CHAPTER 3

Screening and selecting of pure culture for soy isoflavone aglycones producer

3.1 Introduction

Isoflavones are classified as flavonoids which can be found in soybean and soybean products. Their structure and function are similar to estradiol or estrogen hormone. Isoflavones have twelve isomers and can be divided into two main forms including glucosides and aglycones. The glucoside form has nine isomers which are daidzin, genistin, glycitin, acetyldaidzin, acetylgenistin, acetylglycitin, malonyldaidzin, malonylgenistin and malonylglycitin. The aglycone form has three isomers which are daidzein, genistein and glycitein (Liu, 2004; Shimoni, 2004). Generally, isoflavones in soybean seed appear in the form of glucosides whereas the amount of aglycone forms is less. Glucoside form is less estrogenic activity, less antioxidant activity and poorly absorbed in the small intestine comparing to aglycone form (Choi *et al.*, 2002; Setchell, 2000). The glucoside form can be hydrolyzed to aglycone form by β-glucosidase from *Bacillus* spp. such as *Bacillus subtilis*, *B. pumilus*, *B. licheniformis*, *B. circulans*, *B. firmus* and *B. megaterium* (Kudou *et al.*, 1991; Song *et al.*, 1998; Wang and Murphy, 1994; Klump *et al.*, 2001; Lee *et al.*, 2007 and Dajanta *et al.*, 2009). Therefore, the application of pure culture for isoflavone aglycones production is a method that allows transformation process to run efficiently because it can be fully grown and can reduce other unwanted microorganisms.

This chapter was to screen and select *Bacillus* spp. for isoflavone aglycones production. The 41 strains of *Bacillus* spp. used in this study were derived from Traditional Food Research and Development Unit, Science and Technology Research Institute, Chiang Mai University, Thailand. Thirty five strains were isolated from Thai fermented soybean (thua nao) (code: CM, CR, LG, LP, MH, NN, PR and PY) and

six strains were isolated from Japanese fermented soybean (natto) (code: NTA, NTB and NTC). All strains were screened to find efficient strain which produced the highest amount of isoflavone aglycones. The selected strain was identified to specie and used as pure culture for isoflavone aglycones production.

3.2 Methods

3.2.1 Screening and selecting of *Bacillus* spp.

Most of the *Bacillus* spp. strains were maintained and subcultured on nutrient agar (NA) slant at 4°C. Each strain of *Bacillus* spp. was cultured on nutrient broth (NB) and incubated at 37°C, 120 rpm for 18 h. The culture was used as inoculum for soybean fermentation.

For the fermented soybean preparation, soybean [*Glycine* max (L.) Merrill] (Chiang Mai 1 variety) was purchased from the Chiang Mai field crop research center (Chiang Mai, Thailand). Initially, soybeans were washed 2 times with clean water, soaked in clean water for 6 h at ambient temperature (30-35°C) and boiled for 4 h. After draining the water, the pasteurized soybean was hot filled into sterilized polypropylene bag and sealed with cotton wool. After that, the pasteurized soybean was inoculated with 1% (v/w) of *Bacillus* spp. and incubated at 30°C for 72 h. Finally, the fermented soybeans were freeze dried and grinded into powder before isoflavones analysis (see Appendix A). The relative change of isoflavone aglycones was defined. The strain with highest isoflavone aglycones was selected.

3.2.2 Identification *Bacillus* spp.

The selected strain was cultured on nutrient broth (NB) and incubated at 37°C, 120 rpm for 18 h. After that, the selected strain was identified the specie by using biochemical test (see Appendix B) and was confirmed the specie by using double strand 16S rDNA sequencing (see Appendix C).

3.2.3 Isoflavone aglycones production by selected strain

The selected strain of *Bacillus* spp. was used as pure culture for soybean fermentation. The fermentation time was 10 days. Isoflavones content, β-glucosidase activity and viable colony number of *Bacillus* spp. (total bacterial count) were analyzed.

The β-glucosidase activity assay was modified from Yin *et al.* (2004). Five grams of sample was homogenized with 25 ml of 0.2 M acetate buffer, pH 4.5 at 4°C. The slurry was centrifuged at 8000g for 30 min at 4°C and the supernatant was used as a crude enzyme solution. Then 2 ml of 1 mM *p*-NPG solution and 0.5ml of a crude enzyme solution were mixed and incubated at 45°C for 30 min. The reaction was stopped by the addition of 2.5 ml of 1 M sodium carbonate. The resultant color was immediately measured at 405 nm. One unit of enzyme activity was defined as the amount of enzyme which liberated l μm of *p*-nitrophenol per min. For determination of *Bacillus* spp., ten grams of samples was mixed with 90 ml of 0.1% peptone water and suspension was diluted by ten-fold dilution. The diluted suspension (1 mL portions) was pour plate in NA. The plates were incubated at 37 ^{o}C for 24 h and calculated viable colony number.

3.3 Results and discussion

3.3.1 Screening and selecting of *Bacillus* spp.

The isoflavone aglycones content of fermented soybean was shown in Table 3.1. It was found that, each strain differently produced isoflavone aglycones. The LG01, PR03 and NTA02 were able to produce daidzein, genistein and glycitein in the number of 94.81, 53.93 and 11.16 mg/100 g dry weight respectively. Considering from the total amount of isoflavone aglycones, it was found that PR03 is the most effective strain which was able to produce isoflavone aglycones in the maximum number of 146.21 mg/100 g dry weight, or the production amount increased up to 3.81 times of nonfermented soybean. The less effective strains were NTA02 and LG01 which produced in the number of 129.80 and 123.70 mg/100g dry weight respectively. The NN07 and MH05 were the least effective strain. They produced only in the amount of 30.71 and 30.94 mg/100g dry weight respectively. According to the result of the study, *Bacillus* PR03 was the selected as starter culture for isoflavone aglycones production.

Table 3.1 Isoflavone aglycones of fermented soybean from various *Bacillus* spp.

Isolated number	Isoflavone aglycones content (mg/100g dry weight)				Relative change of total isoflavone aglycones*
	Daidzein	Genistein	Glycitein	Total aglycones	
Bacillus PR03	82.48±2.04	53.93±1.34	9.81±0.26	146.22±3.64	3.81
Bacillus NTA02	74.01±0.29	44.63±0.18	11.16±0.12	129.80±0.35	3.27
Bacillus LG01	94.81±1.93	18.96±0.08	9.93±0.22	123.70±2.23	3.07
Bacillus CH09	75.02±0.26	39.18±0.48	9.28±0.09	123.48±0.85	3.06
Bacillus LG03	83.28±0.91	27.50±0.34	8.57±0.11	119.35±1.36	2.93
Bacillus LG09	90.10±1.14	8.92±0.06	9.71±0.03	108.73±1.15	2.58
Bacillus NTA01	58.30±2.39	39.33±4.80	9.66±0.27	107.29±2.68	2.53
Bacillus PY03	79.11±1.32	17.54±0.45	10.60±1.12	107.25±2.89	2.53
Bacillus CR01	98.03±1.57	8.17±0.69	0.65±0.01	106.85±2.26	2.52
Bacillus PR04	62.03±0.09	36.33±0.25	7.26±0.12	105.62±0.04	2.48
Bacillus PR01	65.08±0.38	32.15±0.20	7.80±0.03	105.03±0.55	2.46
Bacillus CR05	83.81±4.34	10.09±0.49	7.67±0.64	101.57±1.22	2.44
Bacillus PY01	70.95±2.45	16.93±0.66	9.71±0.11	97.59±3.23	2.21
Bacillus LP02	74.76±2.03	14.86±0.18	5.85±4.18	95.47±2.18	2.14
Bacillus LP01	74.78±1.30	15.18±0.15	4.02±0.02	93.98±0.78	2.09
Bacillus CH10	66.21±1.28	18.53±0.49	8.97±0.22	93.71±1.99	2.08
Bacillus CH06	73.34±0.01	10.02±0.02	7.57±0.02	90.93±0.01	1.99
Bacillus CH11	82.89±0.41	5.18±5.18	1.62±0.13	89.69±0.67	1.95
Bacillus LP05	68.60±2.04	15.82±0.43	4.70±019	89.12±2.65	1.93
Bacillus PY02	67.30±1.09	11.97±0.21	9.20±0.12	88.47±1.41	1.91
Bacillus LG05	49.48±0.51	33.71±0.40	4.72±0.10	87.91±0.01	1.89
Bacillus LP03	75.87±1.96	5.13±0.20	6.88±0.01	87.88±2.14	1.89
Bacillus LG11	71.73±0.62	5.67±0.20	7.56±0.01	84.96±1.51	1.80
Bacillus LG10	71.71±0.03	6.69±0.01	5.90±0.05	84.30±0.03	1.77
Bacillus PY04	69.58±0.13	5.35±0.05	6.12±0.02	81.05±0.16	1.67
Bacillus CR02	62.78±1.96	14.67±0.24	0.90±0.03	78.35±2.23	1.58
Bacillus CH07	56.95±3.13	18.97±0.49	2.19±0.16	78.11±3.78	1.57
Bacillus MH03	67.18±0.16	4.39±0.02	4.19±0.26	75.76±0.07	1.49
Bacillus MH04	54.16±0.30	11.40±0.03	5.27±0.04	70.83±0.37	1.33
Bacillus CR04	49.83±0.68	15.10±0.27	5.09±0.05	70.02±1.00	1.30
Bacillus NTC02	36.83±0.65	24.49±0.05	8.26±0.28	69.58±0.98	1.29
Bacillus PR02	44.13±1.88	21.69±1.18	3.65±0.70	69.47±3.76	1.29
Bacillus NN10	53.19±2.20	12.45±0.43	3.17±0.01	68.81±1.61	1.26
Bacillus LG07	56.72±1.10	7.15±0.29	3.15±0.05	67.02±1.45	1.21
Bacillus MH02	42.62±0.45	18.45±0.13	4.98±0.01	66.05±0.72	1.17
Bacillus NTC01	38.88±2.26	23.01±0.90	3.18±0.02	65.07±3.14	1.14
Bacillus NTB02	29.55±0.11	19.75±0.13	6.03±0.12	55.33±0.14	0.82
Bacillus CR06	47.70±0.49	1.47±0.01	3.16±0.09	52.33±0.58	0.72
Bacillus NTB01	29.41±0.46	15.61±0.01	4.03±0.34	49.05±0.80	0.61
Bacillus MH05	21.88±0.72	4.96±0.05	4.10±0.25	30.94±1.02	0.02
Bacillus NN07	20.10±0.11	5.10±0.21	5.51±0.05	30.71±0.22	0.01
Nonfermented soybean	23.30±0.21	3.88±0.45	3.20±0.07	30.38±0.30	

Note: * Relative change of total isoflavone aglycones = $(TAf - TAn) \div TAn$

When TAf = Totoal isoflavone aglycones content of fermented soybean and

TAn = Totoal isoflavone aglycones content of nonfermented soybean

3.3.2 Identification of selected strain

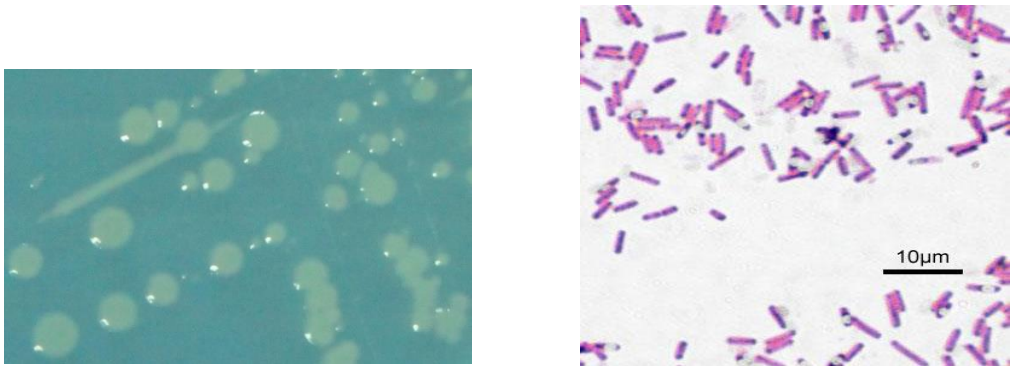
The selected strain, *Bacillus* PR03, was studied morphological characteristic and identified to specie using biochemical test. The result shown in Table 3.2. It was found that the colony's morphology of *Bacillus* PR03 has diameter of 2.0-4.5 mm, undulate margin, effuse elevation, irregular form, rough surface, white pigment and opaque optical. The characteristics of cell's morphology were Gram positive, rod shape, singly or chain, endospore forming was found in the middle of cell (Figure 3.1). Considering from biochemical test found that it had positive reaction in catalase test, Voges-Proskauer test, growth in anaerobe, growth at 50 °C, hydrolysis of starch and width of rod more than 1.0 mm. On the other hand, it was not able to produce acid and gas from glucose fermentation, not able to grow at 65 °C and not able to grow in 7% NaCl medium. When using biochemical test to identify specie followed the method of Norris *et al.* (1981), it found that the strain was *Bacillus coagulans*.

Moreover, the double strands 16S rDNA sequencing was used to confirm the correct specie of *Bacillus* PR03. The nucleotide sequence was shown in Appendix C. The comparison of nucleotide sequences between *Bacillus* PR03 with reference strains (Table 3.3) found that *Bacillus* PR03 was similar to *B. coagulans* (99.93%), and *B. acidiproducens* (97.10%). In considering with phylogenetic tree analysis (Figure 3.2) found that *Bacillus* PR03 was in the same cluster with *B. coagulans* and had genetic distance close to *B. coagulans* in the number of 0.26856. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and were in the units of the number of base substitutions per site. The analysis involved 16 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1359 positions in the final dataset. Evolutionary analyses were conducted in MEGA6. As the result, the *Bacillus* PR03 was identified as *Bacillus coagulans*.

Table 3.2 Morphological characteristics and biochemical test of *Bacillus PR03* compare with *Bacillus coagulans*

Morphology characteristics	***Bacillus PR03***	***Bacillus coagulans****
Colony morphology		
Diameter (mm)	2.0-4.5	1-3
Edge or margin	Undulate	Entire
Elevation	Effuse	Convex
Form	Irregular	Irregular
Surface	Rough	Smooth
Pigment	White	White to cream
Optical	Opaque	Opaque
Cell morphology		
Shape	Rod	Rod
Size W/L (µm)	0.7-1.0/3.0-5.0	0.6-1.0/2.0-5.0
Chain	Singly or in chain	Singly or in chain
Endospore	Middle	Subterminal/terminal/midle
Gram staining	Positive	Positive
Biochemical test Reaction		
Catalase test	Positive	Positive
Voges-Proskauer	Positive	Positive
Acid and gas from glucose	Negative	Positive
Anaerobe growth	Positive	Positive
Growth at 50 °C	Positive	Positive
Growth at 65 °C	Negative	Negative
Width of rod 1.0 µm or greater	Positive	Positive
Hydrolysis of starch	Positive	Positive
Growth in 7% NaCl	Negative	Negative

Note: * data from Norris *et al.* (1981); Logan and Vos (2009).



(a) **(b)**

Figure 3.1 Colony morphology (a) and cell morphology (b) of *Bacillus* PR03.

Table 3.3 Comparison of nucleotide sequences between *Bacillus* PR03 and reference strains

Rank	Name	Reference strain	Accession number	Pairwise similarity* (%)
1	*Bacillus coagulans*	IAM 12463(T)	D16267	99.93
2	*Bacillus acidiproducens*	SL213(T)	EF379274	97.10
3	*Bacillus shackletonii*	LMG 18435(T)	AJ250318	95.71
4	*Bacillus ginsengihumi*	Gsoil 114(T)	AB245378	95.17
5	*Bacillus acidicola*	105-2(T)	AF547209	94.69
6	*Bacillus oleronius*	DSM 9356(T)	X82492	94.59
7	*Bacillus thermotolerans*	SgZ-8(T)	JX261934	94.55
8	*Bacillus methanolicus*	PB1(T)	AFEU01000002	94.49
9	*Bacillus subterraneus*	DSM 13966(T)	FR733689	94.28
10	*Bacillus boroniphilus*	JCM 21738(T)	BAUW01000204	94.21

Note: * % Similarity of 16S rDNA compare with closely related species.

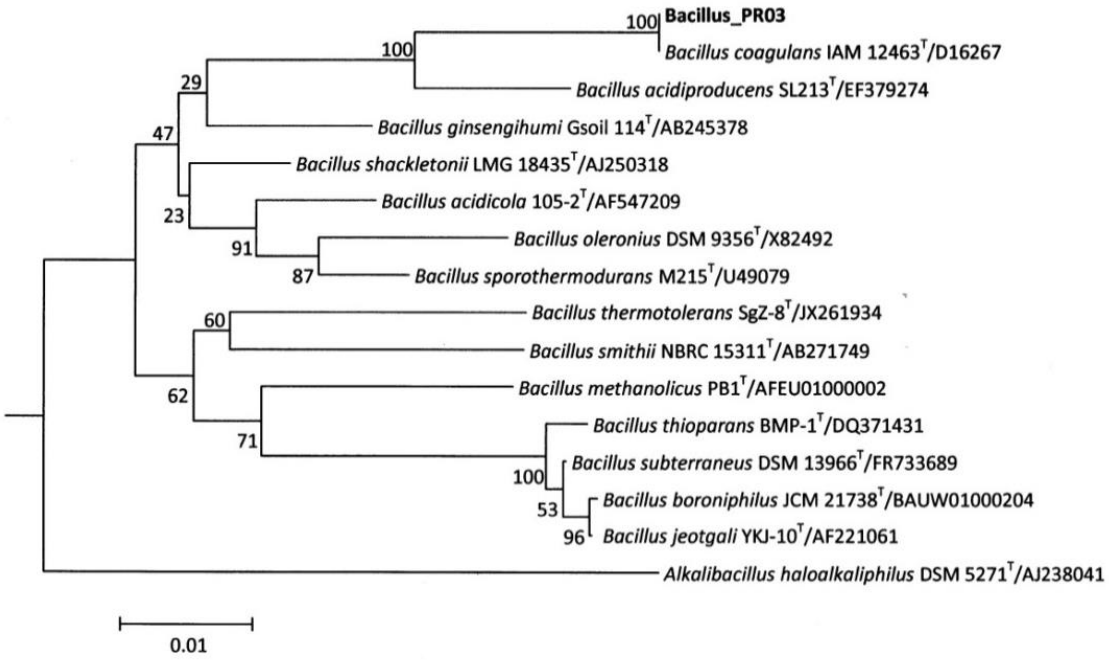


Figure 3.2 Phylogenetic relationships (based on 16S rDNA) of *Bacillus* PR03 between the isolates and closely related species.

Bacillus coagulans has not been reported as predominant bacteria in soybean fermentation. According to the article about bacteria in fermented soybean product, it was mostly found as other *Bacillus* specie. For example, "Thua nao" are *B. subtilis* and *B. megaterium* (Leejeerajumnean, 2003), "Dawadawa" are *B. subtilis*, *B. pumilus* and *B. lichenformis* (Ogbadu and Okagbue, 1988; Omafuvbe *et al.*, 2000) and "Kinema" are *B. subtilis*, *B. lichenformis* and *B. badius* (Sarkar *et al.*, 2002; Sarkar *et al.*, 1994). Therefore, this was the first research using *B. coagulans* as predominant bacteria for producing isoflavone aglycones from soybean.

3.3.3 Isoflavone aglycones production by *Bacillus coagulans* PR03

The selected strain, *B. coagulans* PR03, was used as pure culture for isoflavone aglycones production from soybean. The isoflavones content, β- glucosidase activity and total bacterial count during 10 days of fermentation were investigated.

The isoflavones content, β-glucosidase activity and total bacterial count in fermented soybean by *B. coagulans* PR03 were shown in Table 3.4. Fermentation process had significant effects on the isoflavone aglycones, isoflavone glucosides, β-glucosidase activity and total bacterial count ($P<0.05$). The quantity of isoflavone aglycones tended to increase during the fermentation period. The quantity of daidzein, genistein, glycitein and total aglycones increased from 5.46, 11.69, 1.95 and 19.10 mg/100 g to 99.10, 70.00, 6.35 and 175.51 mg/100 g respectively. While the quantity of isoflavone glucosides decreased during the fermentation which resulted in the value of daidzin, genistin, glycitin and total glucosides decreased from 90.28, 77.54, 6.55 and 174.36 mg/100g to 14.48, 2.99, 1.31 and 18.78 mg/100 g respectively. β-glucosidase activity increased from 8.65 mU/g to the highest value of 32.62mU/g at the fifth day of fermentation process and decreased to17.74 mU/g at the last day of fermentation. Total bacterial count represented cell number of *B. coagulans* PR03 tended to have the same result as β-glucosidase activity. The value increased from 5.85 to 8.95 log cfu/g at the fifth day of fermentation and decreased to 7.35 log cfu/g at the last day of fermentation process.

The changes of isoflavones, β-glucosidase activity and total bacterial count during soybean fermentation by *B. coagulans* PR03 were shown in Figure 3.3. The increasing number of *B. coagulans* PR03 and β-glucosidase activity were relevant. We can see that *B. coagulans* PR03 has grown log phase like until the fifth day of fermentation and β-glucosidase activity would increased as well. The increasing value of *B. coagulans* PR03 and β-glucosidase activity result in hydrolization of isoflavone glucosides in soybean transformed into isofalvones aglycones. After the fifth day of fermentation process, isoflavone glucoside which was the substrate of the strain decreased and eventually the value of *B. coagulans* PR03 and β-glucosidase activity decreased. We can see that isoflavone aglycones value increased only in small number. Therefore, the transformation of isoflavone glucosides into isofalvones aglycones depended on the value of β-glucosidase which *B. coagulans* PR03 produced. The study mentioned above

was relevant to the following researches. Wei *et al.* (2008) reported the isoflavone aglycones content in fermented soybean using *B. subtilis* BCRC14718 was increased significantly after 24 h fermentation. Ibe *et al.* (2001) also reported that β-glucosidase from *B. subtilis* nutto IF9916 has ability to hydrolyzed isoflavone glucosides. Moreover, Kuo *et al.* (2006) reported that isoflavone glucosides (daidzin and genistin) were hydrolyzed into isoflavone aglycones (daidzein and genistein) by β-glucosidase from *B. subtilis* nutto NTU-18 during black soybean fermentation and they indicated that the hydrolysis of isoflavone glucosides started at 8 h after inoculating with *Bacillus* culture.

Table 3.4 Isoflavones content, β-glucosidase activity and total bacterial count in fermented soybean

Time (Day)	Isoflavones content (mg/100 g dry weight)								β-glucosidase activity (mU/g)	Total bacterial count (log CFU/g)
	Aglycones				Glucosides					
	Daidzein	Genistein	Glycitein	Total aglycones	Daidzin	Genistin	Glycitin	Total glucosides		
0	5.46± 0.05^{a}	11.69± 0.79^{a}	1.95± 0.12^{a}	19.10± 0.73^{a}	90.28± 0.03^{h}	77.54± 0.20^{f}	6.55± 0.57^{e}	174.36± 0.80^{h}	8.65±0.36^{a}	5.85±0.02^{a}
1	21.66± 0.64^{b}	28.03± 0.72^{b}	2.98± 0.09^{b}	52.67± 0.17^{b}	74.50± 0.56^{g}	59.61± 0.19^{e}	4.99± 0.38^{d}	139.11± 0.74^{g}	11.21±0.13^{b}	6.57±0.12^{b}
2	44.53± 0.79^{c}	37.60± 0.40^{c}	4.72± 0.16^{c}	86.85± 0.55^{c}	60.88± 0.28^{f}	40.38± 0.43^{d}	2.67± 0.30^{c}	103.92±0.42^{f}	15.81±0.12^{c}	7.10±0.03^{c}
3	74.29± 0.64^{d}	53.90± 0.18^{d}	6.32± 0.05^{d}	134.51± 0.41^{d}	42.06± 0.40^{e}	15.24± 0.54^{c}	2.12± 0.30^{bc}	59.41± 0.64^{e}	19.35±0.20^{f}	7.24±0.02^{d}
4	88.22± 0.75^{e}	62.47±0.79^{e}	6.36± 0.05^{d}	157.05± 0.10^{e}	28.47± 0.45^{d}	4.44± 0.41^{b}	1.77± 0.01^{ab}	34.68± 0.87^{d}	22.40±0.18^{g}	7.38±0.02^{de}
5	94.16± 1.01^{f}	68.29± 0.21^{f}	6.33± 0.02^{d}	168.77± 0.79^{f}	18.73± 0.19^{c}	3.17± 0.49^{a}	1.60± 0.01^{ab}	23.49± 0.30^{c}	32.62±0.38^{j}	8.05±0.07^{h}
6	94.71± 0.57^{f}	68.38± 0.70^{f}	6.31± 0.01^{d}	169.40± 0.13^{f}	16.52± 0.24^{b}	3.06± 0.27^{a}	1.57± 0.05^{ab}	21.15± 0.46^{b}	30.61±0.25^{i}	7.98±0.05^{gh}
7	96.59± 0.99^{g}	68.47± 0.55^{f}	6.31± 0.03^{d}	171.37± 0.41^{g}	14.64± 0.06^{a}	3.05± 0.56^{a}	1.52± 0.01^{ab}	19.20± 0.62^{a}	29.75±0.28^{h}	7.89±0.08^{g}
8	98.28± 0.66^{h}	68.40± 0.30^{f}	6.31± 0.01^{d}	172.99± 0.97^{h}	14.53± 0.07^{a}	3.06± 0.26^{a}	1.46± 0.02^{a}	19.05± 0.31^{a}	19.34±0.20^{f}	7.54±0.09^{f}
9	99.45± 0.33^{h}	68.24± 0.33^{f}	6.36± 0.04^{d}	174.04± 0.04^{h}	14.46± 0.02^{a}	3.07± 0.37^{a}	1.30± 0.30^{a}	18.83± 0.04^{a}	18.23±0.19^{e}	7.51±0.05^{ef}
10	99.16± 0.26^{h}	70.00± 0.10^{g}	6.35± 0.06^{d}	175.51± 0.42^{i}	14.48± 0.02^{a}	2.99± 0.89^{a}	1.31± 0.25^{a}	18.78± 0.61^{a}	16.74±0.16^{d}	7.35±0.04^{d}

Note: Means within same column with different superscripts are significantly different (P<0.05).

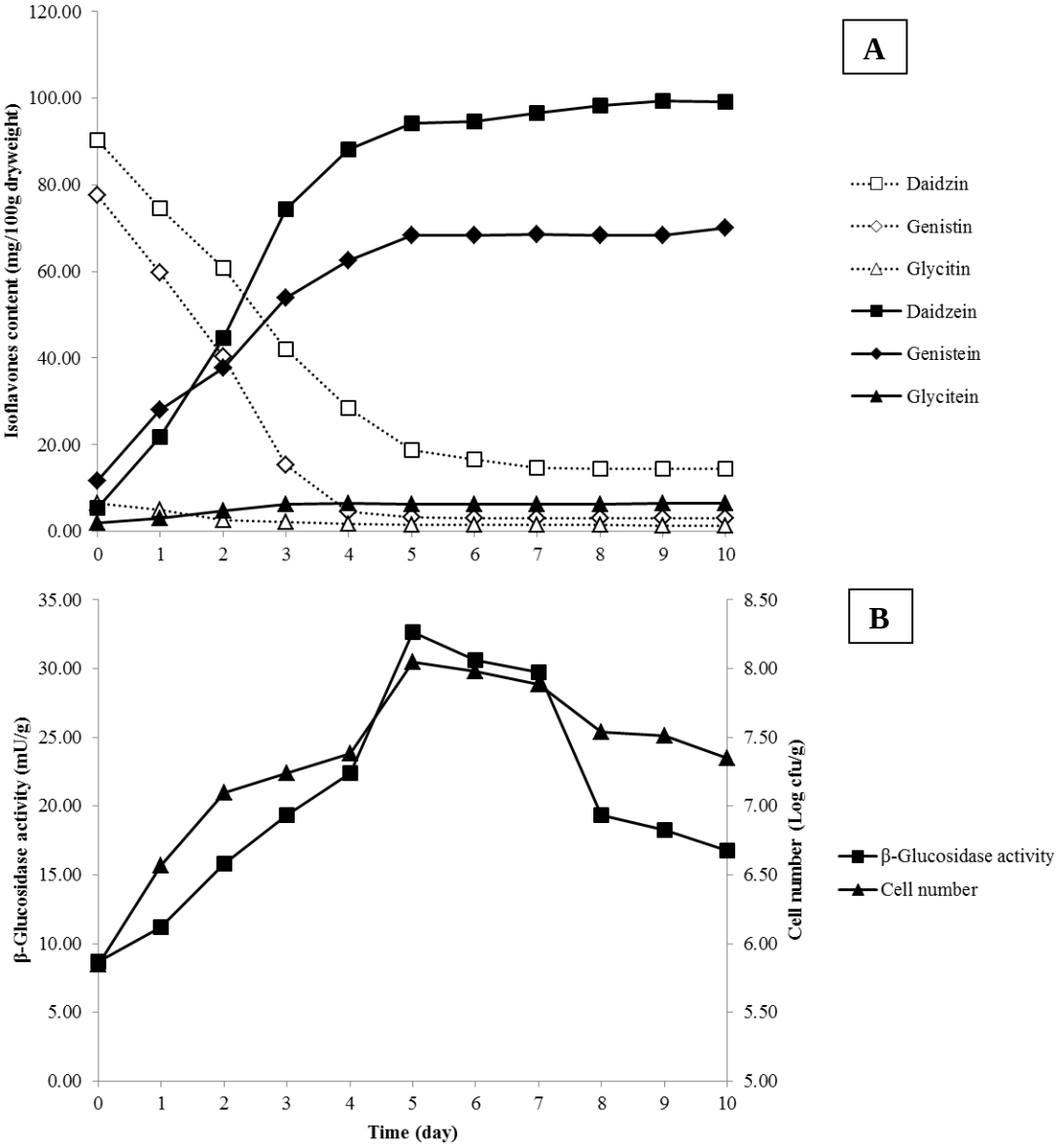


Figure 3.3 Change of isoflavones (A), β-glucosidase activity and total bacterial count (B) during soybean fermentation by *Bacillus coagulans* PR03 incubated at 30-35 ^{o}C.

3.4 Conclusion

The *B. coagulans* PR03, screened from Thai fermented soybean (*thua nao*), was suitable pure culture for soybean isoflavone aglycone production. This was the first research using *B. coagulans* as predominant bacteria for producing isoflavone aglycones from soybean. Moreover, soybean fermentation with *B. coagulans* PR03 revealed high amount of isoflavone aglycones. However, the optimizations of fermentation conditions were studied in the next chapter.