Chapter 5

Phenolic Compounds and Biological Activities in Extracts of *Thua Nao* Produced by *B. subtilis* TN51

5.1 Introduction

Soybean and its products have been widely consumed in various Asian countries as the source of nutritive protein. Besides, it has been reported that soybeans and related products contain phytochemicals which are beneficial for human health. Such phytochemicals including phenolics and isoflavones are well known for their useful biological activity such as anticarcinogenic activity (Gotoh *et al*., 1998; Peterson *et al*., 1998; Jung *et al*., 2006), inhibition of the oxidative damage to low-density lipoprotein (Okamoto *et al*., 1995; Yokota *et al*., 1996; Potter *et al*., 1998; Park *et al*., 2003), improved bone health (Ishimi *et al.,* 2002), eliminated menopause symptoms (Eden, 1998; Kim *et al*., 2006), antimutagenic effects (Park *et al*., 2003), antidiabetic activity (Liu *et al*., 2006), estrogenic effects (Makela *et al.*, 1995) and antioxidant activity (Kwak *et al.*, 2007; Lee *et al.*, 2007a; Moktan *et al.*, 2008; Georgetti *et al*., 2009). A number of researchers have described that traditional soy-fermented foods such as *Chungkukjang*, *Kinema*, *Natto* and *Tempeh* are a good source of antioxidant components (Hattori *et al*., 1995; Shon *et al*., 2007; Moktan *et al*., 2008; Chang *et al*., 2009). Phenolic compounds, one of the most widely studied antioxidants, are beneficial and can react in many biochemical reactions as reducing agents, hydrogen donors, single oxygen quenchers, and metal chelating agents (Rice-Evans *et al*., 1995). The quantity and structural conformation of phenolic compounds have been reported to affect their antioxidant activity. It has also been reported that the content and profile of available phenolic aglycone derivatives are markedly increased in soybean after microbial fermentation and thus exhibit stronger antioxidant activity than those of cooked non-fermented soybean (Kwak *et al*., 2007; Lee *et al*., 2007a; Moktan *et al*., 2008). In addition to phenolic compounds, other antioxidant components such as isoflavones, tocopherols, phospholipids, free amino

acids, oligopeptides and melanoidins (a by-product of Maillard reaction) are present in soy products (Chen *et al*., 1998; Rufian-Henares and Morales, 2007a; Wang, *et al*., 2008). Recently, there are many studies describing phytochemicals and their biological qualities in *Chungkukjang*, *Kinema* and *Natto* (Iwai *et al*., 2002; Kim *et al*., 2008; Moktan *et al*., 2008), but such information of *Thua Nao* remains unknown. This study aims to investigate the content of total phenolic components and their contribution towards antioxidant and antimicrobial effects of *Thua Nao* prepared by pure starter culture of *Bacillus subtilis* TN51.

5.2 Materials and methods

Bacillus subtilis TN51 was prepared as starter culture to ferment soybean cultivar TG145 as described in Section 4.2.2 to 4.2.3. After 72 h fermentations, fermented soybeans were collected and then determined for their antioxidant components as illustrated in Section 3.2.6. In addition, biological activity (i.e., DPPH radical scavenging effect, total antioxidant and antimicrobial activity) of *Thua Nao* and cooked non-fermented soybeans were also investigated as described in Section 3.2.7 to 3.2.8. Data were expressed as means ± standard deviation of triplicate observations. 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 $P \leq 0.05$.

5.3 Results and discussion

5.3.1 Total phenolic contents

According to Esaki *et al.* (1997), methanol was shown to be the best solvent used for extracting antioxidant components from soy-fermented food. This present study thus employed this solvent for extracting antioxidant substances. The contents of phenolic compounds of cooked non-fermented soybeans (CNF) and *Thua Nao* extracts are shown in Table 5.1. It was noted that, when using 80% methanol, antioxidants could be extracted and accounted for 12.22 to 17.97% of lyophilized soybean tested samples. Total phenolic contents of the methanol extracts of fermented

Thua Nao and cooked non-fermented soybeans ranged between 27.67 and 37.29 mg GAE/g of sample extract. After fermentation, total phenolic contents of *Thua Nao* samples fermented naturally (TNMX) and by *B. subtilis* TN51 (TNB51) were 35 and 21% higher than those cooked non-fermented soybeans. Similar results were also found in other fermented soybeans (Lin *et al*., 2006; Moktan *et al*., 2008; Georgetti *et al*., 2009). For example, in *Kinema* production, total phenolic content was increased by 144% after fermentation with *B. subtilis* (Moktan *et al*., 2008). These results may be derived from activity of β-glucosidase responsible for biotransformation of conjugated phenolics to free form derivatives (Kwak *et al.*, 2007; Lee *et al.*, 2007a; Moktan *et al.*, 2008; Georgetti *et al*., 2009). A different result was reported by Shon *et al*. (2007), who identified the similar contents of total phenolics in fermented *Chungkukjang* and their non-fermented soybeans. Compared to TNMX, TNB51 exhibited lower level of total polyphenols. This result is in agreement with the finding of Shon *et al*. (2007) in which lower contents of total phenolics of natural fermented *Chungkukjang* were observed with respect to pure starter fermented products. This is possibly due to the variable selectivity action of β-glucosidase released from various fermenting organisms (Georgetti *et al.*, 2009).

Table 5.1 Yield (%) and total phenolics (mg GAE/g extract) of methanolic extracts of cooked non-fermented soybeans and *Thua Nao*

Sample	**Yield of extract (%)**	**Total phenolics (mg GAE/g ext.)**	**Phenolics/Yield of extract (%)**
Natural fermentation			
CNF1	12.22 ± 0.30^{b}	27.67 ± 1.41^{c}	2.77 ± 0.14^{c}
TNMX	17.97 ± 0.85^{a}	37.29 ± 1.00^{a}	3.73 ± 0.10^{a}
Pure culture fermentation			
CNF2	12.63 ± 1.93^{b}	29.03 ± 0.14^{c}	2.90 ± 0.01^{c}
TNB51	16.53 ± 0.29^{a}	35.18 ± 1.03^{b}	3.52 ± 0.10^{b}

Data are mean ± standard deviation (n = 3). Means in the same column with different letters were significantly different ($P \leq 0.05$). CNF1, CNF2 = Cooked non-fermented soybeans prepared by boiling and autoclaving, respectively; TNMX, TNB51 = *Thua Nao* fermented by naturally occurring bacteria and *B. subtilis* TN51.

5.3.2 Antioxidant activity

In this study, the antioxidant property was assessed by two different assays: lipid peroxidation inhibition by β-carotene-linoleate system and DPPH radicals scavenging effect. In β-carotene-linoleate system, free radicals yielded from linoleic acid oxidation attack unsaturated β-carotene molecules resulting in the decrease of the absorbance at 470 nm. The bleaching of β-carotene can be hindered in the presence of different antioxidants by their neutralising the linoleate-free radical and other free radicals formed in the system (Jayaprakasha *et al.*, 2001). As shown in Table 5.2, *Thua Nao* extracts (TNMX and TNB51) exhibited significantly higher total antioxidant activity than those of their cooked non-fermented soybeans. This observation coincided with amount of phenolic compounds (Figure 5.1). It has shown that high contents of total phenolic compounds in *Chungkukjang*, *Koji* and *Kinema* are responsible for the strong inhibitory of free radicals yielded from linoleic acid oxidation (Kwak *et al*., 2007; Lee *et al*., 2007a; Moktan *et al*., 2008).

Table 5.2 Antioxidant activities of methanolic extracts of cooked non-fermented soybeans and *Thua Nao*

Sample	**Total antioxidant**	**Free radical scavenging activity**		
	(%)	**IC_{50} (mg/ml of ext.)**	**EC_{50} (mg/mg)**	**ARP**
Natural fermentation				
CNF1	54.55 ± 1.16^{d}	3.37 ± 0.05^{b}	114.93 ± 1.82^{b}	0.88 ± 0.01^{b}
TNMX	65.65 ± 0.39^{a}	5.18 ± 0.43^{a}	175.28 ± 14.54^{a}	0.57 ± 0.05^{c}
Pure culture fermentation				
CNF2	58.49 ± 2.30^{c}	2.70 ± 0.01^{c}	91.30 ± 0.40^{c}	1.10 ± 0.00^{a}
TNB51	62.12 ± 0.95^{b}	3.27 ± 0.01^{bc}	110.74 ± 0.41^{bc}	0.90 ± 0.01^{b}

Data are mean ± standard deviation (n = 3). Means within same column with different superscripts of letter are significantly different ($P \leq 0.05$). CNF1, CNF2 = Cooked non-fermented soybeans prepared by boiling and autoclaving, respectively; TNMX, TNB51 = *Thua Nao* fermented by naturally occurring bacteria and *B. subtilis* TN51. Total antioxidant determined by the β-carotene linoleic acid system at 10 mg/ml of dried sample extracts; IC_{50} = Half inhibitory concentration; EC_{50}, half-efficiency concentration = IC_{50}/concentration of DPPH in mg/ml; ARP, anti-radical power = $100/EC_{50}$.

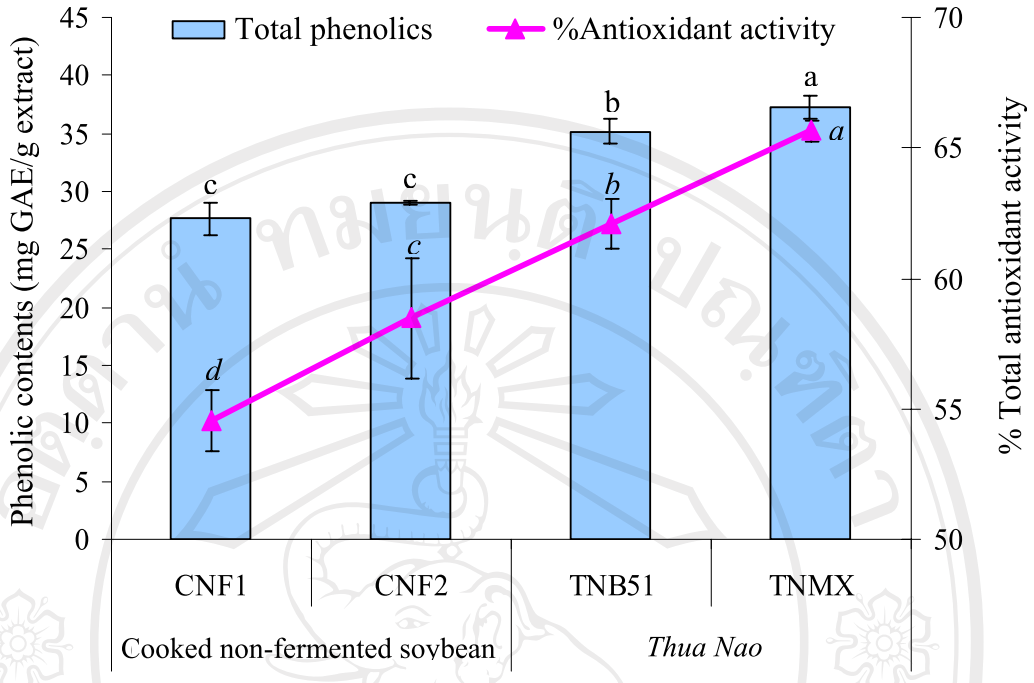


Figure 5.1 Phenolic contents and total antioxidant activities of cooked non-fermented soybeans and *Thua Nao*. CNF1, CNF2 = Cooked non-fermented soybeans prepared by boiling and autoclaving, respectively; TNMX, TNB51 = *Thua Nao* fermented by naturally occurring microbes and *B. subtilis* TN51, respectively. Each value represents mean ± SD (n = 3). Means (bar value) with different letters are significantly different ($P \leq 0.05$).

In addition to phenolic content, high antioxidant activity of *Thua Nao* extracts might give synergistic effects with another antioxidant components like oligoproteins, free amino acids and melanoidins as described in related soy fermented foods (Manzocco *et al.*, 2001; Saito *et al.*, 2003; Delgado-Andrade and Morales, 2005; Delgado-Andrade *et al.*, 2005; Prakash *et al.*, 2007; Wang *et al.*, 2008). Peptides and free amino acids are also known as source of the anti-free radicals and anti-linoleic acid oxidation in Chinese Douchi (Wang *et al.*, 2008). Besides, evidence from the ratio of total polyphenols to the total extractable substances in all soybean samples ranged from 2.77 to 3.73% (Table 5.1), thus around 96% of other than phenolics antioxidant components were expected residues in extractable substances. Melanoidin browning pigments, a product from Maillard reaction, have been indicated to possess

potential antioxidant activity in heated food (Rufian-Henares and Morales, 2007a). In this study, the greater content of antioxidant melanoidins might be expected in autoclaved soybean (121°C) and its fermented product with the effect of higher temperature treatment when compared with boiled soybean (boiling temperature). Previous studies including this investigation have also found the effectively enhanced lipid peroxidation inhibitory activity of pure starter *B. subtilis* fermentation of *Chungkukjang* (Shon *et al*., 2007), *Kinema* (Moktan *et al*., 2008) and *Thua Nao*. Comparing with *Kinema* (Moktan *et al*., 2008), *Thua Nao* extracts also presented higher level of lipid peroxidation inhibition. It may be due to the effect of difference in soybean variety, cooking process, and fermenting *Bacillus* strain in the fermentation process.

DPPH radical scavenging effect has been widely used to evaluate the antiradical power of soybean and another plant extracts (Miliauskas *et al*., 2004; Kwak *et al*., 2007; Shon *et al*., 2007; Georgetti *et al*., 2009). The DPPH radical is a stable free radical that easily accepts an electron or hydrogen radical into a stable molecule. It loses the absorption band at 517 nm after being reduced by antioxidants and this is visually noticeable as a colour change from purple to yellow (Di Mambro and Fonseca, 2005). Antiradical power of soybean extracts is dependent on the concentration of sample extracts. For example, at 5 mg/ml, the various effective inhibitory free radical-scavenging activities of *Thua Nao* and cooked non-fermented soybeans were between 40.20 and 85.17%. The IC_{50} values can then be calculated from the logarithmic graph plotted between the soybean extract concentrations and % scavenging (Parejo *et al*., 2003) (Figure 5.2).

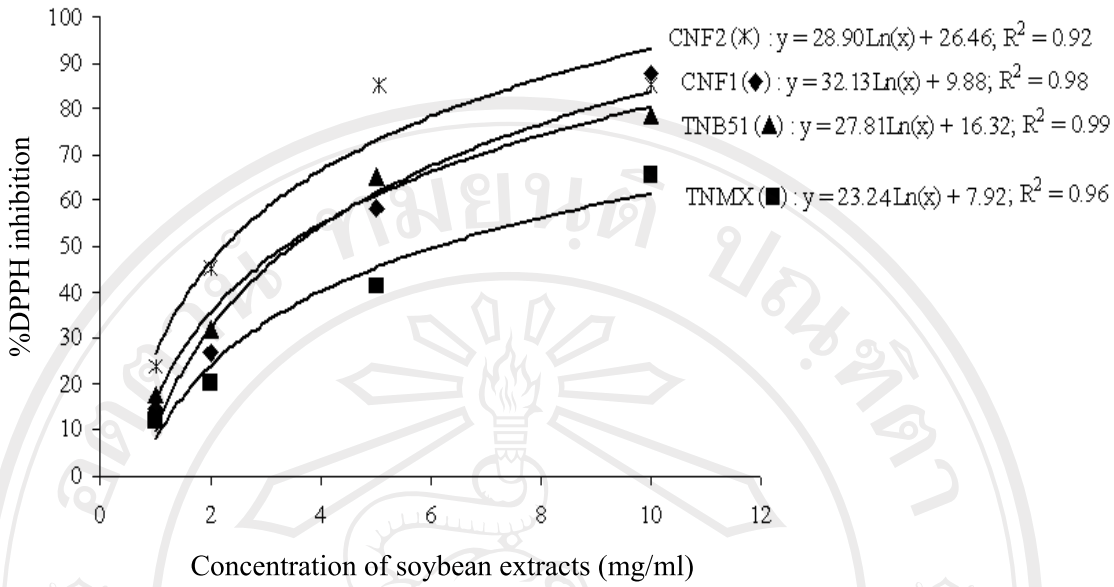


Figure 5.2 Logarithmic graphs between soybean extract concentrations and % scavenging used to determine the IC_{50} values.

According to Table 5.2, all soybean extracts displayed the value of IC_{50} from 2.70 to 5.18 mg/ml of sample extract, EC_{50} range 91.30 to 175.28 mg/mg DPPH, and ARP from 0.57 to 1.10. The extract of autoclaved non-fermented soybean showed the strongest activity of free radical scavenger, as evidenced by the lowest values of IC_{50} and EC_{50} in combination with the highest value of ARP. In this study, although *Thua Nao* extracts contained higher phenolic contents (Figure 5.3), they presented a lower radical scavenging activity when compared with those of non-fermented stages. This is possibly due to the main aglycone phenolics reached in fermented *Thua Nao* possessing a weakness of radical scavenging activity (Mitchell *et al.*, 1998; Jun *et al.*, 2003). In case of high free radical scavenging activity in spite of great level of soy phenolic components have also been reported previously (Lin *et al*., 2006; Prakash *et al*., 2007; Georgetti *et al*., 2009). In contrast, there are reports of stronger radical scavenging activity exhibited in aqueous extracts of *Chungkukjang* and *Tempeh* (Kwak *et al*., 2007; Chang *et al*., 2009). The discrepancy is probably due to different extract solvents and methods used. High polar solvent could extract larger amount of antioxidant component and possess stronger antiradical power (Chang *et al*., 2009).

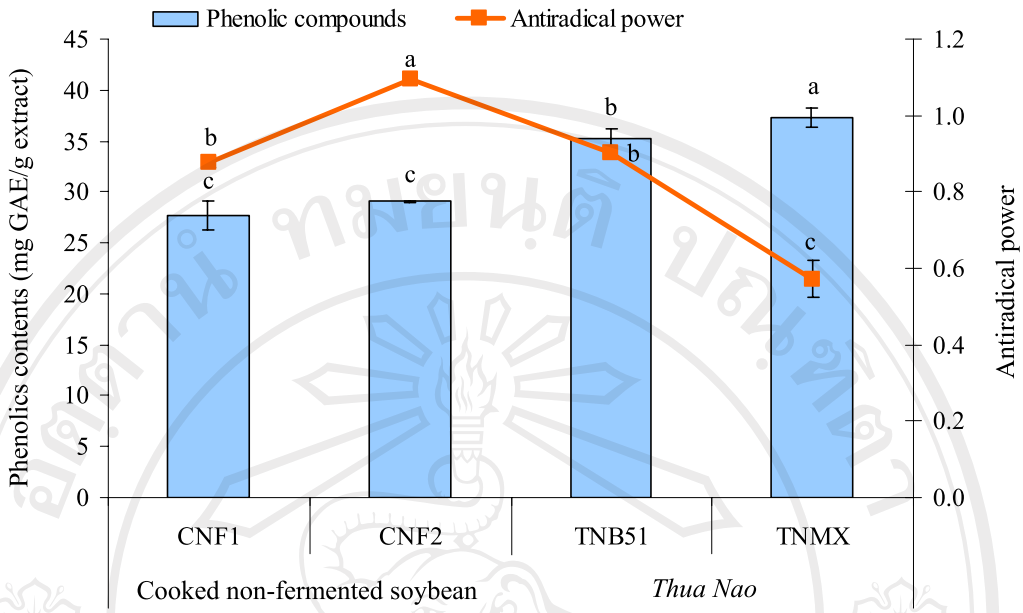


Figure 5.3 Phenolic contents and radical scavenging effects of cooked non-fermented soybeans and *Thua Nao*. CNF1, CNF2 = Cooked non-fermented soybeans prepared by boiling and autoclaving, respectively; TNMX, TNB51 = *Thua Nao* fermented by naturally occurring microbes and *B. subtilis* TN51, respectively. Each value represents mean ± SD (n = 3). Means (bar value) with different letters are significantly different ($P \leq 0.05$).

Phenolics are classified as free radical terminators, even though concentration, the activity of inhibitory free radicals also depends on the structure of phenolics, oxidation condition and nature of the sample oxidized. In soybean, phenolic acids, and isoflavones are major antioxidants phytochemicals. Structural conformations of these components mainly affect the degree of radical-scavenging effect. For instance, syringic acid possessed the highest antiradical power when compared with other phenolic acids of *Douchi* (Chen *et al*., 2005). Also Lee *et al*. (2005b) reported that genistin exhibited stronger DPPH scavenging activity than other glucoside and aglycone derivatives and, in contrast when used lipoprotein oxidation system, the greatest inhibitory isoflavones was genistein.

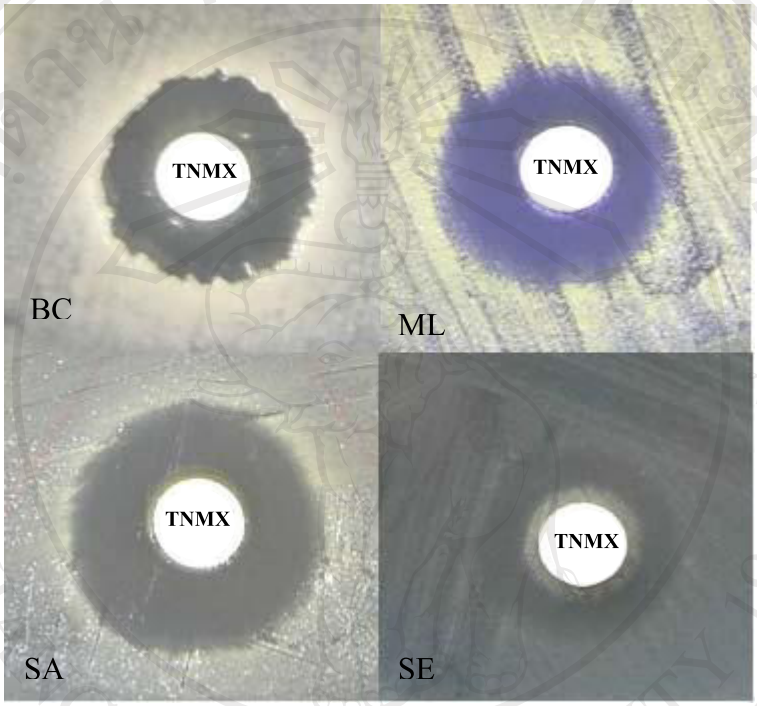
The significant difference of phenolics content, total antioxidant and free radicals scavenger effects were found between natural and pure starter production of *Thua Nao*. It may be expected that the effect of cooking process and fermenting microbial were mainly causing these differences (Lin *et al*., 2006). The result of present work is in agreement with prior studies, microbial fermentation could be improved antioxidant activity of *Chungkukjang* (Shon *et al*., 2007), *Kinema* (Moktan *et al*., 2008) and *Douchi* (Wang *et al*., 2008). On the other hand, almost equal antioxidant activities of *Natto* and non-fermented soybean extracts has also been reported by Esaki *et al*. (1997).

5.3.3 Antimicrobial activity

The antimicrobial activity was determined by the disc diffusion method. The antimicrobial activity against foodborne bacteria and yeasts of all soybean extracts are shown in Figure 5.4 and Table 5.3. Of tested microorganisms, only Gram-positive bacteria i.e. *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Micrococcus luteus*, *Bacillus cereus* and *Listeria monocytogene*s were inhibited by *Thua Nao* extracts. However, TNMX extract (*S. aureus*, *S. epidermidis*, *M. luteus*, and *B. cereus*) showed relatively broader inhibition activity against tested bacteria when compared with TNB51 (*B. cereus* and *L. monocytogene*s) and commercial *Thua Nao* (*B. cereus*) (Table 3.7). The activity of antifoodborne pathogenic bacteria of *Thua Nao* extracts may be related the contents of phenolic components as illustrated in Table 5.1. Since the finding of several investigators revealed the antimicrobial capacity of phenolic phytochemicals (Proestos *et al*., 2005; Pereira *et al*., 2006; 2007; Kwon *et al*., 2007). The potential antimicrobial activity of crude extracts of Chinese fermented seasoning (Zheng and Slavik, 1999), *Chungkukjang* (Kim *et al*., 2004b) and *Doenjang* (Yun, 2005) have been verified. These studies are suggested as the effect of bacteriocins produced during *Bacillus* spp. fermentation of soybeans. Several *Bacillus* sp. isolated from fermented soybeans exhibit antimicrobial activity. Some metabolites derived from these *Bacillus* strains have been characterised and summarised in Table 5.4. Hence, the variation of broad spectrum of inhibition against foodborne bacteria of *Thua Nao* extracts may be caused by the effect of antibacterial substances from other microorganisms present in the products. Besides, the synergistic antimicrobial activity

of melanoidins browning pigments might be also expected in *Thua Nao* extracts, since many studies have verified the potential antibiotic activity of these components (Stecchini *et al*., 1993; Dastidar *et al*., 2004; Rufián-Henares and Morales, 2006; 2007a).

(A)



(B)

Figure 5.4 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 and LM, *Listeria monocytogenes* DMST17303.

Table 5.3 Antimicrobial activity of cooked non-fermented soybeans and *Thua Nao*

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

Data are mean ± standard deviation (n = 3). - = no inhibition zone. Relative magnitude of inhibition = area of inhibition zone of sample/area of inhibition zone of 80%methanol. CNF1, boiled non-fermented soybeans; CNF2, autoclaved non-fermented soybeans; TNB51, *Thua Nao* was prepared by fermentation of autoclaved soybeans with *B. subtilis* TN51; TNMX, *Thua Nao* was prepared by fermentation of boiled soybeans with naturally occurring microbes.

Table 5.4 Antimicrobial activity of metabolite derived from *Bacillus* sp.

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

5.4 Conclusion

From this study it can be concluded that microbial fermentation of soybeans significantly enhances (21 – 35%) the contents of total phenolic components in *Thua Nao* extracts, which contributes to the antioxidant activity development when compared with non-fermented soybeans. The consistent results have been discussed in the production of *Chungkukjang* (Kwak *et al*., 2007), *Koji* (Lee *et al*., 2007) and *Kinema* (Moktan *et al*., 2008). However, only low amounts of phenolics (2.77 to 3.73%) were involved in soybean sample extracts, therefore other antioxidant components such as tocopherols, amino acids, peptides and melanoidins browning pigment may be expected to exert the antioxidant activity of the products. In addition, *Thua Nao* extracts also possess the antimicrobial activity against Gram-positive pathogenic bacteria that might be relative of their phenolics or other antimicrobial substances. The distinct bioactive components need to be identified in further work.