetics of β-glucosidase production

Experiment 3 The optimal condition of isoflavone aglycones production from soy germ

The results from experiments 1 and 2 revealed the optimal method for isoflavone glucosides extraction from soy germ and β-glucosidase production from *B. coagulans* PR03.

3.1 Preliminary the optimal time of isoflavone aglycones production

Study on production time of isoflavone aglycones by preparing isoflavone glucosides: *B. coagulans* PR03: distilled water at 1: 1: 8 was varied with incubation time at 24, 48, 72, 96 and 120 hours using completely randomized design (CRD) (Wiriyacharee, 2012). The solution was shaked 200 rpm at room temperature (30 °C). The isoflavone aglycones was analyzed by Klejdus method (2005) using high performance liquid chromatography (HPLC) at a wavelength of 200-350 nm.

3.2 Optimization of time and temperature on isoflavone aglycones production

In addition, the optimal time and temperature of isoflavone aglycones production were also studied using 2^2 factorial experiment with 2 center points (Wiriyacharee, 2012) was designed with the variation of production time (72.0 - 168.0 hours) and temperature (30.0 - 45.0 °C).

Table 3.5 2^2 factorial central composite design for studying time and temperature of isoflavone aglycones production

Treatments	Production time (hours)	Production temperature (ºC)
1	86.06	32.2
2	153.94	32.2
3	86.06	42.8
4	153.94	42.8
5	72	37.5
6	168	37.5
7	120	30
8	120	45
9	120	37.5
10	120	37.5

Then, isoflavone aglycones content was analyzed by Klejdus method (2005) using high performance liquid chromatography (HPLC) at a wavelength of 200-350 nm.

3.3 Study on isoflavone purification using amberlite XAD-4

For this experiment, the optimal method for purification of isoflavone aglycones using amberlite XAD-4 was investigated (Figure 3.6). The optimal ratio of amberlite XAD-4 and isoflavone aglycones solution was studied using amberlite XAD-4 at 50, 100, 150 and 200 g per 100 ml of isoflavone aglycones solution. The experimental design was completely randomize design (CRD) (Wiriyacharee, 2012).

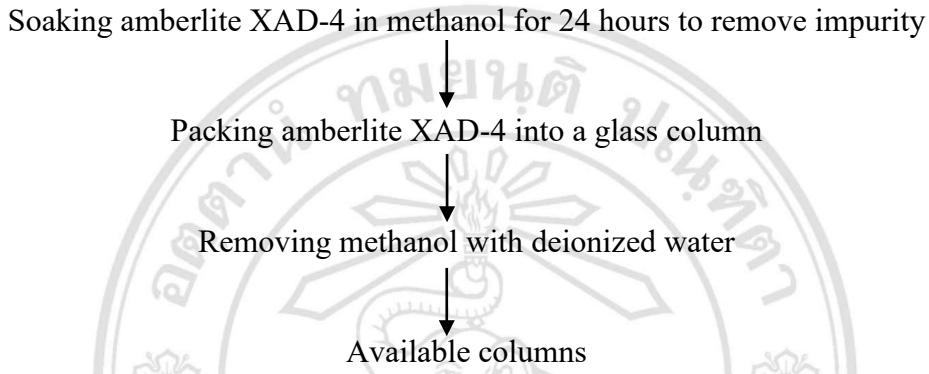

Figure 3.6 Amberlite XAD-4 preparations

The process of purification was modified method of Wu and Lai (2007) that divided into 6 steps (all steps using a flow rate of 1.5 ml/min).

Step 1: crude extract was mixed with 80% ethanol and deionized water in ratio of 1: 2: 4 (w/v/v). Mixed solution was purified by passing through column which isoflavones were absorbed by the resin. The resulting eluent was collected and called fraction 1.

Step 2: column was washed with 400 ml of deionized water to eliminate dissolved solid in water exterior of the resin. The resulting eluent was called fraction 2.

Step 3: column was washed with 20% ethanol and the eluent was collected; fraction 3.

Step 4: column was washed with 40% ethanol and the solution that passed through the column was collected and called fraction 4.

Step 5: column was washed again with 60% ethanol. The resulting eluent (fraction 5) was collected.

Step 6: column was washed with 80% ethanol and the final eluent (fraction 6) was collected.

Then, isoflavone aglycones was analyzed by Klejdus method (2005) using high performance liquid Chromatography (HPLC) at a wavelength of 200-350 nm to analyzed purity of isoflavone aglycones.

Experiment 4 Development of health supplements beverage form isoflavone aglycones

For the experiment 1, 2 and 3, it was found the condition of isoflavone glucosides hydrolysis using β-glucosidase technique was optimized. The prototype of isoflavone aglycones beverage was developed by linear programming. The proportion of passion fruit juice and fructose syrup of prototype product was studied using also 2^2 factorial experiment with 2 center points (Wiriyacharee, 2012) (Table 3.6).

Table 3.6 2^2 factorial experiment with central composite design for study amount of passion fruit juice and fructose syrup

Treatments	Codes	Passion fruit juice (g)	Fructose syrup (g)
1	-1	-1	-1
2	a	1	-1
3	b	-1	1
4	ab	1	1
5	-α a	-1.414	0
6	+α a	1.414	0
7	-α b	0	-1.414
8	+α b	0	1.414
9	Cp1	0	0
10	Cp2	0	0
Level of code	-1.414	60	5
	-1	64.39	6.46
	0	75	10
	1	85.61	13.54
	1.414	90	15

The sensory evaluation for developed products was determined by 60 panelist. Whereas, the final product was evaluated by 104 consumers of 40-60 years old and 96 consumers of over 60 years old and using 7 point hedonic scale and evaluating directions for improvement / development of products. Sensory attribute of color, odor, viscosity, sweetness, sour taste, overall taste and overall acceptance were evaluated.

The developed products were also analyzed for physical, chemical, microbiological, nutritional labeling and packaging as well as testing the acceptance by 200 consumer panelists.