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Antioxidant capacities, total phenolics and flavonoids in black and yellow soybeans fermented by *Bacillus subtilis*: A comparative study of Thai fermented soybeans (*thua nao*)

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Abstract

B. subtilis TN51 was inoculated in cooked black and yellow soybeans, after incubating at 42°C and 4°C for 48 h, the fermented *thua nao* were produced. The antioxidant capacities, total pehenolics and flavonoids of the fermented black (TN-BS) and yellow (TN-YS) soybeans were then examined as compared to their cooked non-fermented (CNF) soybeans. It was found that fermentation enhanced the increase of total phenolic and flavonoid contents (except yellow soybean fermentation) as well as antioxidant activities of anti-DPPH radicals and ferric reducing antioxidant power (FRAP) of the soybean extracts. The extracts of CNF black soybean and TN-BS, showed higher total phenolic content, total flavonoids, anti-DPPH radicals and lipid peroxidation inhibition than those of yellow soybean extracts. While, FRAP value was found higher in the extract of TN-YS than that in TN-BS extract. Correlation studies indicated significant ($P \leq 0.01$) positive correlation between the total phenolic contents and the values of FRAP in soybean extracts ($r = 0.889$). Whereas, the contents of total phenolics ($r = -0.731$) and flavonoids ($r = -0.709$) were negatively correlated ($P \leq 0.01$) with IC_{50} of DPPH radical-scavenging activity. Also, a significant negative correlation ($P \leq 0.01$, $r = -7.39$) was demonstrated between the total flavonoid contents and IC_{50} of lipid peroxidation inhibition. These results show that black soybean *thua nao* produced by powder culture *B. subtilis* TN51 could be used for possible commercial production of functional food to alleviate oxidative stress.

Introduction

It has been reported that oxygen-free radicals and other reactive oxygen species (ROS) may form in human body and in the food system. These radicals can cause oxidative damage by oxidizing biomolecules and thus lead to cell death and tissue damage (Halliwell *et al*., 1995). Many lifestyle-related diseases such as arthritis, cirrhosis, emphysema, atherosclerosis and cancer are believed to be correlated with the oxidative damage induced by these free radicals (Kehrer, 1993; Halliwell *et al.*, 1995). Dietary intake of soybeans and soybean products have been known to a decreased risk of cancer, including breast, colon and prostate cancers, osteoporosis and cardiovascular disease (Lee *et al*., 1991; Naik *et al.*, 1994; Messina and Bennink, 1998; Alekel *et al*., 2000; Tsukamoto *et al.*, 2000; Park *et al*., 2005). Many studies reported that certain components of soybean, especially isoflavone, saponin, vitamin C, tocopherol and phytate, protect against oxidative stress (Diaz-Batalla *et al*., 2006; Kumar *et al*., 2009; Kumar *et al.*, 2010).

Soybean (*Glycine max* L. Merrill) has various color of seed coat, including yellow, red, green and black. Recently, black seed coat soybeans have been found to contain high contents of γ-tocopherol, isoflavones, flavonoids and anthocyanins which possess biological activity (Correa *et al*., 2010; Jeng *et al*., 2010; Kumar *et al.*, 2010). Antioxidant properties of ferric reducing antioxidant power, free radical-scavenging effect and total phenolics have been shown comparative high in black soybean than the yellow soybean (Xu *et al.*, 2007; Xu and Chang, 2008; Kumar *et al*., 2010). In addition, the greater inhibition of low density lipoprotein oxidation of black soybeans also found than those presented in yellow soybeans (Takahashi *et al.*, 2005). Kumar *et al.* (2010) indicated that yellow, green and black soybean seeds were not significant the levels of total phenolics and isoflavones. In China, black soybeans were used to prepare several fermented product such as soy sauce and In-shi (Su, 1980). In addition, black soybeans have been reported to prepare *Bacillus*-fermented soybeans, including *chungkukjang* and *natto* (Shon *et al*., 2007; Juan and Chou, 2010).

In prior researches, a series of studies on

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improving process of *thua nao* by using pure starter culture of *B. subtilis* strain TN51 isolated from commercial product have conducted (Dajanta *et al*., 2009; 2011; 2012). They found that *B. subtilis* TN51 could be improved the 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. Furthermore, a marked increase in the content of total phenolics and antioxidant activity were also found in *B. subtilis* TN51-fermented product as compared to traditional process product and cooked non-fermented yellow soybean. However, such information of the product produced from black soybean has not yet been studied. This study aimed to determine the contents of phenolic, flavonoid and antioxidant capacities in *thua nao* produced from black soybeans fermented by powder culture of *B. subtilis* TN51 in comparison with yellow soybeans and their cooked non-fermented soybeans.

Materials and Methods

Chemicals

2,2-Diphenyl-1-picrylhydrazyl (DPPH), gallic acid, linoleic acid,catechin and trolox were purchased from Sigma-Aldrich Co. (St Louis, MO). Polyoxyethylene sorbitan monopalmitate (Tween 20) and 2,4,6-Tripyridyl-s-triazine (TPTZ) were purchased from Fluka (Germany). Folin-Ciocalteu reagent, ethanol, sodium carbonate, di-sodium hydrogen phosphate, sodium dihydrogen phosphate, potassium dihydrogen phosphate and dipotassium hydrogen phosphate were all obtained from Merck (Germany). Sodium nitrite and aluminium chloride were purchased from Ajax Finechem Pty Ltd. Methanol and sodium hydroxide were purchased from Labscan LTD. (Ireland). Ammonium thiocyanate was obtained from Rankem (India). Ferrous chloride was obtained from QREC (Malaysia). All culture media were purchased from Merck (Germany).

Microorganism and soybeans

Bacillus subtilis TN51 previously isolated from commercial *thua nao* and obtained from Dr. Ekachai Chukeatirote, School of Science, Mae Fah Luang University, Thailand. For inoculums preparation, the activated culture was streaked onto nutrient agar slant, incubated at 37°C for 24 h and then grown in trypticase soy broth at the same condition for 24 h. Medium for the production of primary powder, which consisted of 400 g/l wheat flour, 10 g/l sucrose, 0.5 g/l potassium dihydrogen phosphate and 0.5 g/l dipotassium hydrogen phosphate, was inoculated with 2% broth culture of *B. subtilis* TN51 and incubated at 42°C for 2 days. At the end of fermentation period, the fermented materials were mixed with autoclaved wheat flour (121°C for 15 min) in the ratio 1:1 and then incubated at 42°C for overnight. After oven dried at 100°C for 6 h, cultured wheat flour was pulverized using blender to a fine powder. The obtained powder culture which contains 10^7 CFU/g spore concentration was used for inoculation into fermentation soybeans. Yellow soybean variety Chiang Mai 60 and black soybean variety Sukho Thai 3, which served as the fermentation substrate, were obtained from the Field Crop Research Center, Institute of Agriculture, Chiang Mai, Thailand.

Fermentation of thua nao

Yellow and black soybeans were washed thoroughly two times under tap water and then soaked in distilled water at ambient temperature for 16 h. After decanting the water, soaked soybeans were autoclaved at 121°C for 40 min and cooled to about 55°C. Each steamed soybeans were divided into two portions; one was used for *thua nao* fermentation, while the other was dried directly for the extraction process and used as cooked non-fermented (CNF) sample. Powder culture of *B. subtilis* TN51 (1 g) was mixed with cooked soybeans to a final load of 10^4-10^5 spore cells/g. The inoculated soybeans were incubated in triplicate at 42°C for 24 h and extended for 24 h at 4°C.

Preparation of methanolic extract of soybeans

The yellow soybean *thua nao* (TN-YS), black soybean *thua nao* (TN-BS) and their CNF samples (CNF-YS and CNF-BS) were evaluated. First, all of samples were dried in hot air oven at 60°C for 24 h and pulverized to powder using blender. The ground powder was extracted with 80% methanol (1:20, w/v) for 1 h at room temperature with sonicating. The extraction process was repeated twice, and the extracts were pooled together and filtered using Whatman No. 1 paper. The filtrates were dried at 45°C under vacuum (Heidolph, Germany) and re-dissolved with 80% methanol to final concentration at 200 mg/ml. The extracts were stored in screw-capped amber glass bottle at -20°C until used. Three separate samples of soybeans were extracted (n = 3).

Determination of total phenolics

The total phenolic contents of the soybean extracts were measured according to the method described by Luque-Rodriguez *et al.* (2007). An aliquot of 0.4 ml methanolic soybean extract was

added to 2 ml of 0.25N Folin-Ciocalteu phenol reagents in water. After that, 1.6 ml of 7.5% (w/v) sodium carbonate was added to the mixture and heated in a water bath at 50°C for 5 min. The absorbance was measured at 760 nm by a spectrophotometer (Spectronic Evolution 200 Series, China) after cooling in darkness. The calibration curve was established using gallic acid (10-100 µg/ml) as the standard sample, with the correlation coefficient r = 0.9965. The gallic acid content was used as the total phenolic contents in the soybean sample, and was expressed as milligram of gallic acid equivalent (mg GAE)/g extract.

Determination of total flavonoids

The total flavonoids of the soybean extracts were assayed according to the method described by Yang *et al.* (2009). An aliquot of 0.25 ml of soybean extract was mixed with 1.25 ml of distilled water and 75µl of 5% sodium nitrite. After 6 min, 150 µl of 10% aluminum chloride were added and standing for 5 min prior mixed with 0.5 ml of 1M sodium hydroxide and 775 µl of distilled water. The absorbance of the solution was determined at 510 nm. The calibration curve was established using catechin (50-400 µg/ml) as the standard sample, with the correlation coefficient r = 0.9992. The result of total flavonoid contents in the soybean extracts were expressed as milligram catechin equivalent (mg CE)/g extract.

DPPH radicals-scavenging assay

The antioxidant activity of the soybean extract was assessed on the basis of the radical scavenging effect of the stable DPPH-free radical activity by the method according Nuengchamnong *et al.* (2009). Briefly, 0.2 mM methanolic DPPH solution was prepared and 150 µl of this solution were added to 75 µl sample extracts at different concentrations (0.3-20 mg/ml) in each well of 96 well plate. After incubation in the dark at ambient temperature for 30 min, the absorbance was measured at 515 nm using microplate reader. The scavenging percentage of DPPH was calculated according to the following equation:

$$\% \text{ scavenging effect} = [1- (\text{Absorbance of sample}/ \text{Absorbance of control})] \times 100$$

Percent antioxidant activity was plotted against log concentration (µg/ml).The results were expressed as the half-inhibitory concentration (IC_{50}) in mg/ml.

Lipid peroxidation inhibition assay

The antioxidant activity of the methanolic extract of soybean on inhibition of lipid peroxidation was determined according to the ferric thiocyanate method as reported by Kuo *et al.* (2009). A solution of 0.02 mM linoleic acid emulsions was prepared by dissolving 0.28 g linoleic acid and 0.28 g Tween 20 in 50 ml of 0.02 M phosphate buffer (pH7.0). Aliquots of this emulsion (2.5 ml) and 2 ml of 0.2M phosphate buffer (pH 7.0) were transferred into different test tubes containing 4 ml of various concentrations (5 - 50 mg/ml) of the sample extract in 80% methanol. After incubated at 55°C in darkness for 72 h, 0.1 ml of the mixture was sampled and combined with 4.7 ml of 75% ethanol, 0.1 ml of 30% ammonium thiocyanate, and 0.1 ml of 20 mM ferrous chloride (in 3.5% hydrochloric acid). For control, 80% methanol was used in the reaction instead of the sample extracts. After addition ferrous chloride exactly 3 min, the absorbance of the reaction mixture was measured at 500 nm. The inhibitory effect was calculated according to the following equation:

$$\% \text{ inhibitory effect} = [1- (\text{Absorbance of sample}/ \text{Absorbance of control})] \times 100$$

The percentage of inhibitory effect obtained was subsequently plotted against log sample concentration. The antioxidant activity of soybean extracts was expressed as IC_{50}, which was defined as the concentration in mg/ml.

Ferric reducing antioxidant power (FRAP)

The FRAP assay was performed as previously described by Maier *et al.* (2009). The FRAP reagent was prepared from 2.5 ml of a TPTZ solution (10 mM) in hydrochloric acid (40 mM) and 2.5 ml of a ferric chloride solution (20 mM) mixed with 25 ml of an acetate buffer (0.3 M, pH 3.6). For the determination of the antioxidant capacity, the FRAP reagent (1.5 ml) was mixed with 100 µl of water and 100 µl of the appropriately diluted sample. The mixture was allowed to stand for 4 min at room temperature before the absorption was measured at 593 nm (Spectronic Evolution 200 Series, China). The calibration curve was established using trolox (50-1000 mM) as the standard sample, with the correlation coefficient r = 0.9996. The results were expressed as mole of trolox equivalent per gram of soybean extract (mole TE/g extract).

Statistical analysis

The experimental results were expressed as mean ± SD of triplicate observations made for three parallel extractions and determinations. Data were analyzed statistically by ANOVA and Duncan's multiple range tests and a P-value of less than 0.05 was considered significance.The correlation coefficient methods among the content of each antioxidant component

and the value of each antioxidant capacity were determined by Pearson's correlation coefficient, SPSS® ver. 17.

Results and Discussion

Extraction yield

80% methanol could be used as a solvent for antioxidant extraction which yielded 11.99-22.72% dried samples (Table 1). Among all the examined soybean extracts, the extraction yields of CNF-YS and TN-YS were significantly higher than those observed in black soybeans ($P \leq 0.05$). In addition, the extraction yields of TN-BS showed a significant higher than that of CNF-BS ($P \leq 0.05$), while a comparative yields were found in TN-YS and CNF-YS. For a lowering extraction yield of *thua nao* samples, Lee *et al.* (2008) have explained that it might be due to the rapid decomposition of small substances which occurred immediately after those were degraded from the substrate.

The contents of total phenolics and total flavonoids

As shown in Table 1, CNF-BS exhibited 56% higher level of total polyphenols than those of yellow one. This result is consistent with the finding of Takahashi *et al.* (2005) and Xu and Chang (2008). It is due to the larger content of the compounds in black seed coat. After *B. subtilis*-fermentation, the contents of total phenolics were 217% and 859% higher in fermented black and yellow soybeans than those originated in their CNF soybeans, respectively. The consistent results have been found in the production of other *Bacillus*-fermented soybeans, *kinema* and *natto* (Moktan *et al.*, 2008; Juan and Chou, 2010; Yao *et al*., 2010). Extracting the total phenolics from non-fermented and *Bacillus*-fermented black soybeans with 80% methanol, Juan and Chou (2010) found that total phenolic contents were 15.94 and 23.43 mg GAE/g extract. Comparing with their results, around 2 times lower level of total phenolics was observed in the methanolic extract CNF-BS in this study. However, after fermentation, a slight (15%) increased content was found in TN-BS samples obtained in present investigation. This study also found that the total phenolic content was 33% higher in TN-YS extract with respect to fermented product from black soybean.

The content of flavonoids in soybean extracts varied between 1.38 and 3.50 mg CE/g extract (Table 1). CNF-BS exhibited 41% higher content of total flavonoids than that of CNF-YS. The variable trends of the total flavonoid contents were observed in different type of soybean fermentations. Of black soybean process, 70% of total flavonoids were increased in TN-BS. On the other hand, change in content of total flavonoids was not significantly ($P > 0.05$) observed between CNF-YS and TN-YS. This result is in accordance with that observed by other investigators for higher content of total flavonoids during fermentation of black soybeans by *Bacillus*, yeast and fungi (Juan and Chou, 2010; Yao *et al.*, 2010). Moreover, present work also found that TN-BS extract showed 154% higher content of total flavonoids than that presented in yellow soybean product.

Table 1. Extraction yields and the contents of total phenolics and total flavonoids of methanolic extracts of black and yellow soybean *thua nao* and cooked non-fermented soybeans[1]

Samples[2]	Extraction yield (%)	Total phenolics (mg GAE/g extract)	Total flavonoids (mg CE/g extract)
CNF-YS	22.72 ± 0.02a	3.74 ± 0.88d	1.46 ± 0.00c
CNF-BS	19.45 ± 0.09b	8.54 ± 0.11c	2.06 ± 0.00b
TN-YS	21.94 ± 0.65a	35.88 ± 2.74a	1.38 ± 0.19c
TN-BS	11.99 ± 0.79c	27.05 ± 1.77b	3.50 ± 0.51a

[1]Each value is expressed as mean ± SD (n = 3), and means in the same column with different letters are significantly different ($P \leq 0.05$).
[2]CNF-YS = cooked non-fermented yellow soybean; CNF-BS = cooked non-fermented black soybean; TN-YS = yellow soybean *thua nao*; TN-BS = black soybean *thua nao*.

This study is consistent with the reports of Lee *et al.* (2008) and Juan and Chou (2010) for the significant higher concentration of total phenolics and flavonoids in *thua nao* extracts than the respective extracts of CNF soybeans. *B. subtilis* is capable of producing β-glucosidase during soybean fermentation (Wei *et al*., 2008; Kuo *et al.*, 2006; Wu and Chou, 2009). Catalyzing the release of total phenolics and flavonoids from the soybean substrate due to the action of β-glucosidase is produced by *B. subtilis* TN51 during fermentation. This may thus lead to an increase in the content of those compounds as shown in Table 1.

Scavenging of DPPH radicals

DPPH is a stable lipophilic free radical that is used to determine the proton-scavenging activity of the various soybean extracts. The anti-DPPH radical effects increased as the dosage of the methanol extract of soybeans increased. The prepared methanol extracts of all soybeans, at a concentration of 5 mg/ml, showed various degrees of scavenging effect for DPPH radicals (45.58- 97.09%) depending on type of soybeans.

Table 2 shows IC_{50} value, which is the inhibition concentration of solvent extract required to decrease initial DPPH concentration by 50%. The lower IC_{50} value indicates stronger antioxidant activity in the sample.The extract of CNF-BS showed stronger activity of anti-DPPH radicals than that of CNF-YS extract, as evidenced by a significant lower value of IC_{50} ($P \leq 0.05$). In addition, the IC_{50} values of the *thua nao* extracts were less than those of the respective

Table 2. Scavenging activities on DPPH radicals, lipid peroxidation inhibition and FRAP of methanolic extractsof black and yellow soybean *thua nao* and cooked non-fermented soybeans[1]

Samples[2]	DPPH-antiradical (IC_{50}, mg/ml)	Lipid peroxidation inhibition (IC_{50}, mg/ml)	FRAP (mol TE/g SE)
CNF-YS	4.60 ± 0.68a	13.97 ± 1.67a	8.18± 0.81c
CNF-BS	3.61 ± 0.02b	6.37 ± 0.08b	8.18± 0.81c
TN-YS	3.12 ± 0.21b	16.00 ± 3.03a	13.75 ± 0.66a
TN-BS	2.23 ± 0.09c	5.91 ± 0.25b	10.16 ± 0.33b

[1]Each value is expressed as mean ± SD (n = 3), and means in the same column with different letters are significantly different ($P \leq 0.05$).
[2]CNF-YS = cooked non-fermented yellow soybean; CNF-BS = cooked non-fermented black soybean; TN-YS = yellow soybean *thua nao*; TN-BS = black soybean *thua nao*.

Table 3. Correlations among total phenolics, total flavonoids and antioxidant capacity

Correlations	TPC[1]	TFC[2]	DPPH[3]	LPIA[4]	FRAP[5]
TPC[1]	1.000	0.210	-0.731**	0.189	0.889**
TFC[2]		1.000	-0.709**	-0.739**	-0.182
DPPH[3]			1.000	0.393	-0.368
LPIA[4]				1.000	0.51
FRAP[5]					1.00

*Correlation is significant at the level of $P \leq 0.05$ (two-tailed).
**Correlation is significant at the level of $P \leq 0.01$ (two-tailed).
[1]Total phenolic compounds
[2]Total flavonoid content
[3]DPPH radical-scavenging effect
[4]Lipid peroxidation inhibition activity
[5]Ferric reducing antioxidant power

their CNF soybean extracts. This indicated that fermentation with *B. subtilis* TN51 enhanced the DPPH radicals-scavenging effect of the extract of soybeans. This phenomenon is in accordance with that observed on *natto*, *koji* and *tempeh* (Lin *et al*., 2006; Lee *et al*., 2007; Chang *et al*., 2009; Juan and Chou, 2010). Among TN-BS and TN-YS extracts, TN-BS showed greater anti-DPPH radical effect than the TN-YS. Choung *et al.* (2001) indicated that black soybeans contain a high concentration of anthocyanins, belong to the flavonoid family and act as natural colorants, located primarily in its seed coat. Anthocyanins have been reported to possess the capacity to scavenge free radicals (Kathkonen and Heinonen, 2003). Moreover, it has been reported that *B. subtilis*-fermentation resulted in a significant increase in the anthocyanin content in black soybean (Juan *et al*., 2010). This phenomenon may be attributed to the catalytic release of anthocyanin from the black soybean substrate due to the action of β-glucosidase during fermentation (Wu and Chou, 2009). However, the contents of anthocyanin in soybeans were not examined in present study.

Lipid peroxidation inhibitory activity (LPIA)

The IC_{50} of the soybean extracts for LPIA is also presented in Table 2. It was noted that CNF-BS possessed LPIA of 119% greater than those of CNF-YS. This is in agreement with the finding of Takahashi *et al.* (2005) and Prakash *et al.* (2007). The activities of lipid peroxidation inhibition were no significant difference between extracts of *thua nao* and those of their CNF soybean extracts. It means that *B. subtilis* TN51-fermentation ineffectually improves LPIA of the extracts of soybean. A similar result was also found in methanol extract of *Aspergillus* fermented soybean (Esaki *et al.*, 1997). However, a different scenario was reported by Moktan *et al.* (2008). They found that the methanol extract of kinema showed a higher level of LPIA than the CNF soybean.

Similar CNF soybeans, TN-BS exhibited 171% of lipid peroxidation inhibitory effect which was better than that possessed in TN-YS. Takahashi *et al.* (2005) have suggested that total polyphenols contents in black soybeans seed coat and aglycones, which are rich in fermented soybeans, may play a crucial role in the inhibition of lipid peroxidation.

Ferric reducing antioxidant power (FRAP)

As shown in Table 2, different amounts of FRAP were observed with the various soybean extracts (8.18-13.75 mole TE/g extract). The extracts of *thua nao* showed higher values of FRAP of 24% in TN-BS and 68% in TN-YS when compared to their CNF samples. This clearly demonstrated that *B. subtilis* TN51-fermentation enhances the reducing power of the extracts of soybean. Comparing between *thua nao* extracts, TN-YS showed 34% higher FRAP value than that of TN-BS. Yang *et al.* (2000) indicated that the increased reducing power may be due to the formation of reductants that could react with free radicals to stabilize and terminate radical chain reactions during fermentation. Moreover, they also indicated that other factors, including intracellular antioxidants, peptides of the starter organism and their hydrogen-donating ability, may contribute to this increased reducing effect.

Correlation among parameters

Correlations among antioxidant capacity, total phenolics and flavonoids are statistically analyzed and depicted in Table 3. A strong and positive correlation ($P \leq 0.01$) was observed between total phenolic compounds and FRAP ($r = 0.889$). This result is in agreement with those reported in black soybean (Kumar *et al*., 2010) and soybean by-products (Tyug *et al.*, 2010). The FRAP assay measures ability to electron donors and reduce the oxidized intermediates of lipid peroxidation processes. Therefore, it indicates that extract compounds can act as primary and secondary antioxidants. Present results clearly demonstrated that total phenolics in soybean are the potential electron donors and reduce the oxidized intermediates of lipid peroxidation processes.

Total phenolics ($r = -0.731$) and total flavonoids (r

= -0.709) appear to have a strong negative correlation with IC_{50} of DPPH radical-scavenging activity ($P \leq 0.01$). Also, a significant and strong negative correlation ($r = -7.39$, $P \leq 0.01$) was demonstrated between the total flavonoids and IC_{50} of LPIA. This phenomenon indicates that total phenolics and flavonoids in soybean are a strong scavenger of free radicals and suppresser of lipid peroxidation. Phenolic compounds, even though concentration, the activity of inhibitory free radicals also depends on the structure of phenolics, oxidation condition and nature of the sample oxidized. Mathew and Abraham (2006) reported that beside phenolic compounds, other chemical components presented in the extract may suppress lipid peroxidation through different chemical mechanisms, including free radical quenching, electron transfer, radical addition or radical recombination. Therefore, free radical-scavenging activity and lipid peroxidation inhibition of the extract cannot be predicted on the basis of its content of total phenolics alone. Moreover, a synergism of polyphenolics, with one another, and other components present in an extractmay contribute to the overall observed antioxidant activity (Shahidi *et al.*, 1994).

Conclusion

This study demonstrated that yellow and black soybean fermented with powder culture of *B. subtilis* TN51 to *thua nao* enhanced free radical-scavenging activity and ferric reducing antioxidant power. Furthermore, it also proved that *B. subtilis* TN51 fermentation could be enhanced the contents of total phenolics and flavonoids of *thua nao*. Based on the content of flavonoids and antioxidant effects of DPPH and lipid peroxidation inhibitory activity, *thua nao* produced from black soybeans showed greater qualities than those found in yellow soybean product. Therefore, the product from black soybeans, obtained in this work, may be used for possible commercial production of functional foods in the future. Further research is necessary to study the qualities of anthocyanins and isoflavones that might be masked in the present study.

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