Acta Alimentaria, Vol. 45 (1), pp. 11–19 (2016)
DOI: 10.1556/066.2016.45.1.2

CHARACTERIZATION OF *BACILLUS* SPECIES EXHIBITING STRONG PROTEOLYTIC ACTIVITY ISOLATED FROM *THUA NAO*

A. Kanghae[a], J. Monkai[b], P.D. Eungwanichayapant[a], P. Niamsup[c] and E. Chukeatirote[a]*

[a]School of Science, Mae Fah Luang University, Chiang Rai 57100. Thailand
[b]Scientific & Technological Instruments Centre, Mae Fah Luang University, Chiang Rai 57100. Thailand
[c]Faculty of Science, Maejo University, Chiang Mai 50290. Thailand

(Received: 5 August 2014; accepted: 22 September 2014)

In this study, two bacilli strains, namely TN51 and TN69, previously isolated from *Thua Nao*, a Thai traditional fermented soybean, were studied in terms of their phenotypic and biochemical properties. Initially, both strains were subjected to morphological determination and a series of biochemical tests. Both were Gram-positive, endospore-forming bacilli. Based on 16S rRNA gene sequence analysis, the identities of strains TN51 and TN69 were confirmed as *Bacillus subtilis* and *B. cereus*, respectively. In addition, these two strains were also assessed for their antibiogram profiles. It was found that both strains were susceptible to chloramphenicol, erythromycin, kanamycin, tetracycline, and vancomycin and resistant to ampicillin and intermediately susceptible to bacitracin.

Keywords: *Bacillus*, fermented soybean, proteolytic activity, *Thua Nao*

In Asian culture, there are various kinds of fermented soybeans; these include *Chungkukjang*, *Douchi*, *Kinema*, *Natto*, and *Thua Nao* (Steinkraus, 1996). Such fermented products are popular and have been consumed for several centuries. The production processes are also similar, which includes soaking, boiling, fermenting, and post-fermenting of soybeans. *Thua Nao* is an ethnic, fermented soybean of Thailand. The fermented product can be served as a main dish, although it is more commonly used as a condiment in many local dishes. Of these fermented products, *Natto* is best characterised and has been commercially produced using a pure starter culture (Kiuchi & Watanabe, 2004). *Bacillus* species have been reported as the essential bacteria in the production of these fermented soybeans. Many *Bacillus* species have been isolated during the production process as well as from the final products, suggesting their abundance. *Bacillus subtilis* in particular has been identified in *Chungkukjang* (Lee et al., 2005), *Douchi* (Li et al., 2007), *Kinema* (Sarkar & Tamang, 1995), *Natto* (Kiuchi & Watanabe, 2004), and *Thua Nao* (Chantawannakul et al., 2002). Due to a protein-rich nature of soybean, proteolysis is definitely a key mechanism of the fermentation. Soy protein degradation contributes to distinct characteristics of these fermented soybeans including texture, appearance, flavour, and aroma. As a result, these *Bacillus* strains are hot topics for many scientists studying these fermented soybeans by screening for their proteolytic activity (Chantawannakul et al., 2002; Visessanguan et al., 2005; Dajanta et al., 2009). It is also expected that those *Bacillus* isolates exhibiting a strong proteolytic activity could be used as inoculum, and this thus helps improving the manufacturing process and products. A previous study showed that two *Bacillus* isolates, namely TN51 and TN69, isolated from Thai *Thua*

* To whom correspondence should be addressed.
Phone: +66-5391-6778; fax: +66-5391-6776; e-mail: ekachai@mfu.ac.th

Nao, are of great interest due to their strong proteolytic activity (Dajanta et al., 2009). In this study, some phenotypic and biochemical properties of these two *Bacillus* strains were characterised and reported.

1. Materials and methods

1.1. Bacterial strains

Bacillus strains used in this study were isolated from Asian fermented foods: strains TN51 and TN69 from Thai *Thua Nao* (Dajanta et al., 2009); strains BEST195 and ASA from *Natto* products. *B. subtilis* TISTR008 and *B. cereus* TISTR687 were obtained from Thailand Institute of Scientific and Technological Research.

1.2. Pre-screening of proteolytic activty

These bacilli were screened for their proteolytic activity using casein agar. A single colony of the bacterial culture was inoculated into a test tube containing 4 ml of nutrient broth. After 24 h-cultivation at 37 °C with shaking (150 r.p.m.), 1 ml of cell suspension was transferred to a new microfuge tube and the bacterial cells were removed by centrifugation at 13 000 r.p.m. at 4 °C for 10 min. The supernatant was collected and used as the source of protease enzymes in the proteolytic activity assay. For this, 5 µl of the supernatant was dropped on a casein agar plate. The inoculated plate was then incubated at 37 °C for 24 h. The presence of a clear zone was recorded and used to indicate the bacterial ability to produce proteases.

1.3. Morphology and biochemical profiles

The bacterial strains were characterised by morphological and biochemical properties. These included Gram-staining, presence of spore, oxygen requirement, catalase test, lecithinase test, ability to growth in 5 and 7% NaCl, growth at 50 and 65 °C, IMViC test, nitrate reduction, fermentation of glucose, arabinose, xylose, and sucrose, and starch hydrolysis (MacFaddin, 2000). Additionally, the bacterial species confirmation was performed using the API-50 CHB kit (bioMerieux, Inc.).

1.4. Molecular characterisation

Bacterial genomic DNA was isolated and used as a template to amplify their 16S rRNA genes. Specific primers used were 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 520R (5'-ACC GCG GCK GCT GGC-3') (Lane, 1991). DNA amplification was performed in a 50 µl reaction consisting of 5 µl 10X buffer, 3 µl 25 mM $MgCl_2$, 5 µl 2 mM dNTPs, 1 µl Taq polymerase (Promega), 1 µl of each primer (1 pmol), 33 µl of sterile water, and 1 µl of 500 ng μl^{-1} bacterial DNA. Amplification protocol was as follows: an initial denaturation at 94 °C for 5 min, followed by 25 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, and a final extension at 72 °C for 5 min. The amplified products were visualized after electrophoresis in 1.5% agarose gel, and purified using the TaKaRa SUPRECTM-PCR (Takara, Japan). The purified products were then sent to 1st Base Laboratories Company (Malaysia) for sequencing. The sequencing data were analysed using BLAST (Altschul et

al., 1990) and the closest known species were recorded with percentages of identity. The accession numbers of the *Bacillus* isolates were as follows: GU451310 and GU451311 for strains TN51 and TN69, respectively. Sequence alignment and phylogenetic analysis were performed online using the Phylogeny.fr software (Dereeper et al., 2008).

1.5. Susceptibility tests

Antimicrobial susceptibility was performed using the disc agar diffusion method (NCCLS, 1997). A bacterial culture was prepared in 5 ml of Mueller-Hinton broth and incubated at 37 °C for 24 h with shaking at 150 r.p.m. A sterile cotton swab was dipped into the inoculum (0.5 MacFarland) and applied evenly onto the surface of Mueller-Hinton agar plate. After drying for 5 min, the antibiotic discs (Oxoid, England) were placed and the plates were incubated at 37 °C for 24 h. The zones were measured and expressed as sensitive, intermediate, and resistant as described by Hong and co-workers (2008). Antibiotic discs used were vancomycin (30 μg), tetracycline (30 μg), chloramphenicol (30 μg), erythromycin (15 μg), kanamycin (30 μg), ampicillin (30 μg), bacitracin (10 U), and streptomycin (10 μg).

2. Results and discussion

2.1. Proteolytic activity production of Bacillus *strains*

Bacillus species have been predominantly isolated from the fermented soybeans (Sarkar & Tamang, 1995; Lee et al., 2005; Li et al., 2007). The high numbers of Gram-positive, endospore-forming bacilli were also detected in *Thua Nao* from the beginning throughout the fermentation period (Chantawannakul et al., 2002; Chukeatirote et al., 2006). In this study, two bacilli strains, namely TN51 and TN69, previously isolated from *Thua Nao,* were further characterised because of their proteolytic activity (Dajanta et al., 2009) (see Fig. 1). When using casein agar, the presence of the clear zone was observed; the proteolytic activity was then calculated and expressed as the relative index value (RI, a ratio between the diameter of the clear zone and that of the bacterial colony). From Table 1, the RI values of *Bacillus* strains TN51 and TN69 were 2.0 and 1.8, respectively; these were higher than those of other *Bacillus* strains suggesting superior proteolytic activity.

Table 1. Relative index values (RI) of protease activity of the bacilli strains when cultured in casein agar

Bacterial strains	RI values
TN51	2.0±0.08
TN69	1.8±0.08
BEST195	1.5±0.05
ASA	1.2±0.05
B. subtilis	1.2±0.06
B. cereus	1.1±0.04

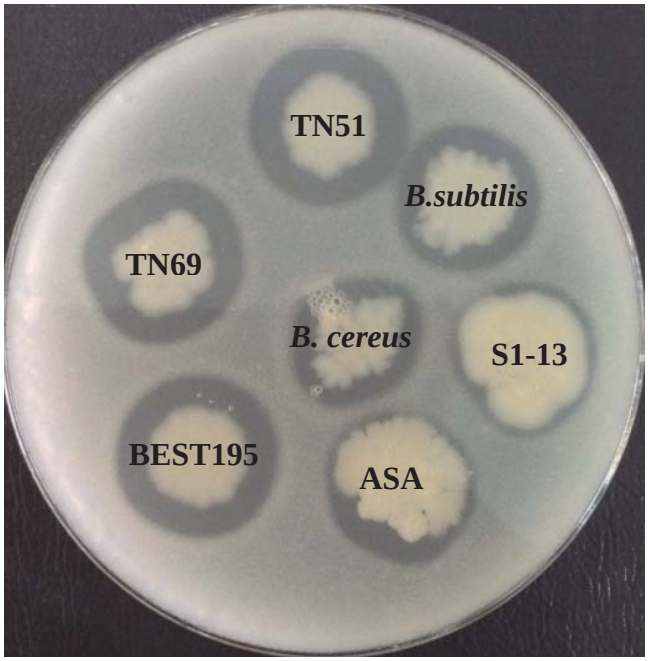

Fig. 1. Proteolytic activity on casein agar of the bacilli strains. Sources of the bacteria are as follows: the isolates TN51 and TN69 from *Thua Nao*; the isolates ASA and BEST195 from *Natto*; the isolates S1-13 from *Terasi* (a shrimp paste); *B. cereus* and *B. subtilis* are reference strains

Soybeans are protein-rich and thus, when fermented, proteolysis is undoubtedly one of the major biochemical changes. Soy proteolysis is caused by microbial activities mainly by the predominant *Bacillus* species. The relatively high proteolytic activity of *Bacillus* strains TN51 and TN69 is useful in hydrolysing soy proteins into small peptides and amino acids. Such proteolysis could enhance the nutritional value of the fermented products. Screening and isolation of protease-producing bacteria from these fermented products are therefore of great interest and are subject to comprehensive study by many scientists (Chantawannakul et al., 2002; Visessanguan et al., 2005; Dajanta et al., 2009). It is evident that these protease-producing bacteria can be used as a pure starter culture, thereby improving the manufacturing process and nutritive quality of the fermented soybean products. For example, the use of *Bacillus* pure starter culture accelerated protein hydrolysis, and hence increased the amount of dialyzable proteins in *Kinema* (Kiers et al., 2000) and *Thua Nao* (Visessanguan et al., 2005).

2.2. Morphological and biochemical characteristics of bacilli isolated

Bacillus strains TN51 and TN69 were then subject to a series of biochemical tests as listed in Table 2. They were confirmed to be *B. subtilis* according to the following taxonomic criteria: Gram-positive bacilli, presence of endospore, facultative anaerobic, catalase and nitrate reductase positive reactions, production of acetylmethylcarbinol and acid from glucose. Besides, the biochemical profiles of both strains are also very similar to those of *B. subtilis*

strains BEST195 and ASA, which were obtained from *Natto* products. Evaluation of substrate utilisation patterns of the two bacterial strains was also determined using the API-50 CHB kit (Table 3).

Table 2. Morphological, physiological and biochemical features of the bacteria used in this study

Characteristics	TN51	TN69	BEST195	ASA	*B. subtilis*	*B. cereus*
Gram	+	+	+	+	+	+
Shape	rod	rod	rod	rod	rod	rod
Presence of spore	+	+	+	+	+	+
Oxygen requirement	facultative	facultative	facultative	facultative	facultative	facultative
Growth in 5% NaCl	+	+	+	+	+	+
Growth in 7% NaCl	–	–	–	–	+	+
NB 50 °C	–	–	–	–	+	–
NB 65 °C	–	–	–	–	–	–
Catalase	+	+	+	+	+	+
Voges Proskauer	+	+	+	+	+	+
Methyl red	+	+	+	–	+	+
Formation of indole	–	–	–	–	–	–
Starch hydrolysis	+	–	+	+	+	+
Citrate utilization	–	–	–	+	+	+
Nitrate reduction	+	+	+	+	+	+
Egg-yolk lechitinase	+	+	+	+	+	+
Acid from glucose	+	+	+	+	+	+
Gas in glucose	–	–	–	–	–	–
Fermentation of arabinose	+	+	+	–	–	–
Fermentation of glucose	+	+	+	+	+	+
Fermentation of xylose	+	+	+	+	+	–
Fermentation of sucrose	+	+	+	+	+	+

Table 3. Fermentation of carbohydrates of TN51 and TN69 using API 50 CHB

No	Carbohydrate	TN51	TN69
1	Glycerol	+	+
2	Erythritol	–	–
3	D-Arabinose	–	–
4	L-Arabinose	–	–
5	D-Ribose	+	+
6	D-Xylose	–	–
7	L-Xylose	–	–
8	D-Adonitol	–	–
9	Methyl β-D-xylopyranoside	–	–
10	D-Galactose	–	–
11	D-Glucose	+	+
12	D-Fructose	+	+
13	D-Mannose	–	–
14	L-Sorbose	–	–
15	L-Rhamnose	–	–

Table 3 continued

No	Carbohydrate	TN51	TN69
16	Dulcitol	–	–
17	Inositol	–	–
18	D-Mannitol	–	–
19	D-Sorbitol	–	–
20	Methyl α-D-mannopyranoside	–	–
21	Methyl α-D-glucopyranoside	–	–
22	N-Acetylglucosamine	+	+
23	Amygdalin	–	–
24	Arbutin	+	+
25	Esculin ferric citrate	+	+
26	Salicin	–	–
27	D-Cellobiose	–	–
28	D-Maltose	+	+
29	D-Lactose (bovine origin)	–	–
30	D-Melibiose	–	–
31	D-Saccharose (sucrose)	+	+
32	D-Trehalose	+	+
33	Inulin	–	–
34	D-Melezitose	–	–
35	D-Raffinose	–	–
36	Amidon (Starch)	–	–
37	Glycogen	–	–
38	Xylitol	–	–
39	Gentiobiose	–	–
40	D-Turanose	–	–
41	D-Lyxose	–	–
42	D-Tagatose	–	–
43	D-Fucose	–	–
44	L-Fucose	w	–
45	D-Arabitol	–	–
46	L-Arabitol	w	–
47	Potassium gluconate	w	–
48	Potassium 2-ketogluconate	–	–
49	Potassium 5-ketogluconate	w	w

Notes: –: negative (no change); +: positive (yellow); w: weak reaction

2.3. Identification of strains TN51 and TN69

To further verify their identity, a DNA fragment containing the 16S rRNA gene of the strains TN51 and TN69 were amplified and sequenced. This sequence information was initially determined using a BLAST search. The 16S rRNA gene sequences of the strains TN51 and TN69 showed close relatedness with *B. subtilis* and *B. cereus*, respectively. Phylogenetic tree was then constructed to evaluate the phylogeny of the strains TN51 and TN69 (Table 4 and Fig. 2). Based on the dendogram, the strain TN51 was grouped with *B. subtilis* and TN69 was closely related to *B. cereus*. However, it should be noted that *B. subtilis* group is complex, consisting of several closely related members (i.e., *B. amyloliquefaciens*, *B. licheniformis*, *B. mojavensis*, and *B. sonorensis*) (Rooney et al., 2009). For many years, it is widely known that

the *Bacillus* species (i.e., *B. subtilis*) exhibited variations in phenotypic characteristics. Besides, phylogenetic analysis of the 16S rRNA gene also fails to distinguish the *Bacillus subtilis* species (Ash et al., 1991). Several techniques have been introduced to study the phylogenetic relationship of the *B. subtilis* group, including fatty acid analysis (Nakamura et al., 1999), multigene analysis of conserved proteins (Borriss et al., 2011), and genome hybridisation (Earl et al., 2007). It should be noted, that each technique has varying degrees of success. This study was therefore our intention to provide a description of phenotypic and biochemical characteristics of the *Bacillus* strains TN51 and TN61. In terms of their identity, we would like to propose that the strain TN51 was classified in *B. subtilis* species complex, whereas the strain TN69 was closely related to *B. cereus*.

Table 4. GenBank accession numbers for the 16S rRNA gene sequence of *Bacillus* species used in this study

Bacteria	Strains	Accession No.
Bacillus subtilis	DSM 10^T	AJ276351
B. stearothermophilus	T10	X57309
B. cereus	IAM 12605^T	D16266
B. licheniformis	DSM 13^T	X68416
B. megaterium	IAM 13418^T	D16273
B. amyloliquefaciens	ATCC 23350^T	X60605
B. thuringiensis	NCIMB 9134^T	X55062
Lactobacillus plantarum	ATCC 14917^T	AJ621668

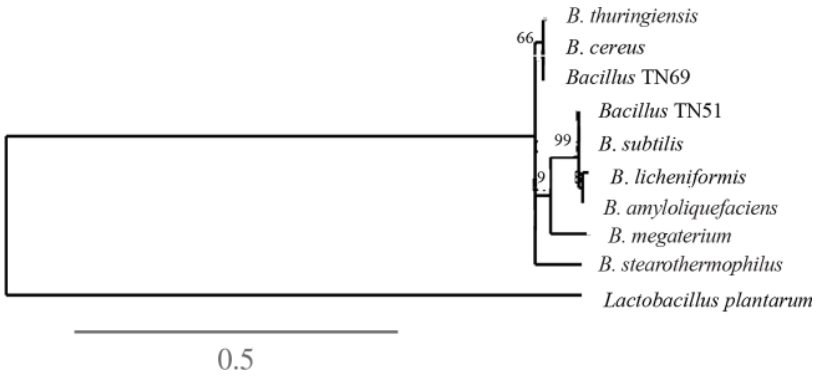

Fig. 2. Phylogenetic relationships based on similarity of the 16S rRNA gene sequences of *Bacillus* strains TN51 and TN69 to reference strains. Bootstrap values greater than 60% were given. The branch length is proportional to the number of nucleotide substitutions per site

2.4. Antimicrobial susceptibility

These *Bacillus* strains were also tested for their antimicrobial susceptibility using the agar disc-diffusion assay as recommended by the NCCLS (1997). This antimicrobial susceptibility test is performed to detect possible drug resistance in common pathogens (Jorgensen & Ferraro, 2009). Our data showed that all *Bacillus* used in this study were sensitive to chloramphenicol, erythromycin, kanamycin, streptomycin, tetracycline, and vancomycin. They are resistant to ampicillin and most of them (except *B. subtilis* type strain) are intermediately susceptible to bacitracin (Table 5). Antibiotic susceptibility of *Bacillus* species (especially for *B. subtilis*) has generally received little attention. This is possibly because *B.*

subtilis is generally regarded as safe (GRAS status). Besides, several *B. subtilis* strains are being used as probiotics (Cutting, 2011) and for producing fermented soybeans and industrial enzymes (Schallmey et al., 2004). There are a few studies dealing with the antimicrobial susceptibility of *B. subtilis* (Hong et al., 2008; Jorgensen & Ferraro, 2009). In general, it is recommended that *B. subtilis* is safe and could be used in food industry. Our data also indicate that the use of the strains TN51 and TN69 is possible. The only issue regarding the safety concern is the resistance to ampicillin, which should be studied further.

Table 5. Antibiotic susceptibility of bacilli strains isolated from *Thua Nao*

Antibiotic discs	TN51	TN69	BEST195	ASA	*B. subtilis*	*B. cereus*
Ampicillin (10 µg)	12.5±0.7 R	14.0±0 R	13.0±0 R	15.0±0 R	14.0±1.4 R	11.5±0.7 R
Bacitracin (10 U)	12.5±0.7 I	12.5±0.7 I	12.5±0.7 I	11.5±0.7 I	14.0±1.4 S	12.5±0.7 I
Chloramphenicol (30 µg)	28.5±0.7 S	29.5±0.7 S	28.0±0.7 S	26.5±0.7 S	21.0±1.4 S	20.0±0 S
Erythromycin (15 µg)	28.5±0.7 S	28.5±0.7 S	27.5±0.7 S	28.5±0.7 S	23.0±0 S	13.0±0 S
Kanamycin (30 µg)	20.5±0.7 S	20.0±0.7 S	21.0±0.7 S	25.0±0 S	25.0±0 S	24.0±0.7 S
Streptomycin (10 µg)	20.5±0.7 S	20.0±0 S	20.5±0.7 S	19.0±0 S	22.0±1.4 S	21.5±0.7 S
Tetracycline (30 µg)	19.5±0.7 S	19.5±0.7 S	19.5±0.7 S	27.5±0.7 S	20.5±0.7 S	19.5±0.7 S
Vancomycin (30 µg)	17.0±0 S	17.0±0 S	17.0±0 S	19.5±0.7 S	17.0±0 S	17.0±0 S

Notes: Data presented were diameters of inhibition zones obtained from three individual experiments. R: resistance; I: intermediate; S: sensitive.

3. Conclusions

Bacillus species are predominant in fermented soybean products. The bacilli strains TN51 and TN69 previously isolated from Thai *Thua Nao* were studied in terms of their phenotypic and biochemical properties. Our data confirmed that identification of *Bacillus subtilis* species is difficult. Besides, it is evident that their characteristics are often species dependent (even subspecies- or strain-dependent in some cases). This study is presented with an expectation to provide some key characteristics of the two Thai *Bacillus* strains in comparison with those isolated from similar fermented products.

*

The authors would like to thank Dr. Mitsuhiro Itaya (Keio University, Japan) for providing the *Bacillus subtilis* (natto) strain BEST195. This work was supported by Mae Fah Luang University under the Project Grant No. 57101010027.

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. (1990): Basic local alignment search tool. *J. Mol. Biol.*, *215*, 403–410.

ASH, C., FARROW, J.A.E., DORSCH, M., STACKEBRANDT, E. & COLLINS, M.D. (1991): Comparative analysis of *Bacillus anthracis*, *Bacillus cereus*, and related species on the basis of reverse transcriptase sequencing of 16S rRNA. *Int. J. Syst. Bacteriol*., *41*, 343–346.

BORRISS, R., CHEN, X.H., RUECKERT, C., BLOM, J., BECKER, A., BAUMGARTH, B., FAN, B., PUKALL, R., SCHUMANN, P., SPROER, C., JUNGE, H., VATER, J., PUHLER, A. & KLENK, H.P. (2011): Relationship of *Bacillus amyloliquefaciens* clades associated with strains DSM 7T and FZB42T: a proposal for *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* subsp. nov. and *Bacillus amyloliquefaciens* subsp. *plantarum* subsp. nov. based on complete genome sequence comparisons. *Int. J. Syst. Evol. Micr.*, *61*, 1786–1801.

CHANTAWANNAKUL, P., ONCHAROAN, A., KLANBUT, K., CHUKEATIROTE, E. & LUMYONG, S. (2002): Characterization of protease of *Bacillus subtilis* strain 38 isolated from traditionally fermented soybean in northern Thailand. *ScienceAsia*, *28*, 241–245.

CHUKEATIROTE, E., CHAINUN, C., SIENGSUBCHART, A., MOUKAMNERD, C., CHANTAWANNAKUL, P., LUMYONG, S., BOONTIM, N. & THAKANG, P. (2006): Microbiological and biochemical changes in *thua nao* fermentation. *Res. J. Microbiol.*, *1*, 38–44.

CUTTING, S.M. (2011): *Bacillus* probiotics. *Food Microbiol*., *28*, 214–220.

DAJANTA, K., WONGKHAM, S., THIRACH, P., BAOPHOENG, P., APICHARTSRANGKOON, A., SANTITHUM, P. & CHUKEATIROTE, E. (2009): Comparative study of proteolytic activity of protease-producing bacteria isolated from *thua nao*. *Maejo Int. J. Sci. Technol*., *3*, 269–276.

DEREEPER, A., GUIGNON, V., BLANC, G., AUDIC, S., BUFFET, S., CHEVENET, F., DUFAYARD, J.F., GUINDON, S., LEFOR, V., LESCOT, M., CLAVERIE, J.M. & GASCUEL, O. (2008): Phylogeny.fr: robust phylogenetic analysis for the non-specialist. *Nucleic Acids Res*., *36*, W465–W469**.**

EARL, A.M., LOSICK, R. & KOLTER, R. (2007): *Bacillus subtilis* genome diversity. *J. Bacteriol*., *189*, 1163–1170.

HONG, H.A., HUANG, J.M., KHANEJA, R., HIEP, L.V., URDACI, M.C. & CUTTING, S.M. (2008): The safety of *Bacillus subtilis* and *Bacillus indicus* as food probiotics. *J. Appl. Microbiol*., *105*, 510–520.

JORGENSEN, J.H. & FERRARO, M.J. (2009): Antimicrobial susceptibility testing: A review of general principles and contemporary practices. *Clin. Infect. Dis*., *49*, 1749–1755.

KIERS, J.L., VAN LAEKEN, A.E., ROMBOUTS, F.M. & NOUT, M.J. (2000): In vitro digestibility of *Bacillus* fermented soybeans. *Int. J. Food Microbiol*., *60*, 163–169.

KIUCHI, K. & WATANABE, S. (2004): Industrialization of Japanese *Natto*. -in: STEINKRAUS, K.H. (Ed.): *Industrialization of indigenous fermented foods*. Marcel Dekker Inc., New York, pp. 193–246.

LANE, D.J. (1991): 16S/23S rRNA sequencing. -in: STACKEBRANDT, E. & GOODFELLOW, M. (Eds) *Nucleic acids techniques in bacterial systematics*. Wiley, New York, pp. 115–175.

LEE, M.Y., PARK, S.Y., JUNG, K.O., PARK, K.Y. & KIM S.D. (2005): Quality and functional characteristics of *Chungkukjang* prepared with various *Bacillus* sp. isolated from traditional *Chungkukjang*. *J. Food. Sci*., *70*, 191–196.

LI, H., FENG, F.Q., SHEN, L.R., XIE, Y. & LI, D. (2007): Nutritional evaluation of different bacterial douchi. *Asia Pac. J. Clin. Nutr*., *16*, 215–221**.**

MACFADDIN, J.F. (2000): *Biochemical tests for identification of medical bacteria*. Lippincott Williams & Wilkins, Philadelphia, USA, 912 pages.

NAKAMURA, L.K., ROBERTS, M.S. & COHAN, F.M. (1999): Relationship of *Bacillus subtilis* clades associated with strains 168 and W23: a proposal for *Bacillus subtilis* subsp. *subtilis* subsp. nov. and *Bacillus subtilis* subsp. *spizizenii* subsp. nov. *Int. J. Syst. Bacteriol*., *49*, 1211–1215.

NCCLS (1997): *Performance standards for antimicrobial disk susceptibility tests: Approved standard M2-A7*. National Committee for Clinical Laboratory Standards, Wayne, PA, USA.

ROONEY, A.P., PRICE, N.P.C., EHRHARDT, J.L., SWEZEY, J.L. & BANNAN, J.D. (2009): Phylogeny and molecular taxonomy of the *Bacillus subtilis* species complex and description of *Bacillus subtilis* subsp. *inaquosorum* subsp. nov. *Int. J. Syst. Evol. Micr*., *59*, 2429–2436.

SARKAR, P.K. & TAMANG, J.P. (1995): Changes in the microbial profile and proximate composition during natural and controlled fermentations of soybeans to produce *kinema*. *Food. Microbiol*., *12*, 317–325.

SCHALLMEY, M., SINGH, A. & WARD, O.P. (2004): Developments in the use of *Bacillus* species. *Can. J. Microbiol*., *50*, 1–17.

STEINKRAUS, K.H. (1996): *Handbook of indigenous fermented foods*. 2nd ed., Marcel Dekker Inc, New York, 776 pages.

VISESSANGUAN, W., BENJAKUL, S., POTACHAREON, W., PANYA, A. & RIEBROY, S. (2005): Accelerate proteolysis of soy proteins during fermentation of *Thua-Nao* inoculated with *Bacillus subtilis.* J. *Food Biochem., 29,* 349–366.