CHAPTER 4

Results and Discussion

4.1 The optimal isoflavone glucosides extraction for precursor to isoflavone aglycones production

4.1.1 Study on isoflavone glucosides extraction from soy germ

For this experiment, three extraction methods were compared: (1) Supercritical carbon dioxide (SCO_2), (2) Supercritical fluid extraction with co-solvent (SFE- CS) and (3) High-power ultra sonication (HPU). The temperature and time of extraction were 55°C for 40 minutes. The extracted isoflavones extract was shown in Table 4.1.

Table 4.1 Isoflavone glucoside content from soy germ by different extraction method

Treatments	Isoflavone glucosides (mg/100g wet weight of soy germ)			
	Daidzin	**Genistin**	**Glycitin**	**Total glucosides**
Supercritical carbondioxide extraction	ND	ND	ND	ND
Supercritical fluid extraction	5.06±0.49	2.53±0.14	1.18±0.38	8.87±0.35
High-power ultra sonication extraction	18.43±0.50	12.46±0.34	4.16±0.14	35.04±0.98

Note: ND; not detected

Comparison with supercritical fluid extraction and high-power ultra sonication extraction (Table 4.1) show that supercritical fluid extraction was not able to extract isoflavone glucosides from soy germ because supercritical fluid extraction can be extracted with low polarity but isoflavone glucosides had a very high polarity. Therefore, the extracted solvent of isoflavone glucosides must be polar solvents such as ethanol or methanol. On the other hand, it was observed that the color of pulp after

extracted related with the isoflavone glucosides content. The color of soy germ pulp extracted from high-power ultra sonication extraction was more brown than soy germ pulp extracted supercritical fluid extraction (as shown in Figure 4.1). Which was relatedly with the higher amount of isoflavone glucosides content (Table 4.1). According to the research of Mauricio (2003), it was found that extraction with high-power ultra sonication extraction with 70% methanol solution for 90 minutes was able to extract all isoflavones up to 311.55 micrograms per gram and the supercritical fluid extraction with methanol solution at 50°C at 360 bar can extract only 86.28 micrograms per gram of soybean flour.

Figure 4.1 Pulp of soy germ after extracted various method

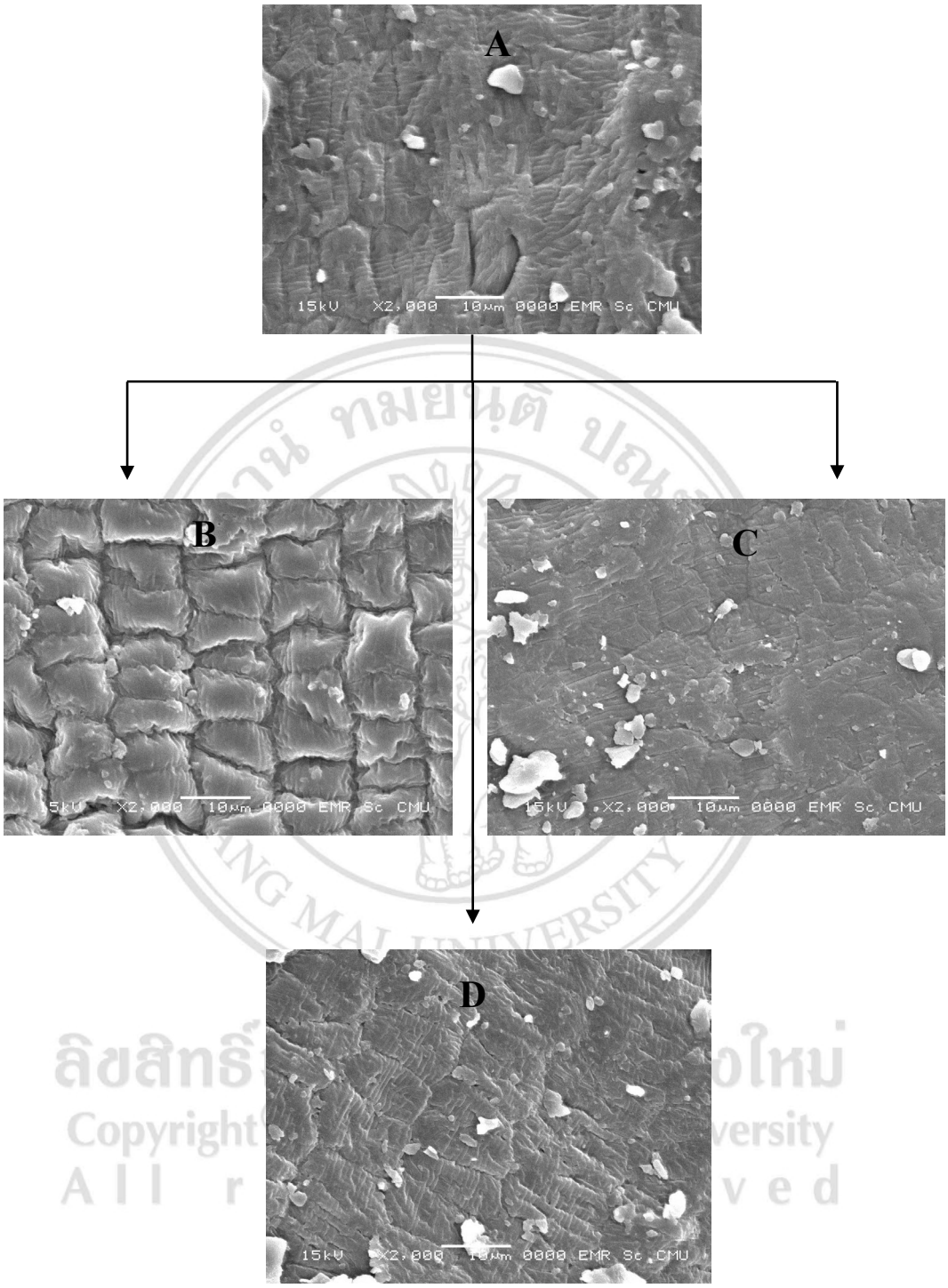

Figure 4.2 Scanning electron microscope (SEM) of soy germ structure was not extracted (A), soy germ structure extracted by high-power ultra sonication extraction with ethanol (B), soy germ structure extracted by supercritical carbondioxide (C), soy germ structure extracted by supercritical fluid extraction with ethanol (D)

Figure 4.2 show the scanning electron microscope of soy germ with different extractions. For high-power ultra sonication extraction method (B), the soy germ structure was fractured when comprised with supercritical carbondioxide (C) and supercritical fluid extraction with ethanol (D). This means that more isoflavone glucosides could be easily liberated from normal soy germ structure (A).Thus, the further experiments are followed by high-power ultra sonication extraction method.

4.1.2 The optimal concentration of ethanol on efficiency isoflavone glucosides extraction

High-power ultra sonication used to extract the isoflavone glucosides in this experiment. Then, the optimal concentration of ethanol for the extraction of isoflavone glucosides was determined. The ethanol concentration varied on 40, 60, 80 and 100%, respectively (Table 4.2).

Table 4.2 Isoflavone glucosides content extracted from ethanol

Concentration of ethanol (%)	**Glucosides' family (mg/100g wet weight of soygerm)**			
	Daidzin	**Genistin**	**Glycitin**	**Total Glucosides**
100	124.63 ± 0.11^{b}	128.32 ± 0.06^{b}	12.19 ± 0.53^{b}	265.17 ± 0.70^{b}
80	130.24 ± 0.45^{a}	137.59 ± 0.49^{a}	16.068 ± 1.27^{a}	280.70 ± 0.43^{a}
60	117.72 ± 0.19^{c}	121.86 ± 0.28^{c}	11.57 ± 0.53^{c}	251.15 ± 0.62^{c}
40	98.45 ± 0.52^{d}	102.76 ± 0.13^{d}	8.04 ± 0.06^{d}	209.25 ± 0.59^{d}
Soy germ	147.53.09+0.52	152.49+0.16	17.67+0.15	317.70 +1.35

Note: Mean within same column with different superscripts were significant difference ($p<0.05$)

Table 4.2 shows that ethanol with concentration of 80% was the best treatment for total isoflavone glucosides extraction which was 280.80 mg/100 g soy germ comprised with the extracted by 100, 60 and 40% of ethanol concentration shows the amount of total isoflavone glucosides 265.17, 251.15 and 209.25 mg/100 g soy germ, respectively. Therefore, 80% ethanol was determined the optimal conditions for extraction of isoflavone glucosides in further experiment.

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

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

Table 4.3 2^2 factorial experiment with central composite design for studied temperature and time of isoflavone glucosides extraction from soy germ

Treatments	Temperature (ºC)	Extraction time (minutes)
1	30	0
2	80	0
3	30	110
4	80	110
5	30	55
6	80	55
7	55	0
8	55	110
9	55	55
10	55	55

Table 4.4 Isoflavone glucosides content of soy germ extract with 80% ethanol at different temperatures and times extraction

Treatments	Glucosides' family (mg/100g wet weight of soy germ)			
	Daidzin	Genistin	Glycitin	Total Glucosides
1	17.72±2.39	9.00±0.39	0.35±0.01	27.55±3.43
2	59.76±2.39	38.40±0.79	1.36±0.15	99.52±3.22
3	149.96±11.44	104.04±1.33	3.11±0.10	257.11±11.78
4	270.23±0.18	247.43±32.32	6.06±0.11	523.72±32.37
5	122.74±0.89	115.33±12.20	2.54±0.01	240.61±11.39
6	189.09±1.51	148.14±6.46	3.45±0.01	340.68±4.95
7	43.07±4.16	31.88±6.67	0.89±0.01	75.84±10.83
8	157.68±1.87	130.29±10.00	2.86±0.08	290.83±11.90
9	128.34±0.28	92.45±0.70	2.43±0.01	223.22±0.99
10	128.44±0.39	86.92±0.81	2.55±0.01	217.90±0.85

The result from Table 4.4, it shows that the amount of isoflavone glucosides composed with daidzin in the range of 17.72-270.23 mg/100g of soy germ, genistin in the range of 9.00-247.43 mg /100 g of soy germ and glycitin in the range of 0.35-6.06 mg /100g soy germ.

The data were analyzed in the form of multiple regressions to describe the correlation between temperature and time at various levels; it was found that the temperature and time of extraction affected the amount of daidzin, genistin and glycitin. It was shown in the regression equation in Table 4.5 and the area of response was shown in Figure 4.3

Table 4.5 The relationship between temperature and time of extraction of isoflavone glucosides

Test parameters	Mathematical model	R^2
Daidzin	$= 105.94 – 3.75 (Temp) + 2.33 (Time) + 0.04 (Temp)^2 - 0.02(Time)^2+0.02(Temp \times Time)$	0.9766
Genistin	$= 133.00 – 4.81 (Temp) + 1.20 (Time) + 0.04 (Temp)^2 - 0.01 (Time)^2 + 0.03 (Temp \times Time)$	0.9468
Glycitin	$= 3.07 - 0.11 (Temp) + 0.03(Time) + 1.12x10^{-3} (Temp)^2 – 2.61 x10^{-4}(Time)^2+ 4.85 x10^{-4} (Temp \times Time)$	0.9343
Total Glucosides	$= 243.27 – 8.69 (Temp) + 3.51 (Time) + 0.09 (Temp)^2 + 0.03 (Time)^2 + 0.05 (Temp \times Time)$	0.9666

Table 4.5 demonstrates that the amount of daidzin, genistin, glycitin and isoflavone glucosides depended on temperature and time of extraction and also relationship interaction between temperature and time of extraction. Increasing of temperature, the amount of daidzin, genistin, glycitin and isoflavone glucosides were decreased. However, when increasing of extraction time, the amount of daidzin, genistin, glycitin and isoflavone glucosides were increased.

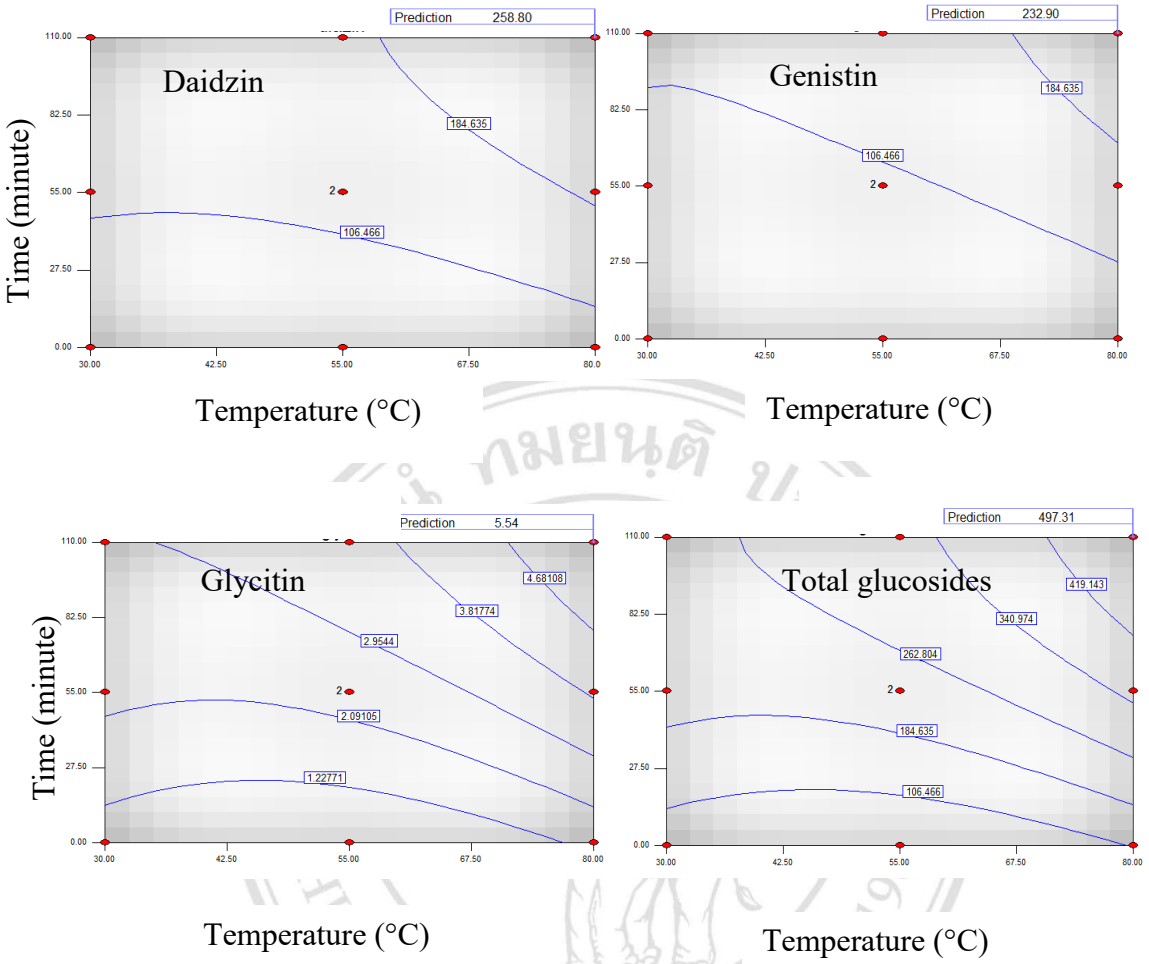

Figure 4.3 Response surfaces of the equation; temperature and time of isoflavone glucosides extraction

Then, the equations given in Table 4.5 were used to determine the optimal temperature and time of extraction. Design Expert 7.10 (Statease Inc., Minneapolis, USA) defines the scoped of the study and the scoped of the desired features (Table 4.6). It was shown that the optimal temperature and time of isoflavone glucosides extraction at 80°C for 110 minutes (Figure 4.4). The responsed suitable area was comprised of daidzin 258.80 mg/100g genistin 232.90 mg/100g glycitin:5.54 mg/100g and total glucosides: 497.31 mg/100g

Table 4.6 Scopes of study factors and required features

Factor	Goal	Lower	Upper	Unit
Temperature	In range	30	80	ºC
Time	In range	0	110	minutes
Parameter	**Goal**	**Lower**	**Upper**	
Daidzin	Maximize	17.72	270.23	mg
Genistin	Maximize	9	104.24	mg
Glycitin	Maximize	0.35	6.6	mg
Total Glucosides	Maximize	27.55	523.72	mg

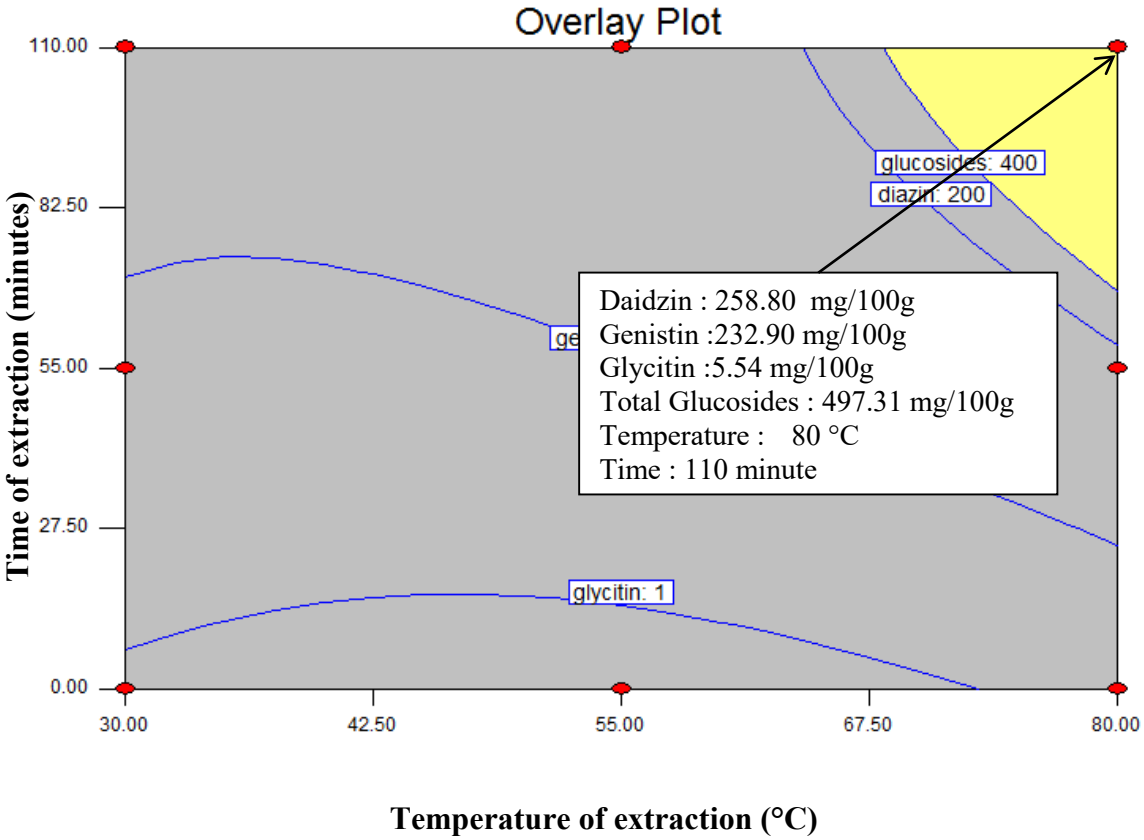

Figure 4.4 Response area of temperature and time extraction on isoflavone glucosides

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

For this experiment, the extraction times using high-power ultra sonication at 80% ethanol (v/v) were studied for in deep in order to get more information about extraction time. The extraction times were varied on 60, 80, 100, 120, 140, 160 and 180 minutes, respectively (Table 4.7). However, the extraction temperature should be controlled at 80 °C as limited of extraction equipment. Which can be conjugated not more than 80 °C.

Table 4.7 The amount of isoflavone glucosides at different extraction time at 80 °C with 80% ethanol

Extraction time (minutes)	**Isoflavone glucosides (mg/100g wet weight of soy germ)**			
	Daidzin	**Genistin**	**Glycitin**	**Total Glucosides**
60	196.55±0.11^{f}	139.32±0.21^{g}	45.09±0.44^{g}	380.96±0.20^{g}
80	216.28±2.89^{e}	152.83±0.31^{f}	49.51±0.22^{f}	418.61±2.42^{f}
100	243.58±3.85^{d}	166.72±0.84^{e}	54.13±0.09^{e}	464.44±4.36^{e}
120	262.87±0.23^{c}	186.34±0.14^{c}	62.25±0.82^{c}	511.45±0.96^{c}
140	268.80±0.89^{b}	190.73±0.59^{b}	63.75±0.48^{b}	523.28±1.87^{b}
160	307.47±0.64^{a}	214.84±0.40^{a}	73.63±0.08^{a}	595.93±1.09^{a}
180	245.16±0.36^{d}	171.12±0.21^{d}	58.46±0.06^{d}	474.74±0.62^{d}

Note: Mean within same column with different superscripts were significant difference (p<0.05)

The results from Table 4.7 shows that the extraction time at 160 minutes was significantly the optimal for isoflavone glucosides extraction, comparing with other extraction times which was 595.93 mg/100 g of soy germ. Therefore, the results could be conclude that the suitable extraction of isoflavone glucosides by high-power ultra sonication which was extracted with 80% ethanol at 80°C for 160 minutes.

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

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

β-glucosidase activity producing from *B. coagulans* PR03 was studied by preliminary screening factors affecting β-glucosidase enzyme formation. The factors composed with beef extract, peptone, magnesium sulfate, glucose, pH and incubation temperature using Plackett and Burman design (n=8) (Wiriyacharee, 2012) with the high (+) and low (-) level of each factor, as shown in Table 3.2 and each treatment shown in Table 3.3.

B. coagulant PR03 (10%) was incubated for 24 hours at anaerobic fermentation and then was added in each treatment. The samples were analyzed for β-glucosidase activity as shown in Table 4.8.

Table 4.8 β-glucosidase activity using Plackett and Burman design (n=8)

Treatments	β-glucosidase activity (mU/ml)
1	3.6
2	0.61
3	1.61
4	0.93
5	2.48
6	2.46
7	0.36
8	0.36

Note: 1 unit of enzyme is the amount of enzyme which can cause *p*-Nitrophenol 1 milligram per minutes from *B. coagulans* PR03 culture in 1 ml media.

Table 4.8 showed that the β-glucosidase activity was in the range of 0.36 - 3.60 mU/ml. The result of the experiment was calculated the effect of each factor on β-glucosidase activities. The result was shown in Table 4.9.

Table 4.9 Effect of *B. coagulans* PR03 on β-glucosidase activity

Factors	β-glucosidase activity (mU/ml)	
	Effect	**Calculated t-test**
A = Beef extract	0.01	3.536*
B = Peptone	-0.003	-1.179
C = Magnesium sulfate	0.009	3.071
D = Glucose	-0.001	-0.5
E = pH value	0.016	5.500*
F =Incubation temperature (˚c)	-0.004	-1.393

Note: *significant difference at 80% (t-table = 3.078)

The results showed that beef extract and pH had a positive effect (p<0.2) on β-glucosidase activity. Therefore, beef extract and acidity condition were also investigated to find the appropriate level the next experiment. Additionally the beef extract and acidity were appropriate factors to study at the higher level.

However, the other factors were non- significant effect (p> 0.20), such as peptone, magnesium sulfate, glucose and incubation temperature. When considering the effect on the β-glucosidase activity, magnesium sulfate had a positive effect; therefore, magnesium sulfate at high levels was chosen. Whereas peptone, glucose and incubation temperature had negative effect, glucose and incubation temperature at low level were selected for the next investigation.

The screening condition of *B. coagulans* PR03 culture affecting on β-glucosidase activity and the optimal condition were summarized find cultural media was comprised of peptone 2.00 %, magnesium sulfate 0. 10 % , glucose 2.00 % and optimal incubation temperature was 30 °C. Factors should be studied in the further experiment were Beef extract (8-15 %) and also pH condition (6-8)

4.2.2 Study on optimal conditions affecting the β-glucosidase production from beef extract and acidification

In the previous experiment, beef extract and pH value were screened for β-glucosidase production. Therefore, this experiment studied both factors using 2^2 factorial experiment with 2 center points (Wiriyacharee, 2012). The analyzed β-glucosidase activity were shown in Table 4.10.

Table 4.10 The β-glucosidase activities from varied beef extract and pH value

Treatment	Beef extract (%)	pH value	β-glucosidase activity (mU/ml)
1	9.03	6.29	1.68
2	13.97	6.29	1.74
3	9.03	7.71	2.35
4	13.97	7.71	3.8
5	8	7	2.15
6	15	7	2.53
7	11.5	6	0.92
8	11.5	8	3.09
9	11.5	7	2.87
10	11.5	7	2.67

Note: 1 unit of enzyme is the amount of enzyme which can cause *p*-Nitrophenol 1 milligram per minutes from *B. coagulans* PR03 cultured in 1 ml media.

Table 4.10 shows that beef extract and pH value affected on β-glucosidase activity which were the range of 0.92 - 3.80 mU/ ml. The regression equation was shown in Table 4.11 and the response area was shown in Figure 4.5.

Table 4.11 Relationship of beef extract and pH value on β-glucosidase activity

Test parameter	Mathematical model	R^2
β-glucosidase activity	$= -25.38 - 0.68\,(\text{beef extract}) + 7.96\,(\text{pH}) - 0.03\,(\text{beef extract})^2 - 0.66\,(\text{pH})^2 + 0.20\,(\text{beef extract})(\text{pH})$	0.9577

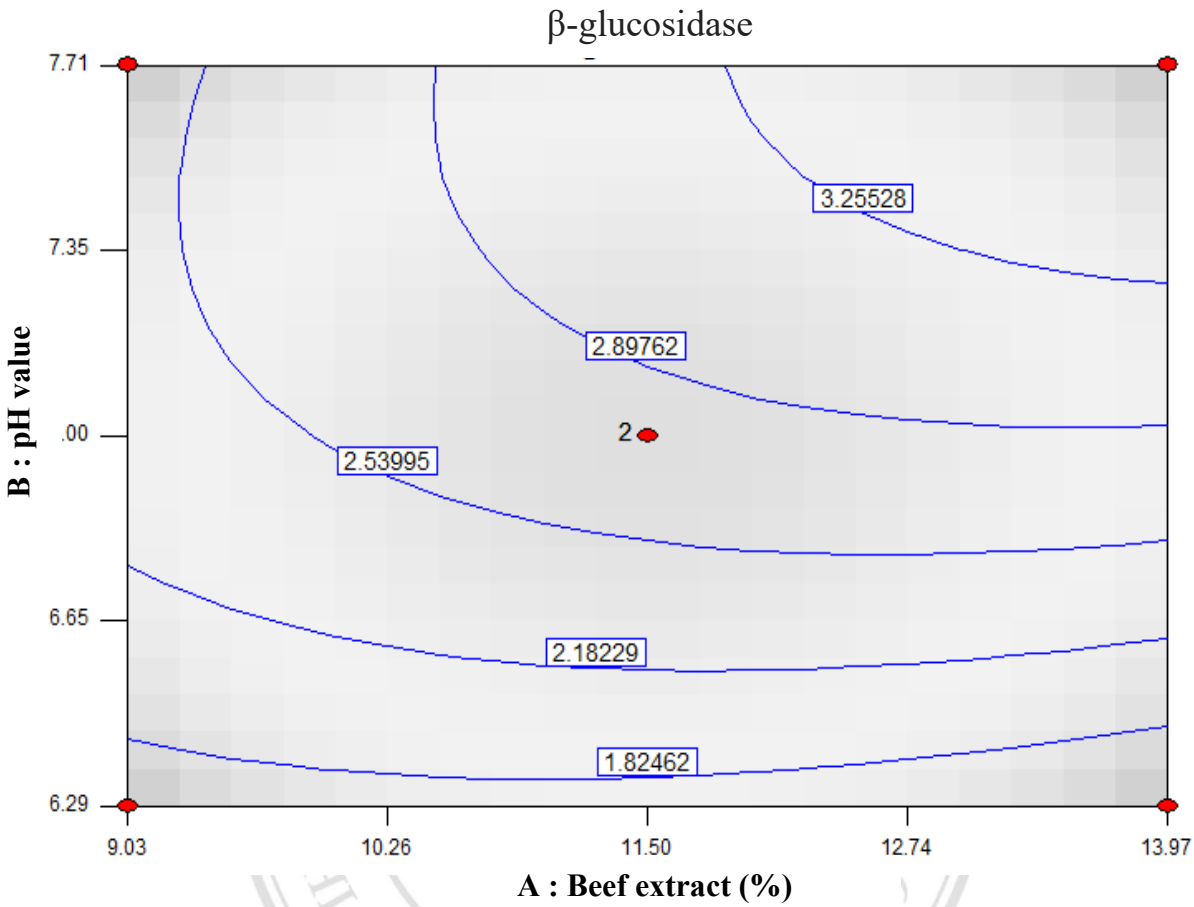

Figure 4.5 Response area of the correlation between beef extract and pH value on β-glucosidase activity

The correlation coefficient of β-glucosidase was shows in Table 4.11 and Figure 4.5. Whereas increasing amount of beef extract and pH value, β-glucosidase activity increased.

The scope of factors and required features of beef extract and pH values was shows in Table 4.12. The results showed that the optimal amount of beef extract and pH value were 14.84 % and 7.96, respectively, as shown in Figure 4.6.

Table 4.12 Scope of factors and required features

Factor	Goal	Lower	Upper	
Beef extract	In range	8	15	%
pH value	In range	6	8	
Factor	**Goal**	**Lower**	**Upper**	
β-glucosidase activity	In range	3	4	mU/ml

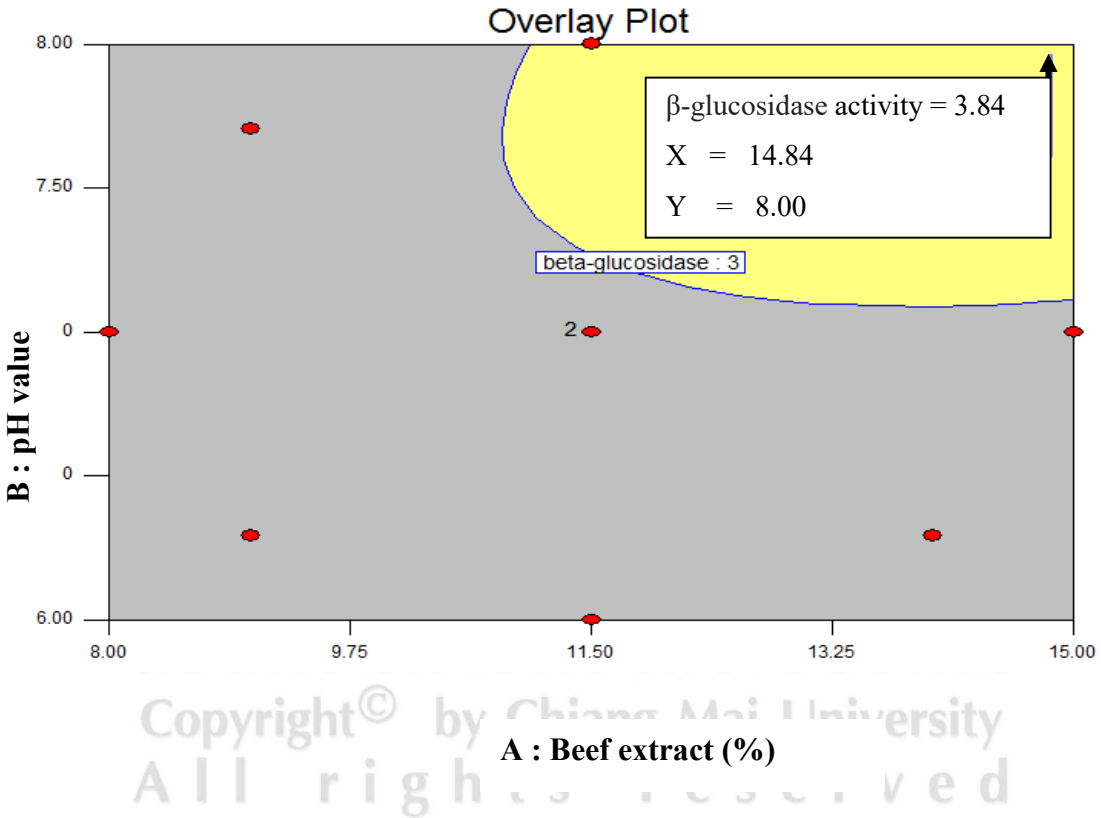

Figure 4.6 Response area of beef extract and pH value on β-glucosidase activity

The results from experiment 4.2.1-4.2.2 showed that the optimal conditions for *B. coagulans* PR03 culture as follows; peptone 2.00%, beef extract 14.84%, glucose 2.00%, magnesium sulfate 0.10%, incubation temperature 30 ° C and adjusted pH value to 8.

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

The kinetics of β-glucosidase production was studied using the optimal conditions for the production from experiment 4.2.1-4.2.2to determine the appropriate incubation time. The β-glucosidase activity was analyzed using optical density (OD) measurement from the spectrophotometer at 620 nm of wavelength. In addition, number of colony was counted to measure the growth rate of the microorganisms every 3 hours. The process of kinetics study was shown in Figure 4.7.

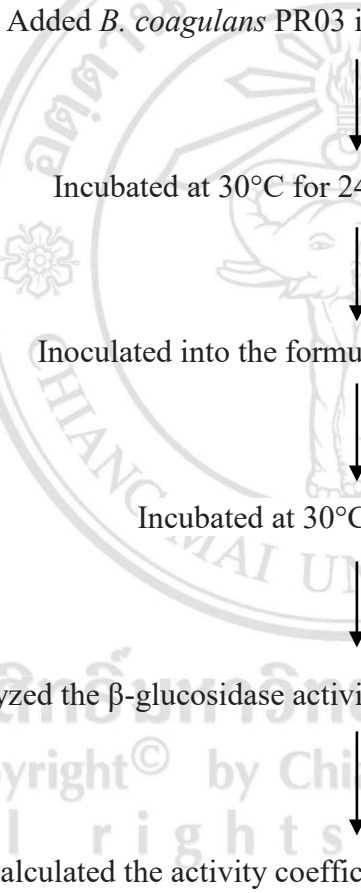

Figure 4.7 The study of the kinetics of β-glucosidase production

Table 4.13 Optical density, number of colony and β-glucosidase activities at different incubation times of *B. coagulans* PR03

Incubation time (hours)	Optical density (wavelength 602 nm)	Number of colony (log CFU/ml)	β-glucosidase activity (mU/ml)
0	0.04±0.01	6.65±0.07	0.40±0.13
3	0.05±0.03	7.45±0.01	1.51±0.12
6	0.16±0.01	8.30±0.09	2.08±0.48
9	0.42±0.04	8.65±0.03	2.67±0.77
12	0.63±0.01	10.46±0.02	2.50±0.32
15	0.70±0.02	10.85±0.10	3.10±0.70
18	0.74±0.03	10.49±0.13	4.01±0.13
21	0.77±0.01	10.11±0.10	3.96±0.19
24	0.75±0.01	9.98±0.03	3.94±0.17

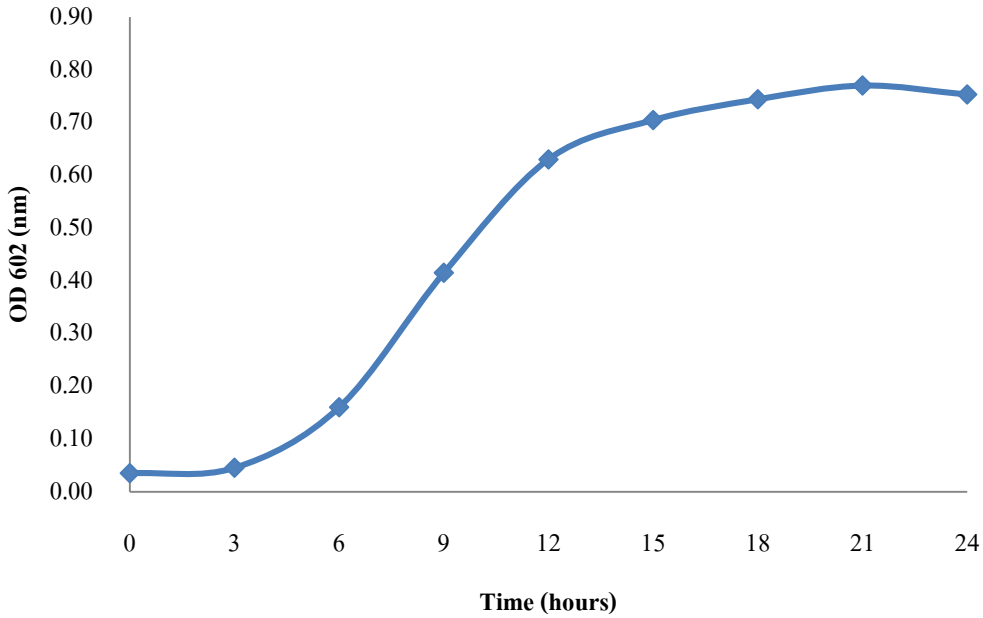

Figure 4.8 Optical densities of *B. coagulans* PR03 at different incubation times

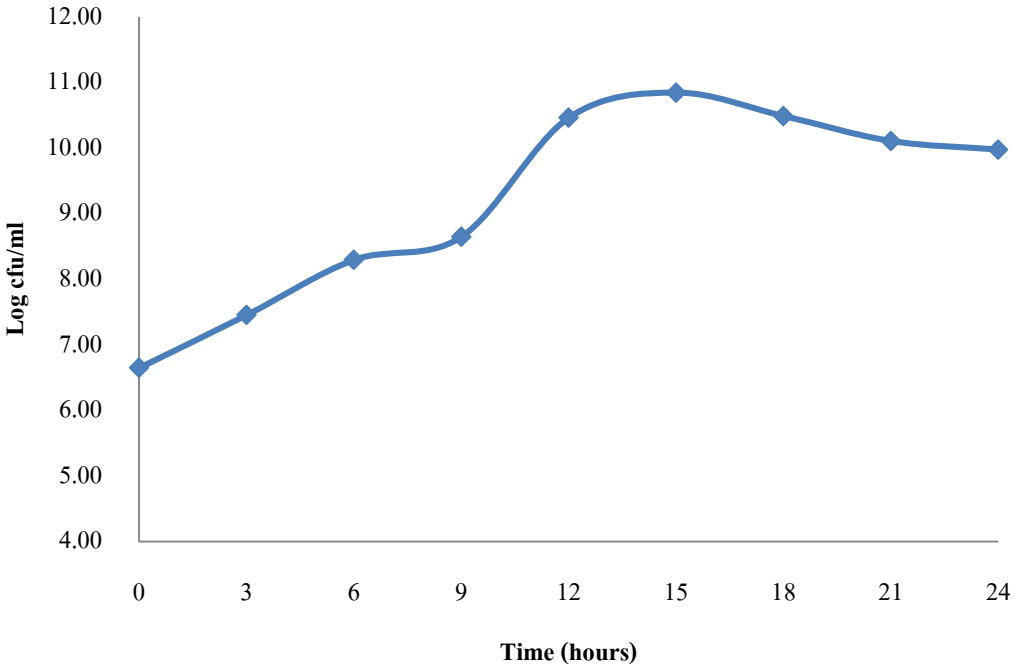

Figure 4.9 Number of colonies of *B. coagulans* PR03 at different incubation time

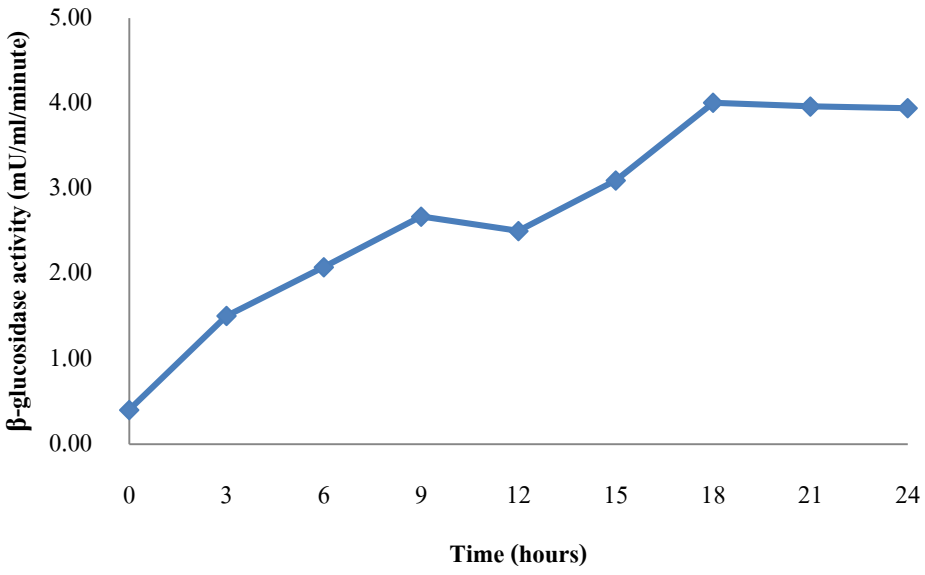

Figure 4.10 β-glucosidase activity at different incubation time

Growth rate of *B. coagulans* PR03

In the study of growth rate of *B. coagulans* PR03, the log phase was in the range of 3-12 hours. Specific growth rate (μ) of *B. coagulans* PR03 was found and the time period affected to cell division in generation time.

Growth rate $dx/dt = \mu X$ ----------Equation 1

Then dx = Increased value of biomass at different times dt

$dx/x = \mu \cdot dt$ ----------Equation 2

When Integrate Equation 2,

$Xt = X_0 \cdot e^{\mu t}$ ---------- Equation 3

Then; X_0 = Biomass at initiate time (t=0)

X_t = Biomass or turbidity value after culture at t hour

The natural logarithm in Equation 3 was

$\ln Xt = \ln X_0 + \mu t$ ---------- Equation 4

Therefore , the equation was $\mu = \ln (X_t/X_0)/t$ ---------Equation 5

μ = Specific growth rate

The specific growth rate (μ) is expressed in Equation 6

Generation time (T) $= \ln 2/\mu$ ---------- Equation 6

Equation 5 was used to calculate specific growth rate of *B. coagulans* PR03 using the data presented in Table 4.13.

Specific growth rate of *B. coagulans* PR03

μ = $[\ln (X_{15}/X_0)] - [\ln (X_3/X_0)] /t$

= $[\ln (0.70/0.04)] - [\ln (0.\ 05/0.04\ )]/(15-3)$

= $0.22\ hr^{-1}$

Generation time *B. coagulans* PR03 = $\ln 2/ \mu$

= $\ln 2/0.22$

= 3.15 hour

Calculations of *B. coagulans* PR03 showed that the growth rate was 0.22 CFU per hour and increased from 10^1 to 10^2 over time, every 3.15 hours.

Kinetics of β-glucosidase activity production.

Efficiency of β-glucosidase activity production.

Process product yield (Y_β) = ($P_{\beta 1}$- $P_{\beta 0}$)----------Equation 7

Then; Y_β = Efficiency of β-glucosidase activity production

$P_{\beta 1}$ = The final of β-glucosidase activity

$P_{\beta 0}$ = Initiate of β-glucosidase activity

Increasing of product = Product creation – Destroyed product

$dP/dt = (qpX)-kP$ ---------- Equation 8

Because the incubation process is a shift. Therefore, the destroyed product is very small compared to the product created during incubation. Therefore, no kP was used in this equation, and the new equation can be written as

$dP/dt = (qpX)$ ----------Equation 9

$$(1/X) \bullet dP/dt = qp \qquad \text{---------- Equation 10}$$

Then; qp = Specific product formation rate

X = The amount of microorganisms

dP/dt = Increasing of product

$$qp/qs = dP/ds \qquad \text{----------Equation 11}$$

And Equation 7 equals Equation 11

$$Yp/s = qp/qs = dP/ds \qquad \text{----------Equation 12}$$

When dividing equation 12 with equation 1

$$qp/\mu = [(1/X)dP/dt]x[X \bullet dt/dX] \qquad \text{----------Equation 13}$$

$$qp/\mu = dp/dX = Yp/x \qquad \text{----------Equation 14}$$

$$qp = (dp/dX) \bullet \mu \qquad \text{----------Equation 15}$$

Then; dp = Rate of change of initiate and final products

dX = Rate of change of volume at 0 to 18 hours

μ = Specific growth rate *B. coagulans* PR03 at 0.22 $hour^{-1}$

Therefore, it was possible to calculate the specific production rate from equation 15 using the results from hours 0 to 18 to calculate the β-glucosidase activity.

$$q_{\beta\text{-glucosidase}} = (dp/dX) \bullet \mu$$

$$= [(4.01-0.40)/(10.49-6.65)] \times 0.22$$

$$= 0.21 \text{ mU/ml/hour}$$

Calculations of *B. coagulans* PR03 showed that the growth rate was 0.22 CFU per 1 hour and *B. coagulans* PR03 was increased from 10^1 to 10^2 cell every 3.15 hours.

The calculation of specific production rate from equation 15 using the experimental results from hours 0 to 18 to calculate the β-glucosidase. It was shown 0.21 mU/ml/hour specific β-glucosidase formation rate for this case.

4.3 The optimal condition of isoflavone aglycones production from soy germ

From experiments 1 and 2 revealed that the optimal method for isoflavone glucosides extraction and β -glucosidase production from *B. coagulans* PR03. However , from the preliminary experiment found that β-glucosidase from *B. coagulant* PR03 was not able to change structure from isoflavone glucosides to aglycones. Therefore, it was decided to use *B. coagulant* PR03 in combination with isoflavone glucosides extract for production.

4.3.1 Preliminary the optimal time of isoflavone aglycones production

The production time of isoflavone aglycones were studied by preparing isoflavone aglycones the ratio of isoflavone glucosides solution: *B. coagulans* PR03 : deionize water at 1: 1: 8, incubation times were varied on 24, 48, 72, 96 and 120 hours using completely randomized design (CRD) (Wiriyacharee, 2012). The solution was shaked 200 rpm at room temperature (°C). Then, isoflavone aglycones were determined by High Performance Liquid Chromatography (HPLC), the result was shown at Table 4.14 and Table 4.15.

Table 4.14 Isoflavone glucosides content at different incubation time

Treatments	Incubation time (hours)	Isoflavone glucosides (µg/ml)			
		Daidzin	Genistin	Glycitin	Total Glucosides
1	0	136.75±1.34^{a}	81.31±0.78^{a}	29.35±0.27^{a}	247.41±0.65^{a}
2	24	131.09±2.84^{b}	83.84±1.93^{a}	27.43±0.91^{b}	242.36±1.85^{a}
3	48	84.21±0.68^{c}	81.98±0.90^{a}	22.41±0.05^{d}	188.60±0.44^{b}
4	72	66.50±1.51^{d}	83.64±1.84^{a}	21.86±0.67^{d}	172.00±0.23^{c}
5	96	69.46±0.77d^{e}	91.75±1.44^{b}	23.52±0.21^{c}	184.73±1.02^{b}
6	120	71.56±1.84^{e}	89.68±2.06^{b}	24.27±0.66^{c}	185.51±1.20^{b}

Note: Mean within same column with different superscripts were significant difference (p<0.05)

Table 4.15 Isoflavone aglycones content at different incubation time

Treatments	Incubation time (hours)	Isoflavone aglycones (µg/ml)			
		Daidzein	Genistein	Glycitein	Total Aglycones
1	0	10.36±0.07^{e}	31.11±0.44^{e}	5.48±0.03^{f}	46.95±0.34^{f}
2	24	16.91±0.49^{d}	35.30±1.03^{d}	9.97±0.29^{e}	62.18±1.29e^{f}
3	48	40.00±0.15^{c}	41.32±0.72^{c}	8.25±0.34^{d}	89.57±0.65^{d}
4	72	52.40±1.74^{b}	50.38±1.66^{b}	17.85±0.67^{c}	120.64±0.66^{c}
5	96	57.25±0.79^{a}	54.36±0.15^{a}	19.40±0.33^{b}	131.01±0.56^{b}
6	120	58.66±1.60^{a}	54.63±1.43^{a}	20.89±0.77^{a}	134.19±1.06^{a}

Note: Mean within same column with different superscripts were significant difference (p<0.05)

The results from Table 4.14 and Table 4.15 showed that increasing of production time, isoflavone glucosides was decreased whereas isoflavone aglycones was increased. The total isoflavone glucosides was reduced at 120 hours from 247.41 µg/ml to 185.51 µg/ml, However, total isoflavone aglycones was increase at 0 hours from 46.95 µg/ml to 134.19 µg/ml.

4.3.2 Optimization of time and temperature on isoflavone aglycones production

The time and temperature of isoflavone aglycones production were studied using 2^2 factorial experiment with 2 center point (Wiriyacharee, 2012) varied by production time from 72.00 - 168.00 hours and temperature of 30 - 45°C.

Table 4.16 2^2 factorial with central composite design for studying the effect of production time and temperature on isoflavone aglycones production

Treatment	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

Table 4.17 Isoflavone aglycones production at different times and temperatures

Treatment	Aglycones' family (µg/ml)			
	Daidzein	Genistein	Glycitein	Total Aglycones
1	30.79±0.18	42.12±0.72	12.36±0.12	85.27±0.35
2	43.71±0.13	45.68±0.15	16.45±0.28	105.84±0.11
3	10.64±1.07	29.87±3.21	6.07±0.41	46.57±0.21
4	11.25±0.12	31.80±1.02	9.40±0.16	52.44±0.32
5	45.42±0.13	43.81±3.78	16.61±2.91	105.84±0.75
6	50.81±0.21	46.65±1.37	13.48±2.46	110.94±0.88
7	45.02±0.34	51.24±0.52	19.89±0.66	116.15±0.45
8	11.93±0.06	33.02±0.65	11.16±0.18	56.11±0.12
9	51.26±0.81	50.28±0.81	12.33±0.20	113.88±0.65
10	51.31±0.06	48.45±0.09	12.15±0.08	111.92±0.33

Table 4.17 showed that isoflavone aglycones content composed of daidzein, genistein, glycitein and total aglycones in the range of 10.64-51.31, 29.87-51.24 , 6.07-19.89 and 46.57-116.15 µg/ml, respectively.

The data was analyzed in the form of multiple regressions to describe the correlation between production time and temperature at various levels. The mathematical model and response surface of correlation of production time and temperature affected to daidzein glycitein and total isoflavone aglycones as follows Table 4.18 and Figure 4.10 respectively

Table 4.18 The relationship between production time and temperature of isoflavone aglycones composition

Test parameters	Mathematical model	R^2
Daidzein	= -759.20 + 1.78 (Time) +39.59 (Temp) – 0.02 (Temp x Time) – 0.004 $(Time)^2$ – 0.53 $(Temp)^2$	0.8515
Glycitein	= -224.70 + 0.88 (Time) + 12.91 (Temp) – 0.02 (Temp x Time) – 0.003 $(Time)^2$ – 0.18 $(Temp)^2$	0.8554
Total isoflavone aglycones	= -921.19 + 2.64 (Time) + 50.45 (Temp) – 0.02 (Temp x Time) – 0.007 (Time)2 – 0.69 (Temp)2	0.8210

Table 4.18 showed that the amounts of daidzein, glycitein and total isoflavone aglycones depended on production time, temperature and interaction between production time and temperature. Increasing of production time and temperature, the amount of daidzein, glycitein and total isoflavone aglycones were increased.

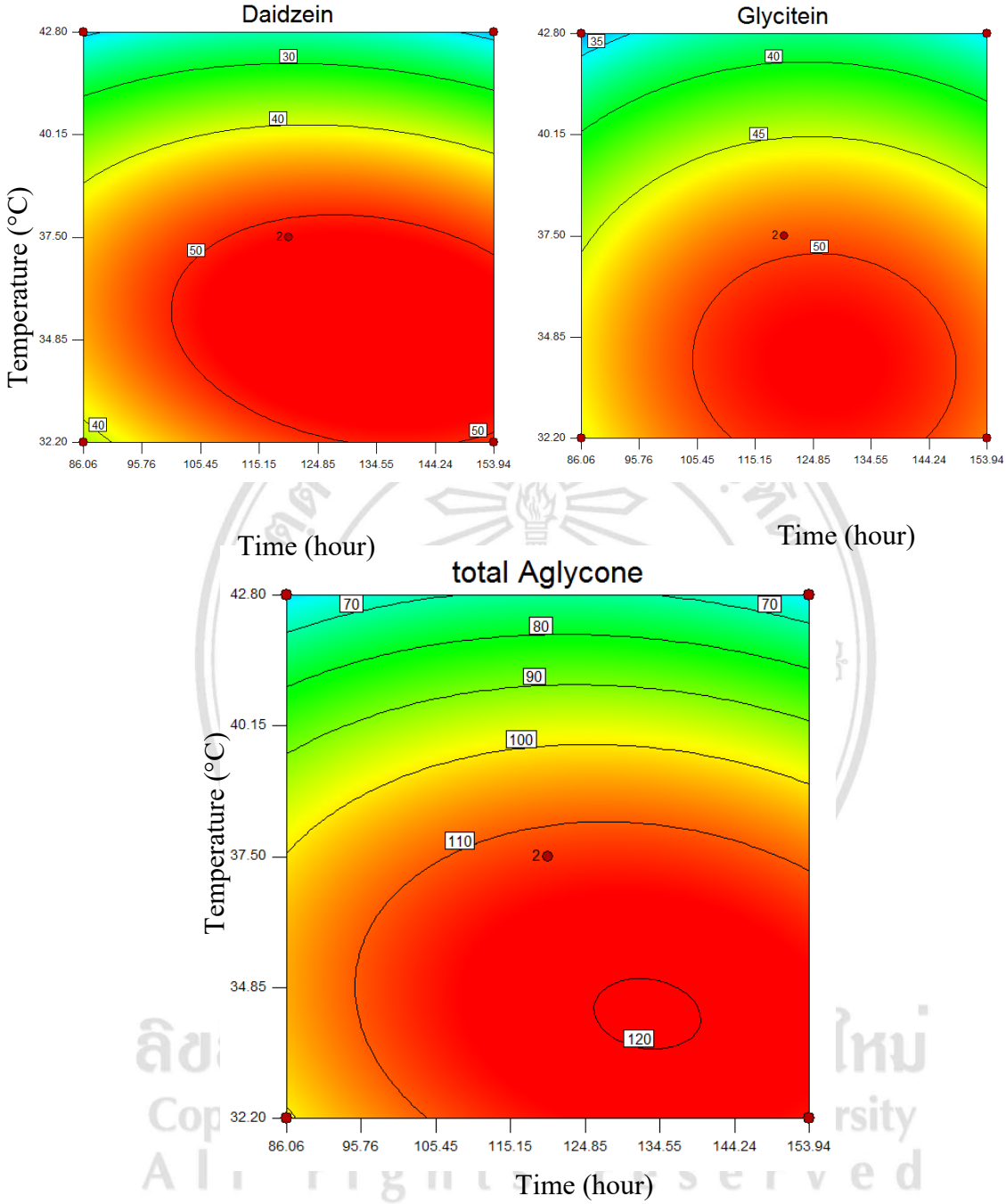

Figure 4.11 Response surfaces of correlation production time and temperature on isoflavone aglycones

The equation given in Table 4.18 showed the suitable production time and temperature of isoflavone aglycones using design expert 7.10 (Statease Inc.,

Minneapolis, USA) which was defined the scope of the study and the scope of properties as Table 4.19. The production time and temperature of isoflavone aglycones were 37.50°C for 120 hours as shown in Figure 4.12.

Table 4.19 Scope of studied factors and properties

Factor	Goal	Lower	Upper	
Time	In range	86.06	153.94	hours
Temp	In range	32.2	42.8	ºC
Factor	**Goal**	**Lower**	**Upper**	
Daidzein	Maximize	31.36	71.2	µg
Genistein	Maximize	40.49	58.24	µg
Glycitein	Maximize	5.07	19.4	µg
Total Aglycones	Maximize	77.8	147.98	µg

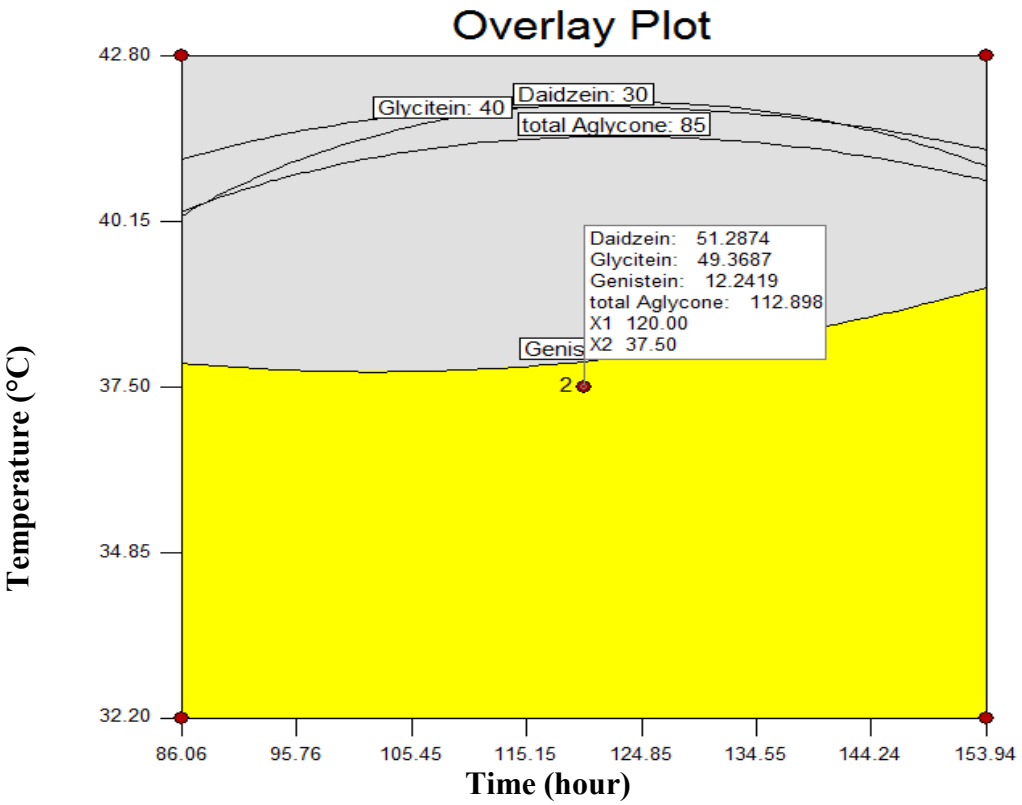

Figure 4.12 Response surfaces of production time and temperature on isoflavone aglycones

4.3.3 Study on isoflavone purification using amberlite XAD-4

For this experiment, the optimal method for isoflavone aglycones purification using amberlite XAD-4 was studied by varying the suitable ratio of amberlite XAD-4 and isoflavone aglycones solution. Amberlite XAD-4 resin was studied at 50, 100, 150 and 200 g per 100ml isoflavone aglycones solution by completely randomized design (CRD). Then, also isoflavone aglycone were analysed by HPLC method and percentage purity of isoflavone aglycones were calculated (Table 4.20 - Table 4.23)

Table 4.20 Percentage of isoflavone aglycones purity at amberlite XAD-4 50 g per 100 ml isoflavone aglycones solution

Amberlite XAD-4 in column 50 g.	Fraction	Operating volume (ml)	Mass of concentrate (mg)	Mass ratio of Concentrate (%)	Mass of aglycones (mg)	Yield of aglycones (%)	Purity of aglycones (%)
Adsorption	1	100	271.06	74.32	5.34	27.87	1.97
Washing (DI water)	2	100	29.03	7.96	0.36	1.87	1.24
Step-gradient elution							
20% ethanol	3	100	4.41	1.21	0.15	0.78	3.4
40% ethanol	4	100	11.2	3.07	2.84	14.87	25.35
60% ethanol	5	100	15.71	4.31	6.75	35.23	42.95
80% ethanol	6	100	33.33	9.14	3.71	19.38	11.13
Total		-	364.74	100	19.16	100	

Note: The initial purity of isoflavone was 5.25%

Adsorption = isoflavone aglycones solution from crude isoflavone aglycones: ethanol 80%: DI water at 1: 2: 4

Operating volume (ml) = solution was used to each columns in each fraction.

Mass of concentrate (mg) = solid in each fraction

Mass ratio of concentrate (%) = (mass of concentrate; mg) /total mass of concentration) x 100

Mass of aglycones (mg) = isoflavone aglycones in each fraction

Yield of aglycones (%) = (mass of aglycones; mg)/total mass of aglycones) x 100

Purity of aglycones (%) = (mass of aglycones; mg/mass of concentrate ; mg) x 100

Table 4.21 Percentage of isoflavone aglycones purity at amberlite XAD-4 100 g per 100 ml isoflavone aglycones solution

Amberlite XAD-4 in column 100 g.	**Fraction**	**Operating volume (ml)**	**Mass of concentrate (mg)**	**Mass ratio of concentrate (%)**	**Mass of aglycones (mg)**	**Yield of aglycones (%)**	**Purity of aglycones (%)**
Adsorption	1	100	271.42	78.92	5.32	26.29	1.96
Washing (DI water)	2	100	20.12	5.85	0.33	1.64	1.64
Step-gradient elution							
20% ethanol	3	100	2.34	0.68	0.14	0.67	5.99
40% ethanol	4	100	11.29	3.28	3.62	17.89	32.05
60% ethanol	5	100	14.55	4.23	7.29	36.02	50.11
80% ethanol	6	100	24.18	7.03	3.55	17.49	14.68
Total		-	343.9	100	20.25	100	

Note: The initial purity of isoflavone was 5.25%

Adsorption = isoflavone aglycones solution from crude isoflavone aglycones: ethanol 80%: DI water at 1: 2: 4

Operating volume (ml) = solution was used to each columns in each fraction.

Mass of concentrate (mg) = solid in each fraction

Mass ratio of concentrate (%) = (mass of concentrate; mg) /total mass of concentration) x 100

Mass of aglycones (mg) = isoflavone aglycones in each fraction

Yield of aglycones (%) = (mass of aglycones; mg)/total mass of aglycones) x 100

Purity of aglycones (%) = (mass of aglycones; mg/ mass of concentrate; mg) x 100

Table 4.22 Percentage of isoflavone aglycones purity at amberlite XAD-4 150 g per 100 ml isoflavone aglycones solution

Amberlite XAD-4 in column 150 g.	Fraction	Operating volume (ml)	Mass of concentrate (mg)	Mass ratio of concentrate (%)	Mass of aglycones (mg)	Yield of aglycones (%)	Purity of aglycones (%)
Adsorption	1	100	293.18	78.94	5.48	27.44	1.87
Washing(DI water)	2	100	23.98	6.46	0.36	1.8	1.5
Step-gradient elution							
20% ethanol	3	100	2.85	0.77	0.16	0.8	5.6
40% ethanol	4	100	9.78	2.63	3.33	16.69	34.09
60% ethanol	5	100	15.15	4.08	7.22	36.14	47.65
80% ethanol	6	100	26.47	7.13	3.42	17.13	12.93
Total		-	371.42	100	19.98	100	

Note: The initial purity of isoflavone was 5.25%

Adsorption = isoflavone aglycones solution from crude isoflavone aglycones: ethanol 80%: DI water at 1: 2: 4

Operating volume (ml) = solution was used to each columns in each fraction.

Mass of concentrate (mg) = solid in each fraction

Mass ratio of concentrate (%) = (mass of concentrate; mg) /total mass of concentration) x 100

Mass of aglycones (mg) = isoflavone aglycones in each fraction

Yield of aglycones (%) = (mass of aglycones; mg)/total mass of aglycones) x 100

Purity of aglycones (%) = (mass of aglycones; mg/mass of concentrate ; mg) x 100

Table 4.23 Percentage of isoflavone aglycones purity at amberlite XAD-4 200 g per 100 ml isoflavone aglycones solution

Amberlite XAD-4 in column 200 g.	Fraction	Operating volume (ml)	Mass of concentrate (mg)	Mass ratio of concentrate (%)	Mass of aglycones (mg)	Yield of aglycones (%)	Purity of aglycones (%)
Adsorption	1	100	256.34	71.69	4.79	23.44	1.87
Washing(DI water)	2	100	38.17	10.67	0.57	2.8	1.5
Step-gradient elution							
20% ethanol	3	100	6.57	1.84	0.37	1.8	5.6
40% ethanol	4	100	7.52	2.1	3.21	15.69	42.65
60% ethanol	5	100	17.14	4.79	7.39	36.14	43.11
80% ethanol	6	100	31.84	8.9	4.12	20.13	12.93
Total		-	357.59	100	20.45	100	

Note: The initial purity of isoflavone was 5.25%

Adsorption = isoflavone aglycones solution from crude isoflavone aglycones: ethanol 80%: DI water at 1: 2: 4

Operating volume (ml) = solution was used to each columns in each fraction.

Mass of concentrate (mg) = solid in each fraction

Mass ratio of concentrate (%) = (mass of concentrate; mg) /total mass of concentration) x 100

Mass of aglycones (mg) = isoflavone aglycones in each fraction

Yield of aglycones (%) = (mass of aglycones; mg)/total mass of aglycones) x 100

Purity of aglycones (%) = (mass of aglycones; mg/mass of concentrate ; mg) x 100

In Table 4.20 - 4.23 showed that different amounts of amberlite XAD-4 have different effects on the efficiency of isoflavone aglycones purification. When compared the purity, the result was as follows Table 4.24

Table 4.24 Percentage yield and percent purity of isoflavone aglycones at different Amberlite XAD-4 resin

Amberlite XAD-4 in column (g)	%yield40-60%^{ns}	%purity 40-60%
50	50.10	35.63^{b}
100	53.91	42.22^{a}
150	52.83	42.31^{a}
200	51.83	42.98^{a}

Note: - The initial purity of isoflavone was 5.25%

- Mean within same column with different superscripts were significant difference (p<0.05)
- ns = Non significant difference (p≥0.05)

The purity of isoflavone aglycones was determined. Amberlite XAD-4 at 100, 150 and 200 g per 100 ml of isoflavone aglycones solution showed no significant difference in purity of isoflavone aglycones. Amberlite XAD-4 at 50 g was the lowest isoflavone aglycones purity only 35.63 %. Thus, the amount of amberlite XAD-4 at 100 g per 100 ml of isoflavone aglycones solution was the suitable condition as save the amount of amberlite XAD-4.

4.4 Development of health supplements beverage from isoflavone aglycones

4.4.1 Development of prototype for isoflavone aglycones beverage

The development of beverages using linear programming techniques to determine the suitable level of studied factors. The constraints of these factors follows as;

1. Isoflavone aglycones solution from soy germ, recommended consume 50-100 mg per day (Kritz *et al*., 2003)
2. Inulin powder is a source of fiber as a prebiotic, adults should consume at 5 grams per day (Tunjor *et al.,* 2010). Inulin can be a food source of microorganisms in the human intestine.
3. The passion fruit juice is representative of sour taste at 7 point hedonic scale.

4. Fructose syrup is representative of sweet taste at 7 point hedonic scale.

The quality target of isoflavone aglycones beverage products from soy germ is about 30 percent of the amount of nutrients that Thai people should receive per day. Therefore, isoflavone aglycones solution was 15-30 mg per day and inulin powder 2 g per day.

Table 4.25 Nutritional value of isoflavone aglycones beverage

Quality	Ingredients per 1 Unit of sample	
	X_1	X_2
Isoflavones (mg)	23.07	
Inulin (g)		0.95

Note : X_1 = Isoflavone solution X_2 = Inulin powder

From Table 4.25, the following equation can be constructed:

Isoflavones solution $23.07(X_1) \geq 18$

Inulin powder $0.95\ (X_2) \geq 2$

To correct the equation with the LP88 program, it was found that the optimum amount of ingredients in the production of isoflavone aglycones beverage from soy germ consisted of isoflavone aglycones solution (X2 $\geq$ 1 ml) and Inulin Powder (X1 $\geq$ 2 g)

The result from linear programming showed the optimal solution was 1 ml isoflavone aglycones and 2 g inulin.

From the previous results, it was found that 1 ml of extracted isoflavone from soy germ, total isoflavone aglycones was 3.197 mg and isoflavone glucosides was 1.341 mg. In addition, the purity of isoflavone aglycones was 42.22%. This means that 1 ml of purified isoflavone aglycones is estimated at 25.71 mg of isoflavone aglycones and isoflavone glucosides at 10.88 mg. Therefore, it should be used 0.7 ml of purified isoflavone aglycones in order to fit the optimal solution from linear programing. However, for the commercial preparation, 1 ml of purified isoflavone aglycone was added in the beverage.

Consequently, in one serving size of isoflavone aglycones beverage (70 ml), it was calculated that 1 ml of extracted isoflavone aglycones, 2 g of inulin and 67 ml of passion fruit juice and fructose syrup. Then, studied the passion fruit juice at 60-90 % and fructose syrup at 5-15 % were varied using 2^2 factorial design with 2 center point (Wiriyacharee, 2012) (Table 4.26). Sensory evaluation was determined using 7-point hedonic scale in term of color, odor, viscosity, sweetness, sourness, overall taste and overall acceptability are shown in Table 4.27.

Table 4.26 2^2 factorial in central composite design for studied of passion fruit juice and fructose syrup

Treatments	Passion fruit juice (g)	Fructose syrup (g)
1	64.39	6.46
2	85.61	6.46
3	64.39	13.54
4	85.61	13.54
5	60	10
6	90	10
7	75	5
8	75	15
9	75	10
10	75	10

Note: each treatment used 67 ml of passion fruit juice and fructose syrup mixed with 1 ml of purified isoflavone aglycones and 2 g of inulin for the total 70 ml (one serving size).

Table 4.27 Sensory evaluation of isoflavone aglycones beverage varied of passion fruit juice and fructose syrup

Treatment	Sensory attributes (7-point hedonic scaling)						
	Color	**Odor**	**Viscosity**	**Sweetness**	**Sourness**	**Overall taste**	**Overall acceptability**
1	6.22±0.88	5.44±0.88	5.11±1.05	3.22±1.03	2.56±0.34	3.89±0.83	4.11±0.62
2	6.11±1.05	5.11±1.30	5.11±1.01	2.89±0.22	3.22±0.39	3.78±0.33	3.89±0.39
3	6.22±0.83	5.44±1.01	5.56±1.03	5.44±1.22	5.00±1.50	5.22±1.12	5.22±1.07
4	6.11±1.05	5.67±1.00	5.22±1.30	5.56±0.88	5.89±0.78	5.33±1.12	5.11±1.17
5	6.00±1.03	5.89±0.78	5.331.12±	4.67±1.00	4.33±1.05	4.89±0.56	5.11±0.34
6	6.22±1.09	5.33±1.12	5.33±0.46	4.33±0.58	3.89±0.76	4.11±0.44	4.11±0.32
7	6.11±1.05	5.56±1.01	5.33±1.12	3.67±0.56	3.78±0.30	4.56±0.51	4.00±0.73
8	6.22±1.32	5.33±1.12	5.22±1.20	6.11±0.60	5.79±0.60	5.33±1.00	5.22±1.09
9	6.11±1.11	5.56±1.01	5.22±0.98	4.44±0.81	4.11±0.69	4.22±0.64	4.33±0.94
10	6.00±1.02	5.44±0.88	5.33±0.67	4.56±0.67	4.22±0.56	4.33±050	4.44±0.81

Note: 7=like very much, 6=like moderately, 5=like slightly, 4=neither like nor dislike, 3=dislike slightly, 2=dislike moderately, 1=dislike very much

Table 4.27 showed that the color, odor and viscosity preference were in range of 6.00-6.22, 5.11-5.33 and 5.11-5.56, respectively. The preference of sweetness, sourness, taste and overall acceptability were in range of 2.89-6.11, 2.56-5.89, 3.89-5.33 and 3.89-5.22, respectively.

The data was analyzed in the form of multiple regressions to describe the relationship between the amount of passion fruit juice and of fructose syrup. It was found that the sweetness, sourness and overall acceptability had significant difference (p<0.05) as shown in Table 4.28 and response area was shown at Figure 4.12.

Table 4.28 Relationship between the amount of passion fruit juice and fructose syrup

Test parameter	Mathematical model	R^2
Sweetness	= 2.15 - 8.17 $x10^{-3}$ (Passion fruit juice) + 0.29 (Fructose syrup)	0.9146
Sourness	= 0.52 + 0.01(Passion fruit juice) + 0.30 (Fructose syrup)	0.8150
Overall acceptability	= 4.66 - 0.02 (Passion fruit juice) + 0.14(Fructose syrup)	0.8996

From Table 4.28, it was found that the sweetness, sourness and overall acceptability were dependent on the amount of passion fruit juice and fructose syrup. Increasing of passion fruit juice, the preference of sweetness taste and overall acceptability was decreased whereas the preference of sourness was increased. Moreover, increasing of fructose syrup resulted in a tendency of increasing preference for sweetness, sourness and overall acceptability.

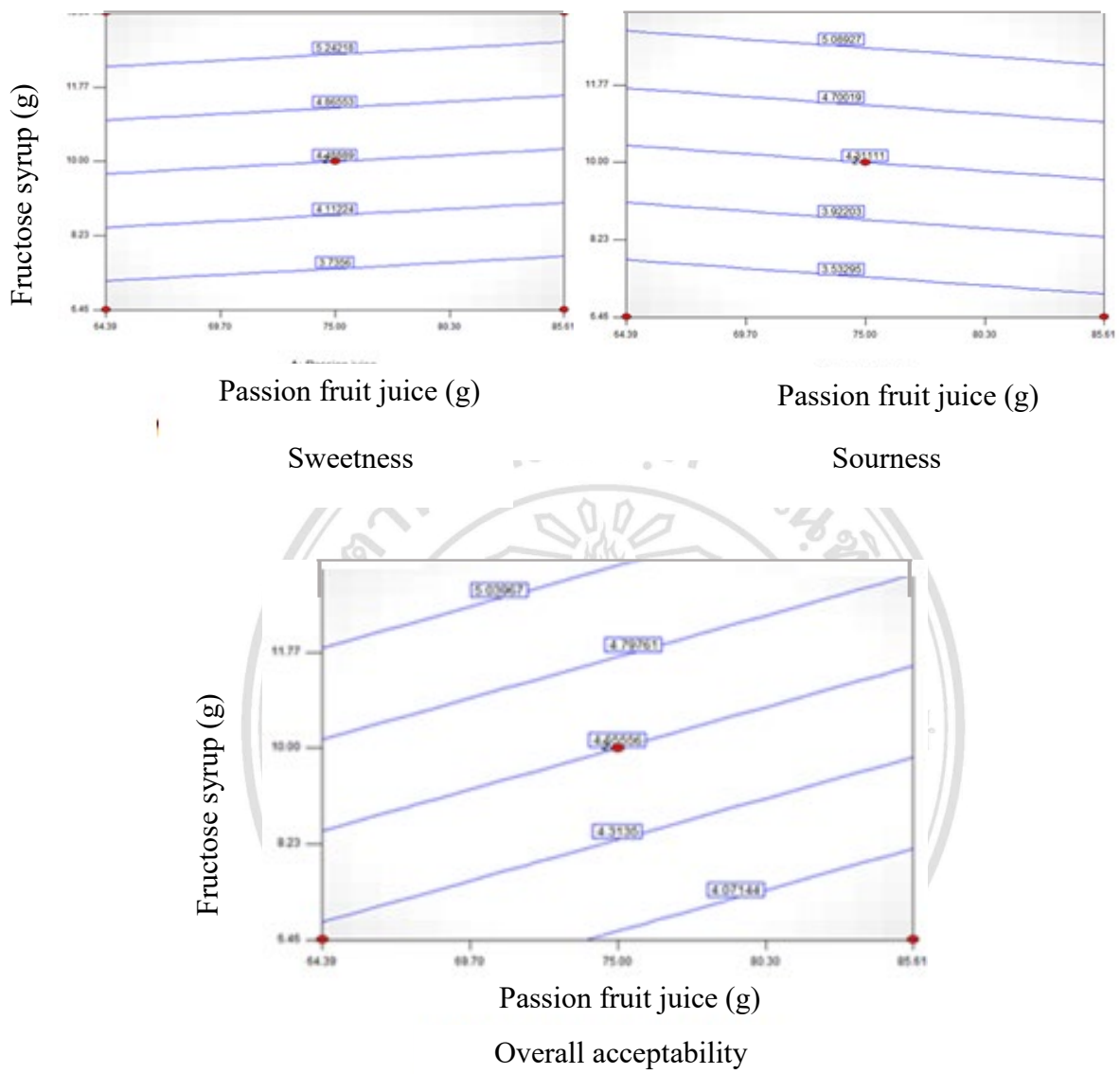

Figure 4.13 Response surface of the relationship of passion fruit juice and fructose syrup for sweetness, sourness and overall acceptance

The equations in Table 4.27 showed the tendency of passion fruit juice and fructose syrup by design expert 7.10 (Statease Inc., Minneapolis, USA). Then the scope of the studied factors and desired features was showed in Table 4.29. The results showed that the suitable amount of passion fruit juice and fructose syrup for the beverage product was 67.28g and 13.54g, respectively as shown in Figure 4.14.

Table 4.29 Scope of studied factors and desired features

Factor	Goal	Lower	Upper	
Passion fruit juice	In range	64.39	85.6	g
Fructose syrup	In range	6.46	13.53	g
Factor	**Goal**	**Lower**	**Upper**	
Sweet	Maximize	2.88	6.11	-
Sour	Maximize	2.55	5.89	-
Acceptance	Maximize	3.88	5.22	-

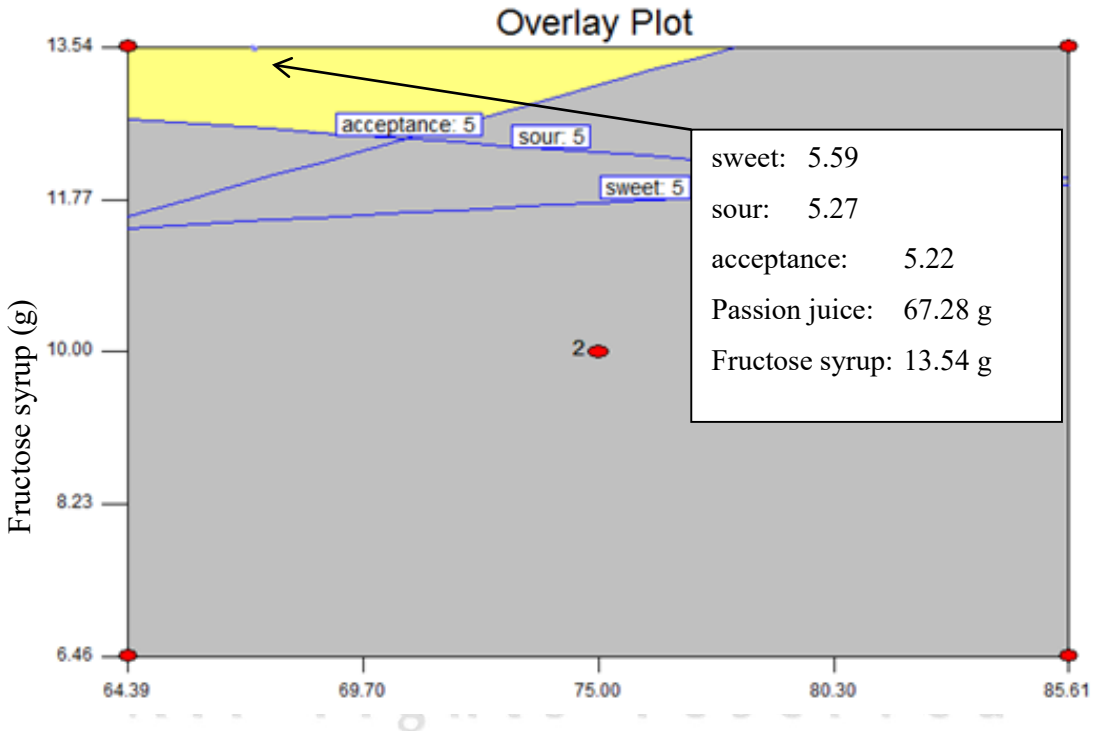

Figure 4.14 Response surfaces of passion fruit juice and fructose syrup

Therefore, it can be concluded that 1 unit (70 ml) of product composed of isoflavone aglycones solution of 1 ml, passion fruit juice 55.78%, fructose syrup 11.22% and inulin powder 2 g.

4.4.2 Study on the quality of isoflavone aglycones beverage products from soy germ

Isoflavone aglycones beverage product from the soy germ has been formulated from previous experiment. Sensory quality, nutritional value, chemical properties and microbiological properties were determined in this experiment and results were shown in Table 4.30. Moreover, consumer acceptability (n=200) consumer panelist, nutritional fact shown in appendix B at Figure B-3 and Figure B-4 and packaging shown in appendix A at Figure A-3 and Figure B-4 of developed product were investigated.

Table 4.30 Nutritional value, chemical properties and microbiological properties of developed products

Quality	Analytical results
Nutrition values	
Moisture content (%)	94.75±0.03
Protein content (%)	0.67±0.01
Total fat content (%)	0.33±0.02
Carbohydrate content (%)	4.02±0.01
Ash content (%)	0.26±0.01
Energy content (Kcal/ 70 ml)	75.39±0.23
Total isoflavone content (mg/70 ml)	23.07±0.65
Chemical values	
Total soluble solid (ºBrix)	25.33±0.07
pH value	3.23±0.02
Color L *	44.19±0.14
Color a*	3.25±0.14
Color b*	26.43±0.65
Viscosity (cp)	98.63±2.31
Microbiological properties	
Total plate count (cfu/ml)	<10
Yeast and mold (cfu/ml)	<10
E. Coli and Coliform (cfu/ml)	Not detected

Table 4.30 shows the nutritional value, chemical and microbiological properties. Isoflavone aglycones beverage composed of moisture content 94.75%, protein content

0.67%, total fat content 0.33%, carbohydrate content 4.02%, ash content 0.26%, energy 75.39 kilocalories, total isoflavones content 23.07 mg, total solids were 25.33° Brix and pH 3.23. Color L * a * b * value were 44.19, 3.25 and 26.43, respectively. Total plate count and yeast mold were less than 10 (cfu/ml) whereas no found *E.* coli and coliforms.

Consumer testing in adult age of more than 40 years was conducted with a total of 200 testers. The results are shown in Table 4.31.

Table 4.31 Data of tester

Data	**%**
Male	22
Female	78
Age	
- 41 – 60 years	52
- > 60 years	48
Education	
- Secondary School	2
- High School	12
- Vocational Certificate	6
- Bachelor degree	50
- Master degree	24
- Doctorate degree	6
Occupation	
- Undergraduate	14
- State enterprise officer	6
- Company employee	24
- Government official	20
- Businessman/ Merchant	12
- Employee	14
- Etc.	10
Rate of salary (Baht/ Month)	
- < 10,000	12
- 10,001 - 15,000	16
- 15,001 - 20,000	16
- > 20,000	56

Table 4.31 shows that the majority of the sample testers were female, 78.00% male were 22.00% comprise of aged 41-60 years and over 60 years was 52% and 48%, respectively. Most of the graduates have a bachelor degree of 50.00%. Most of the occupations are company employees 24.00% and government officials 20.00%. The majority of salary is more than 20,000 baht per month 56.00%.

Considering, the factors that affect the overall acceptability on isoflavone aglycones beverage such as gender, age, education and salaries of the consumers using chi-square test showed that gender, age, education and salary were not correlated on isoflavone aglycones beverage by chi-square ≥ 0.05.

Table 4.32 Consumer acceptance test

Sensory attribute	Scale (7-point hedonic)
Color	6.02±0.91
Odor	5.86±0.56
Viscosity	5.66±0.29
Sweetness	5.52±0.28
Sourness	5.68±0.32
Overall acceptability	5.84±0.65

Note: 7-point hedonic scale sensory analysis (7=like very much, 6=like moderately, 5=like slightly, 4=neither like nor dislike, 3=dislike slightly, 2=dislike moderately, 1=dislike very much)

Consumer acceptability (n=200) of product was shown in Table 4.32. The preference of color was in range of like moderately. While the preference of odor, viscosity, sweetness, sourness and overall acceptability were in range of like slightly to like moderately.