Selection of *Bacillus* spp. for Isoflavone Aglycones Enriched *Thua-nao*, A Traditional Thai Fermented Soybean

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This study aimed to select the strain of *Bacillus* spp. as a starter culture for enriching isoflavone aglycones in traditional Thai fermented soybean (*thua-nao*) production. Forty-one strains of *Bacillus* spp. from Thai *thua-nao* and Japanese *natto* were characterized and divided into 5 groups; *Bacillus licheniformis*, *Bacillus coagulans*, *Bacillus subtilis*, *Bacillus pumilus* and *Bacillus megaterium*. Each strain was used to produce *thua-nao*. As a result, *Bacillus* PR03 produced the highest amount of isoflavone aglycones (146 mg/100g dry weight; 3.81 times higher than the amount in soybean) and was selected as the starter culture. According to 16S rDNA sequencing, *Bacillus* PR03 was identified as *B. coagulans* with 99.93% identical to IAM12463 strain. *B. coagulans* have never been reported as predominant bacteria in soybean fermentation. Therefore, this is the first research using *B. coagulans* as predominant bacteria for producing *thua-nao*. To enrich isoflavone aglycones in *thua-nao* production by *B. coagulans* PR03, it was found that the fermentation time of 5 d resulted in the optimal contents of daidzein, genistein, glycitein and total aglycones; 94.16, 68.29, 6.33 and 168.77 mg/100 g dry weight, respectively. With improved functional properties, the enriched isoflavone aglycones *thua-nao* could be applied as an ingredient in functional food development.

Key words: isoflavones, aglycones, glucosides, fermented soybean, *Bacillus*.

Fermented soybean is high nutrition food which is popular in Asia for a long time. According to the research, it is found that fermented soybean comsumption everyday will help reduce the risk of chronic disease such as cancer[1-4], heart disease[5, 6], osteoporosis[7, 8], reducing hot flash during menopause period in woman[9, 10]. This is because soybean contains the important substance, called "isoflavone" which has the ability of antioxidant[11, 12] and antimutation[13]. Moreover, isoflavone has particular molecule structure which is able to combine with estrogen receptor. Therefore isoflavone is called as phytoestrogen. Isoflavone is flavonoid substance which has two forms including glucoside form (acetyldaidzin, acetylgenistin, acetylglycitin, daidzin, genistin, glycitin, malonyldaidzin,malonylgenistin and malonylglycitin) and also aglycone form (daidzein, genistein and glycitein)[14, 15]. Aglycone forms are easier to absorb in intestine and also has estrogenicity more than glucoside forms. From the study of volunteer who consume each type of isoflavone found that the intensity of aglycone

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forms in plasma of the volunteer is higher than glucoside forms. These show that body is able to absorb isoflavone aglycones faster than isoflavone glucosides[16]. Therefore, human body can use this substance easier. Generally, isoflavones in soybean is often found to be in the glucoside forms, while aglycone forms are absent or present in low concentration[17]. However, isoflavone glucosides will be changed to aglycones form by fermentation process. During fermentation, glucosides will be hydrolyzed by microbial â-glucosidase[18]. Thus, body can absorb isoflavones in fermented soybean better than isoflavones in nonfermented soybean[19]. Fermented soybean product in various country has high isoflavone aglycones such as *thua-nao* (Thailand), *natto* (Japan), *chungkookjang* (Korea), *dawadawa* (Nigeria) and *kinema* (India and Nepal)[20].

Thua-nao, the traditional Thai fermented soybean, is an alkaline fermented product by *Bacillus subtilis* and related bacilli[21, 22]. It is mainly consumed in northern part of Thailand. In the household *thua-nao* production, soybean seeds are cleaned and soaked in water. Soaked seeds are boiled in aluminum pots over wood fires for about 7 h to soften the seeds and water is drained off. After that, seeds are placed in bamboo basket, covered with banana leaves and fermented by *Bacillus* spp. at room temperature for 2-3 days. The product has strong and pungent odor during fermentation. Fresh *thua-nao* is cooked by steaming, seasoned with spice and consumed with sticky rice. Alternatively, fresh *thua-nao* could be smashed, formed into thin disc and dried with sunlight to dried form of *thua-nao* that can be stored for 3-4 months at room temperature. Dried *thua-nao* is used as flavor enhancer in various kinds of local dishes[21, 23].

Thua-nao is non-salted fermented soybean product that similar to Japanese *natto* and Indian *kinema*. The isolation and identification of microorganism in fermentation soybean process found that mostly it is *Bacillus* spp. group. For example, *Bacillus subtilis* and *B. megaterium* are in *thua-nao*[23], *B. subtilis*, *B. pumilus* and *B. lichenformis* are in *cawadawa*[24, 25] and *B. subtilis*, *B. lichenformis* and *B. badius* are in *kinema*[26, 27].

Traditional fermented soybean production naturally processed from different types of microorganism in environment. Therefore, the product has unstable quality affecting the content of isoflavone aglycones in fermented soybean. Using pure culture will help in maintaining the quality of fermentation process and product as pure culture can efficiently grow and reduce other unwanted microorganism[28].

This study aims to selection of suitable *Bacillus* spp. which were isolated from traditional fermented soybean (Thai *thua-nao* and Japanese *nutto*) and used as starter culture for enriched isoflavone aglycones *thua-nao*.

MATERIALS AND METHODS

Chemicals

Nutrient agar (NA), nutrient broth (NB) and peptone water from Difco (Becton, Dickinson and co., USA) were used. Daidzin, daidzein, genistin, genistein, glycitin, glycitein and *p*-nitophenyl-â-D-glucoside (*p*-NPG) were purchased from Sigma (Sigma-Aldrich, USA) also flavone from Fluka (Sigma-Aldrich, USA). High performance liquid chromatography (HPLC) grade acetic acid, acetonitrile and methanol from J.T. Baker (Germany) were applied. Analytical reagent grade hydrochloric acid was also used.

Bacterial strains

Most of *Bacillus* spp. strains utilized 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 *thua-nao* in northern part of Thailand (sample code: CM, CR, LG, LP, MH, NN, PR and PY) and six strains were isolated from Japanese *Natto* (sample code: NTA, NTB and NTC). The strains were maintained and subcultured on NA slant at 4°C.

Physiological and phenotypic characterization of *Bacillus* spp.

Each strain of *Bacillus* spp. was cultured on NA at 37°C for 24 h by streak plate technique. The single colony was studied for colony morphology (diameter, edge or margin, elevation, form, surface, pigment and optical), cell morphology (shape, size, chain, endospore and Gram's staining) and biochemical tests (catalase, Voges-Proskauer, growth in anaerobic agar, growth at 50°C, growth in 7% NaCl, acid/gas from glucose, hydrolysis of starch, growth at 65°C and width of

rod 1.0 mm or greater)[29]. The phenotypic characterization was performed according to Norris *et al*.[29].

Screening and selecting of *Bacillus* spp.

Each strain of *Bacillus* spp. was cultured on NB at 37°C for 24 h and used as inoculum for *thua-nao* fermentation. For the *thua-nao* 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 *thua-nao* was freeze dried in a freezone12 freeze dryer (Labconco, USA) and grinded into powder before isoflavones analysis.

The powder was extracted by solvent extraction, as modified from Murphy *et al*[30]. A gram of freeze dried samples were extracted with 5 ml of acetonitrile plus 110 ml of 0.1 mg/ml flavones, 1 ml of 0.1 N hydrochloric acid and 5 ml of deionized water type I. The extract was stirred for 10 min and sonicated in a S100H sonicator (Elma, German) at 50 – 60 Hz of ultrasonic frequency for 10 min. The extracts were centrifuged in Gyrospin microcentrifuge (Labtech, Korea) for 10 min at 10,000 rpm. Supernatants were filtrated through 0.45 mm nylon syringe filters. Finally the extracts were analyzed by HPLC.

The isoflavones analytical method was modified from that of Klejdus *et al*[31]. Twenty microliter of sample was injected into a C18 Inertsil ODS-3 column (5 mm, 4.6x250 mm, GL Sciences Inc., Japan) with a C18 guard column (Inertsil ODS-3 column (5 mm, 4.0x10 mm, GL Sciences Inc., Japan). Column temperature was constant at 40°C. The mobile phase was 0.1% (v/v) acetic acid (solvent A) and methanol (solvent B). Separation was performed at flow rate 1 ml/min using gradient program. The system was maintained by the solvent B at 30%, then increased to 35% at 5 min, 42% at 8 min, 90% at 21.5 min and 100% at 28 min. At 28.5 min the system recycled back to 30% of solvent B and held at that level for 2.5 min. A total run time was 32 min. Eluted isoflavones were detected at 255 nm. Peaks were integrated into peak area with the LC solution (Shimadzu Corporation, Kyoto, Japan). The concentration of each isoflavone was determined by comparison with a known concentration standard. All measurements were performed in triplicate. The relative change of isoflavone aglycones was defined. The strain with highest isoflavone aglycones was selected for high isoflavone aglycones fermented soybean production.

16S rDNA identification

The selected strain was identified by double strands 16S rDNA sequencing. DNA coding for 16S rRNA regions was amplified by mean of PCR with *Taq* polymerase, as described by Katsura *et al*[32], Kawasaki *et al*[33] and Yamada *et al*[34]. The used primer were 20F (5'-GAG TTT GAT CCT GGC TCA G-3', Brosius *et al*.,[35] and 1500R (5'-GTT ACC TTG TTA CGA CTT-3'; Brosius *et al*.,[35]. The PCR amplification was carried out with DNA Engine Dyad® Thermal Cycler (Bio-Rad Laboratories). One hundred ml of a reaction mixture contained 15-20 ng of template DNA, 2.0 mmoles of each of primers, 2.5 units of *Taq* polymerase, 2.0 mM $MgCI_2$, 0.2 mM dNTP and 10 nl of 10x*Taq* buffer, pH 8.8, containing $(NH_4)_2S0_4$, which was comprised of 750 mM Tris-HCI, 200 mM $(NH_4)_2S0_4$ and 0.1% Tween 20. The PCR amplification was programmed to carry out an initial denaturation step at 94°C for 3 min, 25 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and elongation at 72°C for 2 min, followed by a final amplification step at 72°C for 3 min. The PCR product was analyzed by 0.8% (w/v) agarose gel electrophoresis and purified with a GenepHlow™ Gel/PCR Kit (Geneaid Biotech, Taiwan). The sequencing of the purified PCR products was performed on an ABI Prism® 3730XL DNA Sequence (Applied Biosystems, Foster City, California, USA) by sequencing service provider. The four primers 27F (5'-AGA GTT TGA TCM TGG CTC AG-3'), 518F (5'-CCA GCA GCC GCG GTA ATA CG-3'), 800R (5'-TAC CAG GGT ATC TAA TCC-3') and 1492R (5'-TAC GGY TAC CTT GTT ACG ACT T-3') were used for double strands 16S rDNA sequencing. All of the sequencing data were analyzed using Cap contig assembly program, an accessory application in BioEdit (Biological sequence alignment editor) Program (http://www.mbio.ncsu.edu/ BioEdit/BioEdit.html).

Isoflavone aglycones enrichment in *thua-nao* production

The selected strain of *Bacillus* spp. was utilized as inoculum for *thua-nao* fermentation. The fermentation time was 10 days. Isoflavones content, â-glucosidase activity and viable colony number of *Bacillus* spp. were analyzed. The â-glucosidase activity assay was modified from Yin *et al*.[36]. 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 8,000g 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.

Table 1. Morphological characteristics of *Bacillus* spp. isolated from fermented soybean (*thua-nao* and *nutto*)

	Group				
	I	II	III	IV	V
Number of strains	1	4	30	2	4
Distribution (%)	2.43	9.76	73.17	4.88	9.76
Colony morphology					
Diameter (mm)	3.0-5.0	2.0-4.5	2.0-3.0	3.5-4.0	2.5-4.5
Edge or margin	Lobate	Undulate	Undulate	Entire	Entire
Elevation	Raised	Effuse	Flat	Raised	Convex
Form	Irregular	Irregular	Irregular	Circular	Circular
Surface	Rugose	Rough	Smooth	Smooth	Smooth
Pigment	White	White	Creamy white	White	Yellow-white
Optical	Opaque	Opaque	Translucent and glistening	Opaque	Opaque
Cell morphology					
Shape	Rod	Rod	Rod	Rod	Rod
Size W/L (µm)	0.7-0.8/1.5-2.5	0.7-1.0/3.0-5.0	0.7-0.8/1.5-3.0	0.5-0.7/1.5-2.5	1.2-1.5/3.0-4.0
Chain	Singly or in chain	Singly or in chain	Singly or in chain	Singly or in chain	Singly or in chain
Endospore	Midle	Midle	Midle	Midle	Midle
Gram staining	Positive	Positive	Positive	Positive	Positive

The resultant color was immediately measured at 405 nm. One unit of enzyme activity was defined as the amount of enzyme which liberated l mm 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 °C for 24 h and calculated viable colony number.

Statistical Methods

All data were expressed as mean ± standard deviation. SPSS Statistical software Version 15.0 was performed for calculations. Statistical significance was determined by ANOVA. *P*-values of < 0.05 were considered to be statistically significant difference.

RESULTS AND DISCUSSION

Physiological and phenotypic characterization of *Bacillus* spp.

A total of 41 strains, which isolated from *thua-nao* and *nutto*, were divided into five groups on the basis of morphological, biochemical and physiological characteristics (Table 1 and Table 2). There were 30 strains (73.17%) were found in Group III (LG01, LG03, LG05, LG09, LG10, LG11, MH03, MH04, LP01, LP02, LP05, CR01, CR02, CR04, CR06, PR02, PY04, CH06, CH07, CH09, CH10, CH11, NN07, NN10, NTA01, NTA02, NTB01, NTB02, NTC01 and NTC02). Four strains (9.76%) were found in group II (LG07, PR01, PR03 and PY02). Four strains (9.76%) were found in group V (MH05, LP03, PY03 and PR04). Two strains (4.88%) were in Group IV (CR05 and MH02). One strain (2.42%) belongs to Group I (PY01). All of the isolates were rod shape, spore forming, Gram-positive, catalase-positive, facultatively anaerobic, growth at 50°C and not growth at 65°C. The colony morphology of the strains in group I-III was irregular form while group IV-V was circular form. Colony elevation of group I and IV had similar form which was raised. However, for group II, III and IV was in effuse, flat and convex, respectively. Colony surface of group III-V smooth. On the contrary, group I-II were rugose and rough. Colony edge or margin of group I was lobate. Group II-III were undulate and group IV-V were entire. In term of pigment and optical character, it was found that group I, II, IV and V

Table 2. Biochemical and physiological characteristics of *Bacillus* spp. isolated from traditional fermented soybean (*thua-nao* and *nutto*)

	Catalase test	Voges-Proskauer	Acid and gas from glucose	Anaerobe growth	Growth at 50 °C	Growth at 65 °C	Width of rod 1.0 um or greater	Hydrolysis of starch	Growth in 7% NaCl
Group I	+	+	-	+	+	-	-	+	+
Group II	+	+	-	+	+	-	+	+	-
Group III	+	+	-	-	+	-	-	+	+
Group IV	+	+	-	-	+	-	-	-	+
Group V	+	-	-	-	-	-	+	+	+
Bacillus licheniformis[a]	+	+	-	+	+	-	-	+	0
Bacillus coagulans[a]	+	+	-	+	+	-	0	+	0
Bacillus subtilis[a]	+	+	-	-	+	-	-	+	0
Bacillus pumilus[a]	+	+	-	-	+	-	-	-	0
Bacillus megaterium[a]	+	-	-	-	-	-	+	+	0

Note: + positive reaction, - negative reaction and 0 no data; [a] Data from [23]

Table 3. Isoflavone aglycones content of *thua-nao* from various *Bacillus* spp.

Isolated number	Isoflavone aglycones content (mg/100g dry weight)				Relative change of total isoflavone aglycones[a]
	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:[a] 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

had opaque-white color but group V had slightly yellow color. Group III had translucent and glistening-creamy white color.

The five groups of strains were characterized according to the biochemical and physiological criteria given by Norris *et al*[29]. It was found that, the strains in group I – V were *B. licheniformis*, *B. coagulans*, *B. subtilis*, *B. pumilus* and *B. megaterium*, respectively. The most effective strain which was able to produce highest isoflavone aglycones will be selected and identified by 16S rDNA sequencing to confirm species of the strain.

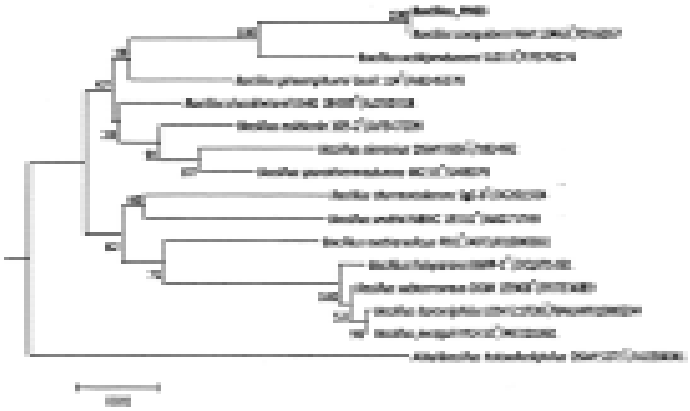

Fig. 1. Phylogenetic relationships (based on 16S rDNA) of *Bacillus* PR03 between the isolates and closely related species. The optimal tree with the sum of branch length = 0.26858774 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Evolutionary analyses were conducted in MEGA6

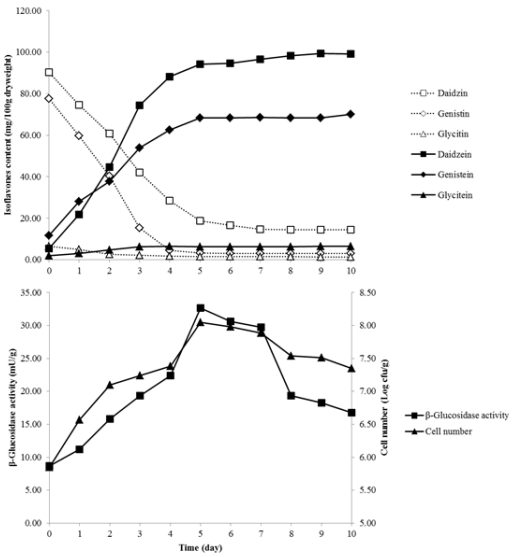

Fig. 2. Change of isoflavones, â-glucosidase activity and total bacteria count during *thua-nao* fermentation by *Bacillus coagulans* PR03

Screening of isoflavone aglycones producing *Bacillus* spp.

The isoflavone aglycones content of *thua-nao* was shown in Table 3. It was found that, each strain differently produced isoflavone aglycones. The LG01, PR03 and NTA02 were able to produce daidzein, genistein and glycitein, 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 3.81times of nonfermented soybean. The less effective strains were NTA02 and LG01 which produced isoflavone aglycones 129.80 and 123.70 mg/100 g dry weight respectively. The NN07 and MH05 were the least effective strain. They produced only 30.71 and 30.94 mg/100g dry weight respectively. According to the result of the study, *Bacillus* PR03 was selected as starter culture for enriched isoflavone aglycones in *thua-nao* production.

16S rDNA identification

The double strands 16S rDNA sequencing was used to confirm the correct specie of *Bacillus* PR03. The comparison of nucleotide sequences between *Bacillus* PR03 with reference strains found that *Bacillus* PR03 was similar to *B. coagulans* strain IAM12463 and *B. acidiproducens* strain SL213 with 99.93% and 97.10% identity. In considering with phylogenetic tree analysis (Fig 1) found that *Bacillus* PR03 was in the same cluster and had genetic distance close to *B. coagulans* IAM12463 in the number of 0.2686. As the result, the *Bacillus* PR03 was identified as *B. coagulans*. The *B. 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*[23], "*dawadawa*" are *B. subtilis*, *B. pumilus* and *B. lichenformis*[24, 25] and "*kinema*" are *B. subtilis*, *B. lichenformis* and *B. badius*[26, 27]. Therefore, this was the first research using *B. coagulans* as predominant bacteria for producing *thua-nao* with high isoflavone aglycones.

Isoflavone aglycones enrichment in *thua-nao* production by selected strain

The selected strain, *B. coagulans* PR03, was used as pure culture for *thua-nao* production. The isoflavones content, â-glucosidase activity and total bacteria count in *thua-nao* using *B. coagulans* PR03 were shown in Fig 2. Fermentation process had significant effects on the isoflavone aglycones, isoflavone glucosides, â-glucosidase activity and total bacteria count ($P<0.05$). The quantity of isoflavone aglycones tended to increase during the fermentation period. As the result, the quantity of daidzein, genistein, glycitein and total aglycones increased from 5.46, 11.69, 1.95 and 19.10 mg/100 g dry weight to 99.16, 70.00, 6.35 and 175.51 mg/100 g dry weight respectively. On the contrary, 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/100 g dry weight to 14.48, 2.99, 1.31 and 18.78 mg/100 g dry weight respectively. â-glucosidase activity increased from 8.65 mU/g to the highest value of 32.62mU/g at the fifth day of fermentation process. After that the value decreased to17.74 mU/g at the last day of fermentation. Total bacteria count represented cell number of *B. coagulans* PR03 tended to have the same result as â-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. Therefore, the suitable fermentation time was 5 d which consist of daidzein, genistein, glycitein and total aglycones as 94.16, 68.29, 6.33 and 168.77 mg/100 g dry weight.

The increasing number of *B. coagulans* PR03 and â-glucosidase activity were relevant. *B. coagulans* PR03 has grown to log phase until the fifth day of fermentation and â-glucosidase activity would increase as well. The increasing value of *B. coagulans* PR03 and â-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 culture decreased and eventually the value of *B. coagulans* PR03 and â-glucosidase activity decreased. Isoflavone aglycones value increased only in small number. Therefore, the transformation of isoflavone glucosides into isofalvones aglycones depended on the activity of â-glucosidase produced by *B. coagulans* PR03. The study mentioned above was relevant to the following researches. Wei *et al*.[37] reported the isoflavone aglycones content in fermented soybean by *B. subtilis* BCRC14718 was increased significantly after 24 h fermentation. Ibe *et al*.[38] also reported that â-glucosidase from *B. subtilis* nutto IF9916 has ability to hydrolyzed isoflavone glucosides. Moreover, Kuo *et al*.[39] reported that isoflavone glucosides (daidzin and genistin) were hydrolyzed to isoflavone aglycones (daidzein and genistein) by â-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.

In conclusion, the *B. coagulans* PR03 was suitable for pure culture inoculum for enriched isoflavone aglycones in *thua-nao* production. This was the first research using *B. coagulans* as predominant bacteria for producing isoflavone aglycones from soybean. Moreover, *thua-nao* fermentation with *B. coagulans* PR03 at 30-35 ^{o}C for 5 d revealed high amount of isoflavone

aglycones (168.77 mg/100 g dry weight).

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