Appendix A

Isoflavones analysis

Chemicals and solvents

Acetonitrile and methanol used were HPLC grade (J.T. Baker, USA). Hydrochloric acid was analytical grade from Merck, Germany. Daidzin (minimum 95%, HPLC), glycitin (>99%, HPLC), genistin (minimum 95%, HPLC), daidzein (minimum 98%), glycitein (minimum 97%, HPLC), genistein (minimum 98%, HPLC), flavone and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich, USA.

Instruments

Analysis of isoflavones was performed by HPLC. A Shimadzu LC-20A series equipped with binary pump LC-20AB, degasser DGU-20A_3, auto sampler SIL-20A, column oven CTO-10AS vp, photo diode array detector SPD-M20A (Shimadzu Corporation, Kyoto, Japan) was used.

Standard solutions

Each isoflavone standard was dissolved in a small amount of DMSO and diluted with methanol to form 5 ml of stock standard solution (1,000-5,000 ug/ml). The appropriate volume of each isoflavone stock standard solution was then diluted with methanol to make a mixture of isoflavones working standard solutions.

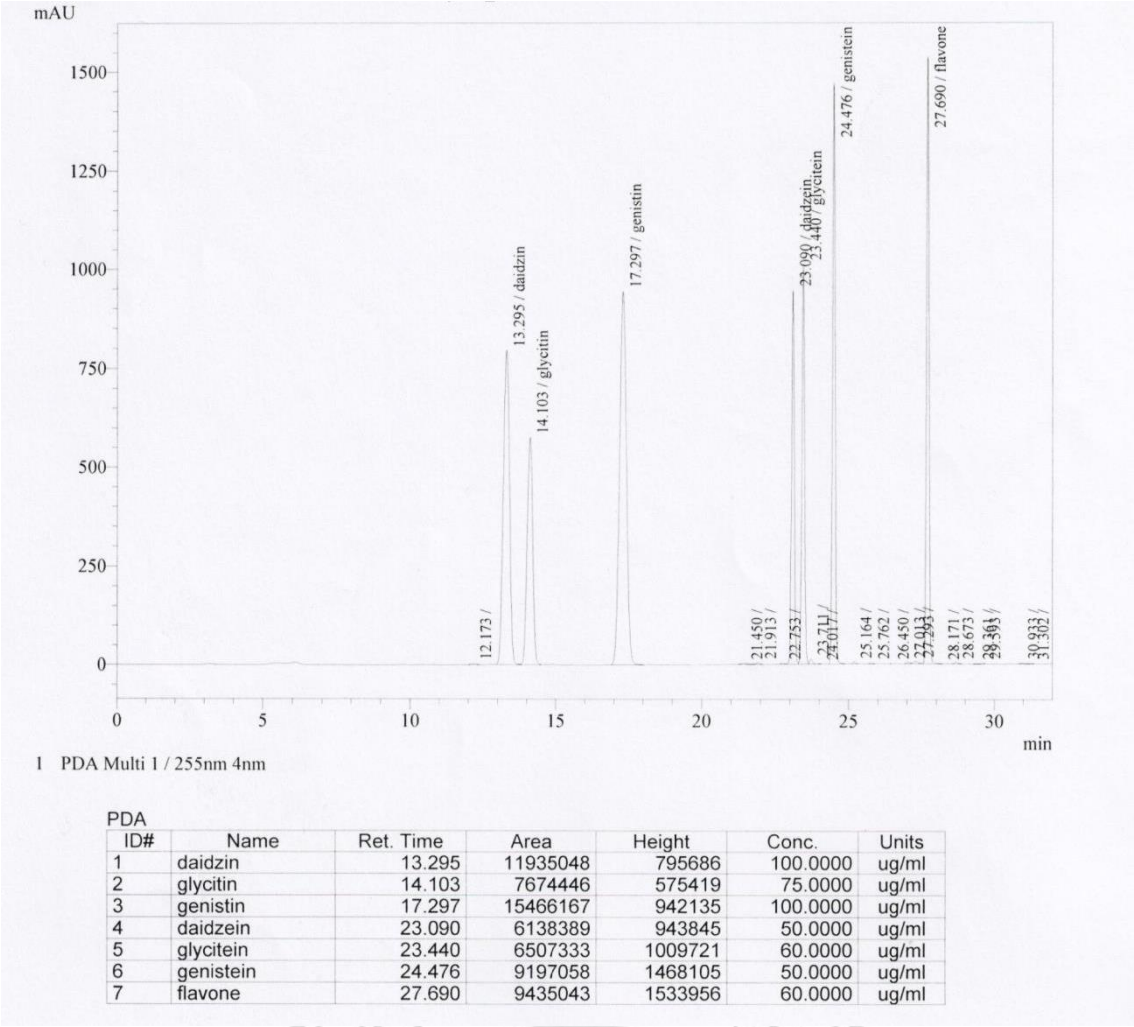
Sample preparation

Freeze dried soybean powder was extracted by solvent extraction. The extraction method was modified from that of Murphy *et al*. (2002). One gram of freeze dried soybean powder was extracted with 5 ml of acetonitrile, 110 µl of 0.1 mg/ml flavone in methanol, 1 ml of 0.1 N hydrochloric acid and 5 ml of water in 250 ml Erlenmeyer

flask. The extract was stirred at room temperature for 10 min and sonicated (S100H, Elma, German) for 10 min. Then an aliquot of extract was centrifuged by microcentrifuge (Gyrospin microcentrifuge, Labtech, Korea) at 10,000 rpm for 10 min. The supernatant was prior filtered through 0.45 μm nylon syringe filter and analyzed by HPLC.

HPLC condition

The isoflavones analytical method was modified from that of (Klejdus *et al.*, 2005). Twenty microliter of sample was injected into a C18 Inertsil ODS-3 column (5 μm, 4.6x250 mm, GL Sciences Inc., Japan) with a C18 guard column (Inertsil ODS-3 column (5 μm, 4.0x10 mm, GL Sciences Inc., Japan). Column temperature was constant at 40^{o}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 at 30% B, then increased to 35% B at 5 min, 42% B at 8 min, 90% B at 21.5 min and 100% B at 28 min. At 28.5 min the system recycled back to 30% 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 of standard. All measurements were performed in duplicate.



PDA

ID#	Name	Ret. Time	Area	Height	Conc.	Units
1	daidzin	13.295	11935048	795686	100.0000	ug/ml
2	glycitin	14.103	7674446	575419	75.0000	ug/ml
3	genistin	17.297	15466167	942135	100.0000	ug/ml
4	daidzein	23.090	6138389	943845	50.0000	ug/ml
5	glycitein	23.440	6507333	1009721	60.0000	ug/ml
6	genistein	24.476	9197058	1468105	50.0000	ug/ml
7	flavone	27.690	9435043	1533956	60.0000	ug/ml

Figure A.1 The representative of HPLC showing isoflavone content

APPENDIX B

Biochemical and physiological tests for *Bacillus* spp. identification

B.1 Culture Media

All culture media were dissolved in distilled water and sterilized at 121 °C for 15 minutes.

1. Nutrient broth (NB) and nutrient agar (NA)

Beef extract	3 g
Peptone	5 g

To prepare Nutrient agar (NA), agar was added to the prior mixture with solid to liquid ratio of 15 g/L.

2. MR-VP medium

Peptone	7 g
Potassium phosphate	5 g
Dextrose	5 g

3. Nitrate broth (Nitrate reduction test medium)

Potassium nitrate	0.2 g
Peptone	5 g

4. Starch agar

Beef extract	3 g
Peptone	5 g
Soluble starch	10 g
Agar	15 g

5. Fermentation carbohydrate medium

Beef extract	3 g
Peptone	5 g
Glucose	10 g
1.6% Bromothymol blue	4 ml

Test tube containing Durham tube was sterilized at 150 °C for 3 hours before adding 6 mL of the sugar solution. The tube was sterilized at 10 psi and immediately put in cool water baht to prevent the sugar from breaking down.

B.2 Chemicals

1. Phosphate buffer 0.05 M pH 7.0

Solution A: KH_2PO_4 9.073 g/L

Solution B: Na_2HPO_4 . $2H_2O$ 11.87 g/L

Distilled water 1 L

A 100 mL of phosphate buffer pH 7.0 was prepared by mixing 41.3 mL of Solution A with 58.7 mL of Solution B.

2. 3% Hydrogen peroxide solution

Hydrogen peroxide (H_2O_2) 3 g (calculate from the label)

Distilled water 100 mL

3. Voges-Proskauer test solution

Solution A: α-napthol 10 g

Ethanol 95% 100 ml (Kept in brown bottle)

Solution B: KOH 20 g

Distilled 100 ml (Kept in brown bottle)

4. 1.6% bromothymol blue solution

Bromothymol blue 1.6 g

Ethyl alcohol 100 mL

B.3 Biochemical test

1. Catalase test

Catalase enzyme contains hematin which can transform hydrogen peroxide (H_2O_2) into oxygen (O_2) and water (H_2O). The reaction is shown in the following equation

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$

Because catalase enzyme only existed in living microorganisms, the test was performed using fresh culture (less than 24 hours) to prevent an error measurement. Furthermore, using microorganism cultured in blood containing media was avoided. Since the culture would contain catalase enzyme in high quantity, the use of such culture could cause a spill to testing staff due to rapid generation of oxygen.

Test methods

1. Added 1 drop of 3% H_2O_2 on the plate

2. Equally distributed the solution using inoculation loop

Positive (+): Bubbles formed

Negative (-): No bubbles formed

2. Voges-Proskauer test (VP)

Some bacteria can generate butylene glycol and ethanol via butylene glycol fermentation of glucose. The reaction also generates acetyl methyl carbinol or acetoin which can be tested

Test methods

1. Inoculated the culture in MR-VP medium and incubate at 37 °C for 48 hours

2. Transferred 1 mL of the resulting culture into a sterile tube.

3. Added 0.6 mL of 5% α-naphthol solution

4. Added 0.2 mL of 40% KOH solution

5. Thoroughly mixed the solution

Positive (+): The solution turned red in 5 minutes

Negative (-): The solution remained yellow

3. Test method for anaerobic bacteria

Inoculated the culture sample in a nutrient agar and incubated in anaerobic jar for 24 hours. For control, another portion of the sample was inoculated in a nutrient agar but incubated under aerobic condition for 24 hours. The anaerobic jar contained Anaerocult® A (filled with water and immediately put in the anaerobic jar) with a resazulin as an oxygen indicator (white color in anaerobic condition, but turned pink in the presence of oxygen). The result was determined by comparing the growth of bacteria with control.

4. Bacterial classification method by growth temperature

Bacteria can be classified into mesophile, psychrophile and thermophile according their growing temperature.

Test method

1. Inoculated the test sample into nutrient broth or onto nutrient agar
2. Incubated the sample at designed temperature
3. Inspected the results every 7 days by turbidity of the broth or appearance to a streak on the agar

Positive (+) Nutrient broth became turbid or streak appeared on the agar

Negative (-) No turbidity in nutrient broth or no streak on the agar

5. Test method for bacterial growth in sodium chloride

This test determined the growth of bacteria in sodium chloride (NaCl) solution along with it level of tolerance.

Test method

1. Inoculated test culture in nutrient broths with sodium chloride concentration of 5%, 7% and 10%

2. Incubated the samples at 37 °C for 24 hours

3. Determined the growth of bacteria using turbidity of the broth

Positive (+) Turbid

Negative (-) Not turbid

6. Bacterial classification method by carbohydrate fermentation

Carbohydrates are compounds with basic molecular structure of CH_2O with various sizes. However, in order for bacteria to utilize the carbohydrate with molecule size of disaccharide or bigger, the bacteria must be able to produce permease enzymes to digest the carbohydrate to a smaller molecules that can be absorbed into the cells. Different type of bacteria has different method for utilization of carbohydrates. Thus, this test classified bacteria according to how it ferments carbohydrates by using glucose as a test compound.

Test method

1. Inoculated test bacteria in a fermentation carbohydrate medium

2. Incubated the sample at 37 °C for 18-24 hours

3. Verified the result by the change in color of test medium and gas generation in Durham tube.

Positive (+)

1. Test medium color changed to yellow but no gas in Durham tube indicated that the bacteria can ferment carbohydrates and the fermentation resulted in acids

2. Test medium color changed to yellow with gas in Durham tube indicated that the bacteria can ferment carbohydrates and the fermentation resulted in both acids and gasses

Negative (-)

Test medium color remained green indicated that the bacteria cannot ferment carbohydrates

7. Test method for starch digestion

Some bacteria can produce amylase enzyme to digest starch

Test method

* Test medium must have 0.2% soluble starch in its composition.

1. Inoculated the test culture into the medium and inoculated at suitable temperature until fully growth
2. Added a few drops of iodine solution into the medium, immediately recorded the result

Positive (+) No color change

Negative (-) Medium color changed to blue

B.4 Simplified key for the tentative identification of typical strains of *Bacillus* species

Table B.1 Simplified key for the tentative identification of typical strains of *Bacillus* species

1. Catalase:	positive	2
	negative.....	17
2. Voges-Proskauer:	positive	3
	negative	10
3. Growth in anaerobic agar:	positive.....	4
	negative	9
4. Growth at 50°C:	positive	5
	negative	6
5. Growth in 7% NaCl:	positive	*B. licheniformis*
	negative	*B. coagulans*
6. Acid and gas from glucose (inorganic N):	positive.....	*B. polymyxa*
	negative.....	7
7. Reduction of NO3 to NO2:	positive.....	8
	negative	*B. alvei*
8. Parasporal body in sporangium:	positive	*B. thuringiensis*
	negative	*B. cereus*
9. Hydrolysis of starch:	positive	*B. subtilis*
	negative.....	*B. pumilus*
10. Growth at 65°C:	positive	*B. stearothermophilus*
	negative	11
11. Hydrolysis of starch:	positive	12
	negative.....	15
12. Acid and gas from glucose (inorganic N):	positive.....	*B. macerans*
	negative	13
13. Width of rod 1.0µm or greater:	positive	*B. megaterium*
	negative.....	14
14. pH in V-P broth <6.0	positive	*B. circulans*
	negative	*B. firmus*
15. Growth in anaerobic agar:	positive.....	*B. laterosporus*
	negative	16
16. Acid from glucose (inorganic N):	positive	*B. brevis*
	negative	*B. sphaericus*
17. Growth at 65°C:	positive	*B. stearothermophilus*
	negative	18
18. Decomposition of casein:	positive.....	*B. larvae*
	negative.....	19
19. Parasporal body in sporangium:	positive	*B. popilliae*
	negative	*B. lentimorbus*

Note: Numbers on the right indicate the number (on the left) of the next test to be applied until the right-hand number is replaced by a species name (Norris *et al.*, 1981; Logan and Vos, 2009)

APPENDIX C

16S rDNA sequencing and phylogenetic tree analysis

1. PCR amplification of 16S rDNA

DNA templates for PCR amplification were prepared by using "Genomic DNA mini kit (Blood/culture cell)" (Geneaid Biotech Ltd., Taiwan). DNA coding for 16S rRNA regions was amplified by means of PCR with *Taq* polymerase, as described by Kawasaki *et al.* (1993), Yamada *et al.* (2000) and Katsura *et al.* (2001). A PCR product for sequencing 16S rDNA regions was prepared by using the following two primers, 20F (5'-GAG TTT GAT CCT GGC TCA G-3', positions 9-27 on 16S rDNA by the *E. coli* numbering system; Brosius *et al.* (1981)) and 1500R (5'-GTT ACC TTG TTA CGA CTT-3', position 1509-1492 on 16S rDNA by the *E. coli* numbering system; Brosius *et al.* (1981)). The PCR amplification was carried out with DNA Engine Dyad* Thermal Cycler (Bio-Rad Laboratories). One hundred nl of a reaction mixture contained 15-20 ng of template DNA, 2.0 nmoles each of the two 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 Ltd., Taiwan). The purified PCR product was stored at -20°C for further step.

2. Direct sequencing of 16S rDNA

Direct sequencing of the single-banded and purified PCR products (ca. 1500 bases, on 16S rDNA by the *E. coli* numbering system; Brosius *et al.* (1981)) was carried out. Then, 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') for double strands 16S rDNA sequencing were used.

3. Sequence analyses

The nucleotide sequences obtained from all primers were assembled using Cap contig assembly program, an accessory application in BioEdit (Biological sequence alignment editor) Program (http://www.mbio.ncsu.edu/ BioEdit/BioEdit.html). The identification of phylogenetic neighbors was initially carried out by the BLASTN (Altschul *et al.*, 1997) program against the database containing type strains with validly published prokaryotic names (Kim *et al.*, 2012). The top thirty sequences with the highest scores were then selected for the calculation of pairwise sequence similarity using global alignment algorithm (Myers and Miller, 1988), which was implemented at the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim *et al.* (2012)).

4. Phylogenetic tree analysis

The DNA sequences determined and obtained from databases were aligned with a program CLUSTAL X (version 1.8) (Thomson *et al*., 1997) in BioEdit program (Hall, 1999). Alignment gaps and unidentified bases were eliminated. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004). Phylogenetic trees of 16S rRNA genes were constructed by the neighbor-joining method of Saitou and Nei (1987). The robustness of individual branches was estimated by 1000 replications bootstrapping (Felsenstein, 1985) with the program MEGA Version 6.0 (Tamura *et al*., 2013).

4. Nucleotide sequence (5' -> 3') of *Bacillus* PR03

Table C.1 Nucleotide sequence of *Bacillus* PR03

Nucleotide region	Nucleotide sequence (5' -> 3')
16S rDNA	>Bacillus PR03 AACGCTGGCGGCGTGCCTAATACATGCAAGTCGTGCGGACCTTTTA AAAGCTTGCTTTTAAAAGGTTAGCGGCGGACGGGTGAGTAACACGT GGGCAACCTGCCTGTAAGACTGGGATAACGCCGGGAAACCGGGGCT AATACCNGATNGTTTTTTCCTCCGCATGGAGGAAAAAGGAAAGGCG GCTTCGGCTGCCACTTACAGATGGGCCCGCGGCGCATTAGCTAGTTG GCGGGGTAACGGCCCACCAAGGCAACGATGCGTAGCCGACCTGAGA GGGTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACG GGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGG AGCAACGCCGCGTGAGTGAAGAAGGCCTTCGGGTCGTAAAACTCTG TTGCCGGGGAAGAACAAGTGCCGTTCGAACAGGGCGGCGCCTTGAC GGTACCCGGCCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCG GTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAG CGCGCGCAGGCGGCTTCTTAAGTCTGATGTGAAATCTTGCGGCTCAA CCGCAAGCGGTCATTGGAAACTGGGAGGCTTGAGTGCAGAAGAGGA GAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGG AACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTGAG GCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCC ACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTA GTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCG CAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGT GGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGT CTTGACATCCTCTGACCTCCCTGGAGACAGGGCCTTCCCCTTCGGGG GACAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGA GATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGACCTTAGTTG CCAGCATTGAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCG GAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTG GGCTACACACGTGCTACAATGGATGGTACAAAGGGCTGCGAGACCG CGAGGTTAAGCCAATCCCAGAAAACCATTCCCAGTTCGGATTGCAG GCTGCAACCCGCCTGCATGAAGCCGGAATCGCTAGTAATCGCGGAT CAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCC GTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCT TTACGGAGCCAGCCGCCAAAGGTGGGACAGATGATTGGGGTGAA

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