

Improvement of *thua nao* production using protein-rich soybean and *Bacillus subtilis* TN51 starter culture

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Abstract *Thua nao*, a Thai traditional fermented soybean, is locally produced, of irregular quality and under risk of contamination with spoilage and pathogenic microorganisms during the artisanal process. This study aims to select a protein-rich soybean variety as a material for *thua nao* fermentation and improve organoleptical quality of the product by fermenting sterilized soybean with pure starter of *Bacillus subtilis* TN51 previously isolated from a traditional *thua nao*. Changes in microbiological and physicochemical qualities of soybean were monitored through fermentation period for 72 h. The completely fermented products were also subjected to sensory evaluation and antimicrobial activity against 13 microbial pathogens and two fermentative yeasts. Of five soybean cultivars tested (i.e., CM60, Pabong7, Kunpae, TG55 and TG145), based on seed size, production yield, protein and fat contents, TG145 was the most suitable for production of *thua nao* due to its high protein content (45.8%), lowest fat (15.6%), small seed size (11.1 g/100 seeds) and potent production yield (358 kg/rai). *Thua nao* produced by fermented autoclaved soybean with pure starter culture of *B. subtilis* TN51 (TNB51) could be improved considerably for its microbiological and chemical

qualities, especially using a smaller amount of ammonia that led to a more organoleptically acceptable product than *thua nao* produced by naturally occurring microbial fermentation (TNMX). Of tested microorganisms, only Gram-positive bacteria (i.e., *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Micrococcus luteus*, *Bacillus cereus*, and *Listeria monocytogenes*) were inhibited by *thua nao* methanolic extracts.

Keywords Fermented soybean · Soybean · *thua nao* · *Bacillus subtilis* · Starter culture

Introduction

Thua nao, a traditional fermented soybean of northern Thailand, is typically present in two forms: fresh *thua nao* (*thua nao mer*) and dried (*thua nao kab*). Traditional fermentation of *thua nao* is as follows: soybeans are cooked by boiling and fermented spontaneously at ambient temperature for 3–4 days. The completion of fermentation is indicated by the formation of a dominant ammoniacal smell, a typical umami taste and a white viscous fluid on the beans. Like other traditional soy-fermented products, *thua nao* is currently produced basically in a traditional manner. This household practice often yields inconsistent quality products, a strong ammoniacal smell, a risk of foodborne disease and a short shelf-life. A number of spoilage and foodborne pathogenic organisms have been identified in the traditional *kinema* (Nout et al. 1998), *soy-dawadawa* (Jideani and Okeke 1991; Dike and Odunfa 2003) and *thua nao* (Leejeerajumnean 2003; Chukeatirote et al. 2006). In order to eliminate these problems, there is a need for scientific investigation to improve production methods. In recent years, many attempts are made by

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several researchers aiming to develop a starter culture(s). A series of successful scenarios have been described in various products such as *natto* (Wei et al. 2001; Wei and Chang 2004), *kinema* (Sarkar and Tamang 1995; Tamang and Nikkuni 1996) and *soy-daddawa* (Omafuvbe et al. 2002; Omafuvbe 2008). As reported in these studies, the use of a potentially pure starter culture and a controlled fermentation process appears to be the gold standard toward improvement of fermented soybean products. In addition to the starter organism, there are many factors affecting the quality of fermented soybean such as soybean cultivar and processing conditions (i.e., soaking, cooking, and fermentation) (Wei et al. 2001; Wei and Chang 2004). Small types of soybeans with high protein and low fat contents are suggested for producing the quality *natto* products (Taira and Suzuki 1983; Steinkraus 1983; Ohta 1986; Griffiths and Wiedermann 1991; Hosoi and Kiuchi 2003). Soybean with high carbohydrate content correlated with a lesser ammonia content in *natto* products (Ohta 1986). The positive correlation of ammonia production with the hardness of steamed soybeans has also been reported by Taira et al. (1987).

The attempts of using pure starter culture to improve the quality of *thua nao* production have also been investigated in Thailand. Changes in proteolytic activity and some biochemical qualities during fermentation of *thua nao* by using pure starter culture of *Bacillus subtilis* strain 38 (Chantawannakul et al. 2002) and BIOTEC7123 (Visessanguan et al. 2005) have been studied. In addition, according to Tangjitjaroenkun et al. (2004), the mixed cultures of *Bacillus amyloliquefaciens* and *Klebsiella* sp. KB2 isolated from *thua nao* could enhance vitamin B12 contents in the fermented soybean. However, the nutritive value, organoleptical quality and antimicrobial quality of the products remain unclear and further study is hence required. In our previous study, proteolytic activities of bacteria isolated from *thua nao* were screened; *Bacillus subtilis* TN51 is the highly active proteolytic activity strain (Dajanta et al. 2009). Therefore, the objective of this study was to propose *B. subtilis* TN51 as starter culture for the fermentation of suitable soybean cultivar into *thua nao* with a high nutritive value. Changes in biochemical quality (i.e., pH, ammonia concentration, and proximate compositions) of soybean to form *thua nao* were monitored during controlled fermentation. Overall acceptability and antimicrobial activity of these products were also involved in this investigation.

Materials and methods

Soybean cultivars

Five soybean cultivars namely CM60, Pabong7, Kumpai, TG55 and TG145 were obtained from the Field Crop Research

Center, Institute of Agriculture, Chiang Mai, Thailand. These soybeans were initially characterised based on their production yield and size. The production yield was calculated as weight per area (kg/rai, 1 rai=1,600 m²) in which the soybean weight used was 12% dry matter of moisture, and these data were obtained from 4 experimental replications in the dry season (December 2007 to March 2008) at the Field Crops Research Center. For soybean size, 100 seeds were randomly selected and used for the analysis. In addition, protein and fat contents were analysed based on the AOAC's protocol (AOAC 2000).

Bacterial strain

Bacillus subtilis TN51 previously isolated from commercial *thua nao* was used as pure starter culture due to its high proteolytic activity (Dajanta et al. 2009). The bacteria were routinely cultured on nutrient agar and, for stock culture, a 20% glycerol bacterial culture was prepared and kept at -20°C. For inoculum preparation, the bacteria were grown in nutrient broth at 37°C for 24 h. The cells were then harvested, resuspended in sterile distilled water and adjusted to 10⁶–10⁷ CFU/mL. This cell suspension was ready to serve as inoculum for soybean fermentation.

Preparation of *thua nao*

Protein-rich soybeans variety TG145 obtained from the Field Crop Research Center, Institute of Agriculture, Chiang Mai, Thailand, were washed and soaked in tap water for 16 h at ambient temperature (~25°C). Before fermentation, different methods were used to prepare cooked non-fermented soybeans (CNF). Soaked soybeans were cooked by boiling for 4 h (CNF1) and left to ferment by naturally occurring microorganisms (TNMX). In contrast, for *Bacillus*-inoculum fermentation, soaked soybeans were sterilised at 121°C for 40 min (CNF2), and inoculated with 10⁴ CFU (per g of sterilised soybeans) of *B. subtilis* TN51 starter culture (TNB51). The fermentation process was then allowed to proceed at 42°C for 72 h. Changes in microbiological and physicochemical qualities of soybean during the fermentation were then determined every 24 h for 3 days.

Microbiological analysis (total viable bacterial count)

Changes in total viable count, spore count and total yeasts and moulds of soybeans were determined during fermentation. Fermented soybeans (5 g) were homogenised with 45 mL of sterile peptone water (0.1% w/v) (Merck, Germany) by stomaching for 2 min. Serial dilutions were prepared in peptone water (0.1% w/v) and 1 mL of appropriate dilutions were poured in duplicate plates of plate count agar (Merck) for viable counts of aerobic mesophilic bacteria, and yeast malt extract agar (HiMedia

M424, India), pH 3.5 for yeasts and moulds. Spore counts were also determined with plate count agar with suspensions heated at 85°C for 20 min. Cultures were incubated in 37°C for 2 days (plate count agar for bacteria), and 25°C for 3–5 days (yeast malt extract agar for yeasts and fungi). The colonies were then counted and expressed as logarithmic colony forming units per gram (log CFU/g) of sample.

Physicochemical analysis

Changes in proximate composition of soybeans during fermentations were determined using the standard AOAC methods (AOAC 2000), No. 955.04 for total nitrogen content, which was then used to calculate the protein content by multiplication by 6.25 (the nitrogen conversion factor of soybean), No. 905.02 for fat content, No. 945.46 for ash measurement, and No. 990.19 for moisture content. Ammonia concentration was also determined by titrating 1 g sample with 0.1 M HCl after steamed distillation with a Kjeltac Distillation Unit. Reducing and total sugars were determined using the dinitrosalicylic reagent method (Miller 1959). For pH, approximately 5 g of fermenting soybeans (wet weight) were homogenised in a blender with 50 mL of distilled water for 15 s and the pH value of the suspension was measured with a pH meter (Consort C830, CE, Belgium). The colour of soybean surface was determined in the L* a* b* system by colorimeter Minolta Data Processor DP-301 (Chroma Meter CR-300 Series, Japan).

Sensory evaluation

Steamed (30 min) *thua nao* products (TNMX and TNB51) and commercial product purchased from Mae Hia market (MH), Chiang Mai, Thailand, were evaluated for consumer's acceptability by 50 untrained local panelists who are familiar with this product using a 7-point hedonic scale test with 7 being extremely good and 1 being extremely poor. Each panelist, after consuming the products, filled in the questionnaire to show their preference of each sensory attribute (colour, flavour, odour, and texture).

Antimicrobial activity assay

The ground powder of the lyophilised sample (30 g) was extracted with 300 mL of methanol (80% v/v) (Merck) for 24 h at room temperature with continuous shaking (Lee et al. 2007). The extracts were filtered through Whatman No.1 paper, concentrated under vacuum (Büchi Rotavapor R-200) at 40°C and freeze-dried (LABCONCO, FREEZONE 4.5).

The microorganisms for testing obtained from the Thailand Institute of Scientific and Technological Research were *Staphylococcus aureus* TISTR118, *S. epidermidis* TISTR518, *Micrococcus luteus* TISTR884, *Bacillus cereus* TISTR687,

Escherichia coli TISTR780, *Pseudomonas aeruginosa* TISTR781, *Salmonella typhimurium* TISTR292, *Enterobacter aerogenes* TISTR1468, *Candida albicans* TISTR5779 (ATCC10231), *Candida famata* TISTR5098, *Candida glabrata* TISTR5006, *Saccharomyces cerevisiae* TISTR5049 (ATCC4105), and *Saccharomyces ellipsoideus* TISTR5194. *Listeria monocytogenes* DMST17303 and *Salmonella enteritidis* DMST15676 were obtained from the Department of Medical Science, Thailand.

The freeze-dried *thua nao* extracts were dissolved in the methanol (80% v/v) to a final concentration of 500 mg/mL, centrifuged at 2,000g for 30 min and then a filtered supernatant of the sample was passed through 0.2-µm Millipore filters (Minisart, Sartorius, Germany). Antimicrobial tests were then carried out by the paper disc diffusion method (Kim et al. 2004). The tested microorganisms were adjusted their turbidity to 0.5 McFarland standards from overnight agar cultures and swabbed over the dried surface of trypticase soy agar (Merck) plate for bacteria and yeast malt agar (Merck) for yeasts. The antimicrobial activity was determined by the paper disc. Discs (6 mm diameter; Macherey-Nagel, Duren, Germany) were placed onto an agar plate inoculated with a test organism and loaded immediately of *thua nao* extracts fluid (20 µL). The diameter (mm) of inhibition was measured after incubation for 16–18 h at 35°C in an incubator. Ofloxacin (5 µg/6 mm disc) and Netilmicin (30 µg/6 mm disc), purchased from Oxoid, UK, were used as standard antibiotics to compare the sensitivity of *thua nao* extracts against test microorganisms, and the negative control was methanol (80% v/v). All determinations were made in separated triplicate. The antimicrobial potential of *thua nao* extracts were expressed in relative magnitude of inhibition (RMI) calculated as the ratio of area defined between zone of inhibition including disc of sample and the zone of negative control.

Statistical analysis

All data except those of sensory evaluation were expressed as means±standard deviation of triplicate observations. The results from sensory evaluation were calculated from the mean values of the 50 panelists. The data were also subjected to analysis of variance (ANOVA), *t* test, and Duncan's multiple range tests. The significant differences between means were defined at $P<0.05$.

Results and discussion

Quality of soybeans

Five soybean cultivars namely CM60, Pabong7, Kunpae, TG55 and TG145 were selected for this study. In general,

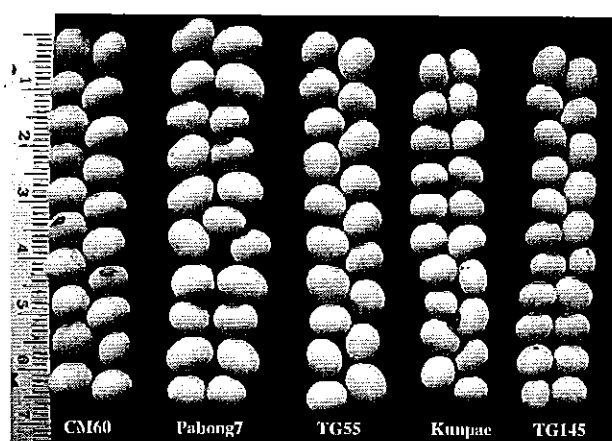


Fig. 1 Appearances of soybean cultivars used in this study

their seeds are oval and round with yellow seed colour and dark brown hila (Fig. 1). In this study, some nutritive qualities such as protein and fat contents, and the production yield and seed size were used as criteria to select the potential soybean cultivar as raw material for producing *thua nao* with a high nutritive value. As shown in Table 1, the protein contents of the five soybean cultivars were between 39.38 and 45.85% which were similar to those of soybean cultivars used to produce *natto* (39.30–45.31%) as reported by Taira and Suzuki (1983). It has also been reported that peptides and amino acids derived from the activities of fermenting microorganisms affect the flavour appeals of *natto*, *douchiba*, and soy source (Hosoi and Kiuchi 2003; Qin and Ding 2007; Yanfang and Wenyi 2009). Therefore, the use of protein-rich soybeans is usually preferred as raw material to produce such products with high quality.

This study also revealed that the fat contents of five soybean cultivars ranged from 15.59 to 19.91% which were consistent with those of suitable soybean cultivars for *natto* processing (18.71–20.51%) as described by Taira and Suzuki (1983). Besides, small soybean seed types having the weights of 100 seeds between 8.1 and 18.2 g (with

moisture content of 11.08–13.02%) were suggested to use in quality *natto* production (Taira et al. 1987). In this present study, the seed size of five tested soybean cultivars, based on the weight of 100 seeds (moisture contents around 12%), displayed in the range 11.1–15.9 g with the smallest one being TG145 and the biggest one being CM60 (Table 1; Fig. 1). It has been reported that the suitable characteristics of soybean seed for making quality *natto* should be a small and round type with light yellow hilum, and a brilliant light yellow or yellow, smooth seed coat (Steinkraus 1983; Ohta 1986; Hosoi and Kiuchi 2003). Small soybeans are desirable because they have high water-absorbing capacity, are easier to cook, and provide a better aerobic fermenting environment (Teng et al. 2004). In addition, soybeans with a protein level of 43% or greater and low fat content could also help improve the nutritional quality of *natto* (Griffis and Wiedermann 1991).

Of the five soybean cultivars tested, the TG145 cultivar displayed the highest protein content and lowest fat content. For production yield, there was no significant difference among these cultivars ($P>0.05$). As a result, the soybean TG145 cultivar was selected for further study as a desirable raw material for producing high nutritional value *thua nao* based on its distinct characteristics (high protein, low fat content, high production yield and smaller seed size).

Appearance of *thua nao* products

The overall appearance of the non-fermented and fermented soybean products is shown in Fig. 2. In general, the colour of autoclaved soybean (CNF2, Fig. 2b) appeared to be slightly darker than that of the boiled soybean (CNF1, Fig. 2a) due to high temperature treatment (further discussion is provided in "Physicochemical changes of fermented soybeans"). After 72 h fermentation, it was found that *thua nao* prepared by *B. subtilis* TN51 was sticky, as it was covered with a white viscous substance (TNB51, Fig. 2d). The distinct odour of *natto*-like was also

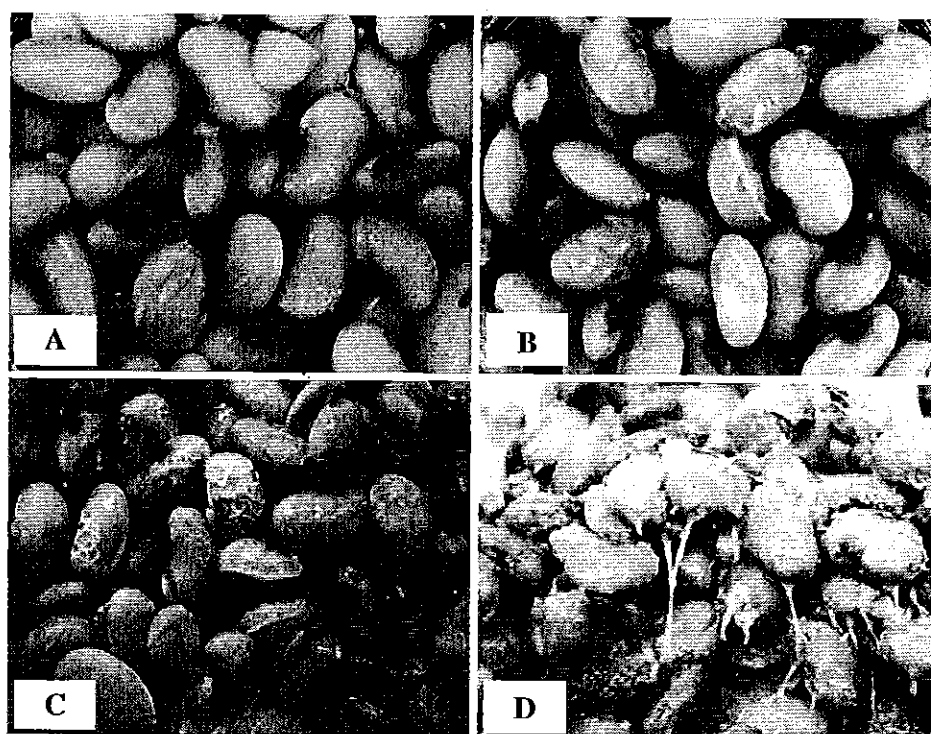
Table 1 Some qualitative data of soybean cultivars

Soybean variety	Protein ^a (% dw)	Fat ^a (% dw)	Production yield ^b (kg/rai)	Weight of 100 seeds ^b (g)
TG145	45.85 ± 0.75 a	15.59 ± 0.02 d	358	11.1
TG55	40.70 ± 0.24 c	18.06 ± 0.43 b	347	14.5
Kumpae	39.38 ± 0.63 c	17.07 ± 0.37 c	366	12.9
Pabong7	43.01 ± 1.37 b	17.00 ± 0.05 c	360	13.6
CM60	39.48 ± 0.16 c	19.91 ± 0.43 a	371	15.9

^a Data are mean ± standard deviation ($n=3$). Means within a column with a different letter are significantly different ($P<0.05$)

^b Data calculated from 4 replications in the dry season (December 2007 to March 2008) were obtained from the Field Crop Research Center, Institute of Agriculture, Chiang Mai, Thailand; 1 rai=1,600 m²

Fig. 2 Appearance of cooked non-fermented and fermented soybeans. **a** Boiled non-fermented soybeans (CNF1); **b** autoclaved non-fermented soybeans (CNF2); **c** naturally fermented *thua nao* (TNMX); **d** *B. subtilis* TN51 fermented *thua nao* (TNB51)



observed in the TNB51 product. In contrast, traditionally fermented soybean did not have such characteristics (TNMX, Fig. 2c). Similar results have been reported in Japanese *natto* and Korean *chungkukjang* when the use of pure starter of *B. subtilis* was introduced (Wei et al. 2001; Lee et al. 2005). The sticky substances comprising of polyglutamic acid (PGA) and levan (β -2,6-fructan) are produced by certain *Bacillus* strains during soybean fermentation (Kunioka and Goto 1994; Ogawa et al. 1997). The presence of this mucilage is one of the important criteria in considering whether the *natto* product is of high quality (Wei et al. 2001). In addition, the benefits of this material have been reported as dietary fiber to reduce the cholesterol level in serum (Tsuiji and Tsuiji 1986) and as

antibiotic agents against food spoilage and pathogenic bacteria such as *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli* O157, and *Salmonella* (Youn et al. 2001). In contrast, the absence of such a sticky substance was observed in the TNMX product. A possible explanation is that, due to traditional fermentation by naturally occurring microorganisms, some bacteria might have secreted enzymes (i.e., γ -glutamyl hydrolases) to degrade this mucilage (Chunhachart et al. 2006).

Bacterial and pH evolution during *thua nao* fermentation

The changes in microbial number and pH were monitored during the fermentation. As shown in Table 2, both batches

Table 2 Relationship between bacterial population and pH during soybean fermentation

Fermentation time (h)	Total viable count		Spore count		pH value	
	TNB51	TNMX	TNB51	TNMX	TNB51	TNMX
0	4.09 $\pm$ 0.09 cA	2.80 $\pm$ 0.14 cB	4.08 $\pm$ 0.07 cA	1.23 $\pm$ 0.21 cB	6.39 $\pm$ 0.01 cB	6.58 $\pm$ 0.01 dA
24	8.89 $\pm$ 0.04 aA	8.89 $\pm$ 0.11 bA	8.36 $\pm$ 0.13 bA	8.17 $\pm$ 0.03 bA	7.66 $\pm$ 0.03 bA	7.30 $\pm$ 0.10 cB
48	8.91 $\pm$ 0.03 aB	9.57 $\pm$ 0.13 aA	8.66 $\pm$ 0.14 aB	9.58 $\pm$ 0.02 aA	8.01 $\pm$ 0.08 aA	7.97 $\pm$ 0.06 bA
72	8.62 $\pm$ 0.17 bB	9.67 $\pm$ 0.02 aA	8.59 $\pm$ 0.03 aB	9.62 $\pm$ 0.07 aA	8.00 $\pm$ 0.02 aB	8.33 $\pm$ 0.11 aA

Data are mean $\pm$ standard deviation (n=3) and expressed as log CFU/g wet weight. Means within a column with different lowercase letters are significantly different within interval samples ($P < 0.05$) and means within a row with different uppercase letters are significantly different between types of *thua nao*, analysed by the *t* test method ($P < 0.05$).

TNB51 *thua nao* prepared by fermentation of autoclaved soybeans with pure starter culture of *B. subtilis* TN51; TNMX *thua nao* prepared by fermentation of naturally occurring microorganisms of boiled soybeans.

Table 3 Changes in physicochemical qualities of soybean during fermentation

	Fermentation time (h)	TNB51	TNMX
Moisture content (% dw)	0	57.60 ± 0.60 aB	66.51 ± 0.22 aA
	24	57.18 ± 1.18 aB	67.10 ± 0.64 aA
	48	56.53 ± 0.04 aB	66.77 ± 1.68 aA
	72	56.74 ± 0.84 aB	68.19 ± 0.49 aA
Ammonia (mg/mL)	0	0.03 ± 0.00 cA	0.04 ± 0.01 cA
	24	0.04 ± 0.00 cB	0.21 ± 0.02 bA
	48	0.18 ± 0.02 bB	0.42 ± 0.03 aA
	72	0.29 ± 0.01 aB	0.42 ± 0.01 aA
Ash (% dw)	0	6.29 ± 0.49 aA	4.93 ± 0.08 cA
	24	6.45 ± 0.47 aB	8.40 ± 0.23 aA
	48	6.17 ± 0.25 aB	8.27 ± 0.10 aA
	72	6.43 ± 0.19 aB	7.58 ± 0.07 bA
Fat (%dw)	0	18.47 ± 0.55 aA	18.54 ± 0.26 bA
	24	16.93 ± 0.56 aA	22.08 ± 0.70 aA
	48	16.53 ± 0.69 aA	21.72 ± 0.73 aA
	72	16.40 ± 0.13 aA	19.06 ± 0.72 bA
Protein (% dw)	0	45.74 ± 0.31 aA	45.27 ± 0.77 aA
	24	46.19 ± 1.06 aA	45.74 ± 0.35 aA
	48	46.00 ± 0.17 aA	46.73 ± 1.00 aA
	72	45.35 ± 0.28 aA	44.91 ± 0.12 aA
Total sugar (% dw)	0	11.61 ± 0.15 aA	4.32 ± 0.08 aB
	24	5.90 ± 0.12 bA	2.73 ± 0.15 bB
	48	5.53 ± 0.16 cA	2.78 ± 0.14 bB
	72	6.02 ± 0.07 bA	2.64 ± 0.06 bB
Reducing sugar (% dw)	0	3.27 ± 0.37 bA	1.54 ± 0.01 cB
	24	3.97 ± 0.12 aA	2.53 ± 0.06 abB
	48	4.04 ± 0.02 aA	2.78 ± 0.14 aB
	72	3.59 ± 0.14 abA	2.47 ± 0.14 bB
Colour L*	0	45.96 ± 1.89 aB	59.37 ± 0.89 aA
	24	41.49 ± 1.81 bB	55.79 ± 1.77 abA
	48	38.70 ± 1.46 bcB	55.21 ± 2.43 abA
	72	36.42 ± 2.98 cB	53.74 ± 3.25 bA
Colour a*	0	8.76 ± 0.45 bA	5.08 ± 0.30 bB
	24	7.56 ± 0.24 cA	5.42 ± 0.08 bB
	48	8.90 ± 0.58 bA	5.54 ± 0.61 bB
	72	10.11 ± 0.85 aA	6.62 ± 0.63 aB
Colour b*	0	20.10 ± 0.78 aA	22.75 ± 1.78 aA
	24	14.55 ± 1.69 bB	20.91 ± 1.14 abA
	48	12.55 ± 0.10 bB	19.12 ± 0.72 bA
	72	14.57 ± 3.99 bA	20.06 ± 2.12 abA

Data are mean ± standard deviation ($n=3$). Means within a column with different lowercase letters are significantly different within interval samples ($P<0.05$) and means within a row with different uppercase letters are significantly different between types of *thua nao*, analysed by the *t* test method ($P<0.05$).

TNB51 *thua nao* prepared by fermentation of autoclaved soybeans with pure starter culture of *B. subtilis* TN51; *TNMX* *thua nao* prepared by fermentation of naturally occurring microbes of boiled soybeans

only differed at the beginning of the fermentation in terms of bacterial population. The bacterial cells of the TNB51 batch were much higher than those of the TNMX batch. This is expected due to a bacterium inoculums addition of *B. subtilis* TN51. The bacterial population of both batches appeared to increase remarkably within 24 h and then entered a stationary phase. The bacterial numbers of both TNMX and TNB51 were nearly consistent during 24 and 72 h fermentation. Spore-forming bacteria were predominant in *thua nao* production (Table 2). This is evident considering that more than 90% of SPC were identified throughout the fermentation process in both batches. Our result is in agreement with the findings of other researchers reporting that spore-forming bacteria play a major role in *thua nao* production (Sundhagul et al. 1972; Visessanguan et al. 2005; Chukeatirote et al. 2006). It should also be noted, however, that the bacterial cell number of the TNMX batch was slightly higher than that of the TNB51 from 48 h fermentation. Other *Bacillus* species, such as *B. licheniformis*, *B. pumilus*, *B. megaterium* and *B. cereus* and including other organisms (i.e., *Lactobacillus* spp. and other Gram-positive cocci), have been reported in spontaneously fermenting *thua nao* (Leejeerajumnean 2003; Chukeatirote et al. 2006). The presence of small amounts of yeast and mould (< 2 log CFU/g) was also detected throughout the fermentation processes. In contrast, a large number of fungi (~ 13 log CFU/g) were found by Chukeatirote et al. (2006) at 72 h of traditional *thua nao* fermentation, implying a safety risk of the product.

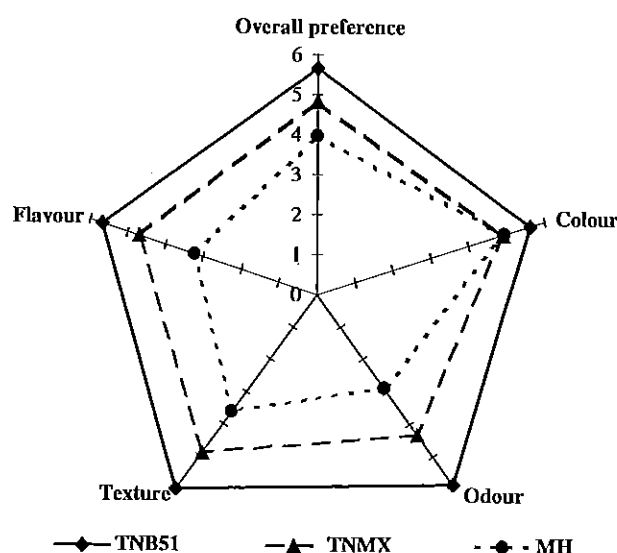


Fig. 3 Sensory evaluation of *thua nao* products produced by pure starter *B. subtilis* TN51 (*TNB51*), natural fermentation (*TNMX*), and commercial product (*MH*)

The pH values increased with fermentation time in both TNB51 and TNMX batches. The final pH values were 8 and 8.33 for TNB51 and TNMX, respectively. This indicates the alkaliphilic state of *thua nao* products after fermentation. According to Table 2, it is likely that the increase in bacterial cell number caused a pH change. One of the possible explanations is that, during the soybean fermentation, the microorganisms including *Bacillus* species utilised soy proteins leading to release of amines and ammonia causing a rise of pH value (Terlabie et al. 2006; Omafuvbe 2008). The strong proteolytic activity of *B. subtilis* TN51 has been investigated previously by Dajanta et al. (2009), and their report also showed that 99% of bacterial strains isolated from traditional *thua nao* were capable of producing protease enzymes and around 76% of these were identified as endospore-forming bacilli.

Physicochemical changes of fermented soybeans

Table 3 presents the proximate composition as well as other physicochemical characteristics of the TNB51 and TNMX

thua nao. The moisture contents in fermented *thua nao* showed little change throughout fermentation (57.6 to 56.74% for TNB51 and 66.51 to 68.19% for TNMX). However, the TNMX product showed a higher moisture content throughout fermentation process ($P<0.05$) than that of the TNB51. The ammonia content of both products increased as the fermentation proceeded. However, it should be noted that the final ammonia content of the TNMX product was two times higher than that of the TNB51 product. This finding is in agreement with the study of Wei et al. (2001) describing that the introduction of bacterial pure culture affects ammonia content of the *natto* products.

Protein, ash and fat contents of *thua nao* were nearly constant throughout fermentation, except the ash and fat contents of the TNMX product which increased significantly in the first 24 h of fermentation and then dropped at the end of fermentation ($P<0.05$). These different patterns may be due to a difference of capabilities to utilise lipid or carbohydrate of starter microbial organisms during *thua nao* fermentation. In the literature, the variable trends of fat

Fig. 4 Inhibitory activity of *thua nao* crude extracts against foodborne pathogenic bacteria. **a** Naturally fermented *thua nao* (TNMX); **b** *B. subtilis* TN51 fermented *thua nao* (TNB51). **BC** *Bacillus cereus* TISTR687; **ML** *Micrococcus luteus* TISTR884; **SA** *Staphylococcus aureus* TISTR118; **SE** *Staphylococcus epidermidis* TISTR518; **LM** *Listeria monocytogenes* DMST17303; **MeOH** methanol

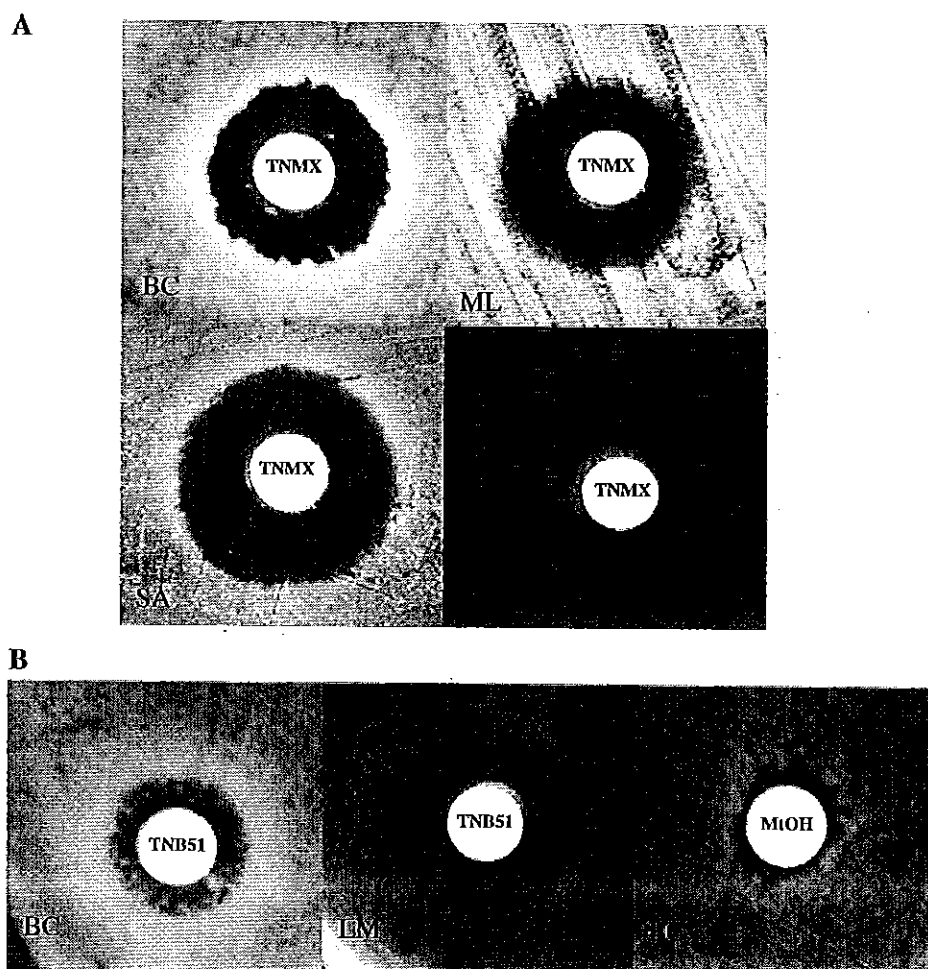


Table 4 Antimicrobial activities of cooked non-fermented soybeans and *thua nao*

Microorganisms	Relative magnitude of inhibition			
	CNF1	CNF2	TNB51	TNMX
<i>Staphylococcus aureus</i> TISTR118	-	-	-	2.4 ± 0.1
<i>Staphylococcus epidermidis</i> TISTR518	-	-	-	1.9 ± 0.2
<i>Micrococcus luteus</i> TISTR884	-	-	-	2.6 ± 0.6
<i>Bacillus cereus</i> TISTR687	-	-	1.6 ± 0.1	1.9 ± 0.3
<i>Escherichia coli</i> TISTR780	-	-	-	-
<i>Pseudomonas aeruginosa</i> TISTR781	-	-	-	-
<i>Salmonella typhimurium</i> TISTR292	-	-	-	-
<i>Salmonella enteritidis</i> DMST15676	-	-	-	-
<i>Enterobacter aerogenes</i> TISTR1468	-	-	-	-
<i>Listeria monocytogenes</i> DMST17303	-	-	1.9 ± 0.1	-
<i>Candida albicans</i> TISTR5779	-	-	-	-
<i>Candida famata</i> TISTR5098	-	-	-	-
<i>Candida glabrata</i> TISTR5006	-	-	-	-
<i>Saccharomyces cerevisiae</i> TISTR5049	-	-	-	-
<i>Saccharomyces ellipsoideus</i> TISTR5194	-	-	-	-

Data are mean ± standard deviation ($n=3$). Relative magnitude of inhibition=area of inhibition zone of sample/area of inhibition zone of 80% methanol

- no inhibition zone

CNF1, boiled non-fermented soybeans; CNF2, autoclaved non-fermented soybeans; TNB51, *thua nao* prepared by fermentation of autoclaved soybeans with *B. subtilis* TN51; TNMX, *thua nao* prepared by fermentation of boiled soybeans with naturally occurring microbes

contents have also been recorded in the production of *natto* (Taira and Suzuki 1983; Wei and Chang 2004)

The contents of total and reducing sugars in both *thua nao* products presented a similar pattern of change in both production processes. The total sugar content tended to decrease significantly whereas the reducing sugar content increased at the beginning of the fermentation (0–24 h) and dropped slightly at the end of the process. It is worth noting that the period of changes in total sugar and reducing sugar levels coincides with the period of changes in the TVC and SPC (Table 2). These observations may indicate that the total sugars of soybeans were used by fermenting microorganisms causing an increase of reducing sugars. The high amounts of these reducing sugars were subsequently utilised by a large number of microbial population causing their decrease in late fermentation. Similar trends of these values have been reported in soy-daddawa production (Omafuvbe 2008). Compared with the TNB51 *thua nao* product, significantly lower contents of total and reducing sugars were found throughout the fermentation of the TNMX product. This is possibly due to the effect of the

cooking process. The TNMX product was prepared by boiling and thus some of these components may be lost while boiling soybeans in water at the initial stage. The faster fermentation rate based on an increase of pH values was also found in the TNB51 product. This may be related to a higher content of reducing sugar. Taira (1990) has reported that there is a strong relationship between the composition of free sugars, the taste of *natto*, and the speed of fermentation.

Colour values (L^* , a^* , and b^*) of the fermented soybeans were also evaluated during the fermentation (Table 3). There was a marked increase in the lightness as indicated by the L^* value of both *thua nao* products from the values recorded at the beginning of the fermentation. In addition, the L^* value of the TNB51 product is usually lower than that of the TNMX product throughout the fermentation period. The effect of cooking (boiling and autoclaving) is possibly a major cause of difference in lightness as observed by the L^* values at 0 h. In contrast, the a^* value (green to red) showed a significant increase whereas the b^* value (blue to yellow) tended to decrease

Table 5 Antimicrobial activity of metabolite derived from *Bacillus* spp

Fermented soybean	<i>Bacillus</i> spp.	Metabolites	Inhibition activity	References
Chungkukjang	<i>B. licheniformis</i>	Phenylacetic acid	<i>Staphylococcus aureus</i> , <i>Escherichia coli</i> , <i>Candida albicans</i>	Kim et al. (2004)
Chinese seasoning	<i>B. subtilis</i>	Bacillocin22	<i>Bacillus cereus</i> , <i>Listeria monocytogenes</i>	Zheng and Slavik (1999)
Soumbala	<i>B. subtilis</i> , <i>B. pumilus</i>	Lipoprotein, bacteriocins	<i>Bacillus cereus</i> , <i>Staphylococcus aureus</i> , <i>Escherichia coli</i>	Ouoba et al. (2007)

after 72 h fermentation. These colour changes are responsible for the appearance of a visibly darker brown colour of the fermented soybean products (Fig. 2c, d). In addition, when compared to the TNMX product, the lower values of lightness and yellowness were observed in the TNB51 product throughout the fermentation period. This is mainly due to the high temperature treatment by the autoclaving practice used in cooking soybeans before fermentation. According to the effect of the heat treatment, melanoidins (a dark brown nitrogen-containing pigment) are formed from the Maillard reaction and Strecker degradation. It has been shown that several factors can influence the higher rate of Maillard reaction including high temperature, low moisture levels, alkaline pH condition and large levels of reducing sugars (Hurrell 1982). For these reasons, autoclaved non-fermented soybean and its fermented product, TNB51, showed a significantly darker brown colour throughout the process of fermentation ($P < 0.05$).

Sensory evaluation

Figure 3 illustrates the sensory evaluation of the TNMX, TNB51 and commercial MH products. The overall acceptability, colour, texture, flavour and aroma attributes of *thua nao* products were assessed by 50 untrained panelists who were familiar with this product using a 7-point hedonic scale test. A high acceptability by consumers was observed as shown by highest scores ($P < 0.05$) in all attributes of the TNB51 product. Of these sensory attributes tested, the soft texture and mild aroma of TNB51 was most acceptable, whilst a strong ammoniacal smell of the TNMX and MH products was regarded as the key factor affecting consumer's acceptability. Previous investigations including this study have also demonstrated that the organoleptical qualities of *kinema*, *soy-daddawa* and *thua nao* can be improved by using sterilised soybean inoculated with the controlled starter of *B. subtilis* (Sarkar and Tamang 1995; Omafuvbe et al. 2002).

Antimicrobial activity

The antimicrobial activity against foodborne bacteria and yeasts of all soybean extracts are shown in Fig. 4 and Table 4. Of the tested microorganisms, only Gram-positive bacteria (i.e., *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Micrococcus luteus*, *Bacillus cereus*, and *Listeria monocytogenes*) were inhibited by both *thua nao* extracts. However, it should be noted that the TNMX extracts exhibited a broader spectrum against bacterial pathogens than those of the TNB51 extracts. The antimicrobial activity of crude extracts of Chinese fermented seasoning (Zheng and Slavik 1999), Korean *chungkukjang* (Kim et al. 2004) and *doenjang* (Yun

2005) have been verified. These studies suggest the potential role of bacteriocins produced by *Bacillus* spp. during the soybean fermentation. Several *Bacillus* spp. isolated from fermented soybeans are also found to exhibit antimicrobial activity and their metabolites have been characterised (Table 5). Hence, variation of spectrum of *thua nao* extracts may be caused by the effect of antibacterial substances from other microorganisms present in the products. In addition, the synergistic antimicrobial activity of melanoidins browning pigments is also expected in these *thua nao* extracts, since many studies have described the potential antibiotic activity of such components (Stecchini et al. 1993; Dastidar et al. 2004; Rufian-Henares and Morales 2006, 2007).

In conclusion, this present study clearly shows that the use of pure starter culture (i.e., *B. subtilis* TN51) can help improve the fermentation of *thua nao*. Furthermore, *thua nao* product prepared by *B. subtilis* TN51 is of great quality in terms of microbiological and chemical characteristics. It is worth mentioning that the introduction of *B. subtilis* TN51 as inoculum also causes mucilage and less ammonia content, generally regarded as important criteria for the quality of fermented soybean products. The process using pure culture is also considered as a controlled fermentation to produce a safer and more acceptable *thua nao* product. In addition, *thua nao* extracts also possess the antimicrobial activity against Gram-positive pathogenic bacteria. The bioactive components need to be identified in further work. Soy-fermented food has been claimed to be a functional food due to its pharmacological and antioxidant properties (Gotoh et al. 1998; Potter et al. 1998; Park et al. 2003; Jung et al. 2006). Therefore, further studies are required to evaluate the antioxidant activity of *thua nao* prepared by pure starter *B. subtilis* TN51.

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