Chapter 9
Changes in Microbiological and Nutritional Qualities of *Thua Nao* During Shelf-life Storage

9.1 Introduction

Fermentation is one of the oldest food preservation methods and has developed based on the knowledge of native people of many areas. The knowledge and skills of fermentation are propagated by word-of-mouth, and this has led to the cultural advances of the regions. For example, fermented milk is believed to be one of the oldest fermented foods dating back to 7000 B.C., followed by fermented vegetables, meats and cereal-legume (Prajapati and Nair, 2004). Soy-fermented foods, widely consumed by people in several Asian countries, are present in a variety of products such as soy sauce, *Miso*, *Tempeh*, *Natto* for over 1000 years. During fermentation, the fermenting microorganisms can help improve the product quality by releasing organic acids to reduce pH, limiting oxygen condition and producing antibiotic substances to inhibit foodborne pathogens. Besides, these fermenting microbes are also responsible for development of flavours and aromas that are unique to these products.

Thua Nao, a traditional soy-fermented food of Thailand, originated in the Northern part of Thailand with the native people's knowledge for preserving and developing a new product as an ingredient of main local dishes and as a flavour enhancing agent. However, the shelf-life of the product is rather limited with only 2 days at room temperature if stored in its fresh form. In order to extend the shelf-life, a dried form is developed consequently by exposing a tip of mashed *Thua Nao* in sun light and this product is called *Thua Nao Kab* that could be kept for several months. Although a quality of *Thua Nao* is produced by the improved methods developed in this thesis, the spoilage or undesired characteristics could occur under unsuitable storage conditions. The deterioration of foods have been suggested as a result of

biochemical changes during storage, such as growth of undesirable microorganisms and acceleration of the Maillard reaction (Nam and Ahn, 2003; Kolapo *et al.*, 2007; Atrea *et al.*, 2009). These changes lead to an unacceptable sensory sensation of the storage product including darkening of the beans, off-flavour, rancid or putrid smell, appeared of mould or liberated mould odour accompanied by a foreign pigment slimy material on the beans. Besides, nutritional loss is another important factor to affect the shelf-life storage of foods (Garcia-Alonso *et al.*, 2009; Murcia *et al.*, 2009). Therefore, this study aims to preserve the quality of cooked *Thua Nao* by vacuum packing and evaluate their shelf-life based on the biochemical and nutritional qualities changes during storage in refrigerated conditions.

9.2 Materials and methods

9.2.1 Shelf-life storage of *Thua Nao* products

Initially, *Thua Nao* was prepared as described in Section 4.2.2 to 4.2.3. For shelf-life storage study, three treatments were used as follows: i) TNB51, autoclaved soybeans fermented with *Bacillus subtilis* TN51; ii) TNMX, boiled soybeans fermented with naturally occurring microbes and iii) MH, commercial *Thua Nao* product purchased from Mae Hia Market, Chiang Mai, Thailand in February, 2009. Prior to storage, all samples were cooked by steaming for 30 min, and after cooling, 100 g of sample was packed in polyethylene (PE) plastic bags for normal package or in PETNPP plastic bags for vacuum package. The PETNPP film is a three-layered laminate that are outer layer, polyethylene terphthalate (PET) with a thickness of 12 μm, middle layer (nylon with a thickness of 15 μm) and the inner layer, polypropylene with a thickness 70 μm. The PE bags were sealed using an impulse heat-sealing machine (Master Num Charoen, Thailand) and the PETNPP bags were vacuum packaged using a vacuum sealing machine (Packmart Supervac®). The samples were prepared in triplicates for each treatment.

Packaged products were then divided into two groups and stored at 4 and 40°C for 60 days or until they were spoiled indicating by strong dark brown colour, swelling package, putrid smell and other deterioration characteristics. During storage

(0, 5, 10, 20, 40 and 60 days), three packages of these products were randomly selected and determined microbiological and physicochemical qualities.

9.2.2 Determination of physicochemical quality during shelf-life storage

Moisture, pH and surface colour of stored samples were measured as illustrated in Section 3.2.3. Also, the lipid oxidation rancidity of cooked *Thua Nao* products during shelf-life storage was determined by thiobarbituric acid (TBA) value based on the method of Kirk and Sawyer (1991). *Thua Nao* paste (10 g) was crushed with 50 ml of distilled water for 20 min and then washed into a distillation flask with 47.5 ml of distilled water followed by added 2.5 ml of 4 N hydrochloric acid and few glass beads. The sample was heated until 50 ml of distillate was collected. Aliquot 5 ml of this distillate was mixed with 5 ml of 0.2883% (w/v) 2-thiobarbituric acid (Fluka, Germany) solution in 90% glacial acetic acid in glass-stopper tube. The covered reaction tube was shaken well and heated in a boiling water bath for 35 min. A blank tube was prepared similarly instead distillated sample with 5 ml of distilled water. After cooling for 10 min in cold water, the absorbance (*A*) was measured against the blank at 538 nm using a UV-VIS spectrophotometer (Perkin Elmer UV WINLAB version 2.85.04) and the value of TBA rancidity was calculated as milligrams of malondialdehyde per kilogram of dry sample (mg MDA/kg), which equals to 7.8 times *A*.

9.2.3 Analysis of antioxidant quality and free amino acid contents

Contents of phenolics compounds were evaluated by a colourimetric assay using Folin-Ciocalteu's phenol reagent as described in Section 3.2.6. Total antioxidant activity and inhibitory effect of DPPH-radicals were analysed as described in Section 3.2.7 with some modifications. In this case, DPPH-radical scavenging activities of storage *Thua Nao* were expressed as milligram of butylated hydroxytoluene (Sigma, St. Louis, MO) equivalent per gram of dried sample extracts (mg BHTE/g extract). Correlation coefficient (R^2) of 0.99 was obtained in a standard curve of the scavenging activity of BHT on DPPH. The advantage of using a BHT equivalent expression is to reduce the effects of analysis conditions such as the

volume ratio of DPPH and sample or concentration of DPPH. The contents of free amino acids in storage *Thua Nao* samples were also assessed by reversed-phase high performance liquid chromatography (RP-HPLC) after pre-column derivitisation with 9-fluorenylmethyl chloroformate (Fmoc-Cl) (see Section 3.2.4).

9.2.4 Determination of microbiological quality during shelf-life storage

A number of total viable count, spore-forming bacteria and total yeast and mould count were examined as described in Section 3.2.3.

9.2.5 Statistical analysis

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 $P \leq 0.05$.

9.3 Results and discussion

9.3.1 Physicochemical quality during shelf-life storage

In this study, three *Thua Nao* samples including two laboratory produced products, *Thua Nao* produced by pure starter of *B. subtilis* TN51 (TNB51) and natural fermentation (TNMX), as well as a commercial *Thua Nao* (MH) were studied for changes of physicochemical and microbiological qualities during storage under normal and vacuum packaging at 4 and 40°C. In general, we define the spoilage of interval *Thua Nao* packages by using criterion of deterioration characteristics such as strong dark brown or undesirable colour of the beans, swelling of package, accompanied the foreign pigment slimy material on the beans (Figure 9.1) and liberated putrid smell when the package is opened.

The storage temperature was shown to be the major factor affecting the quality of shelf-life cooked *Thua Nao*. Our data showed that refrigerated temperature was able to prolong shelf-life storage of packed *Thua Nao* products up to 40 days (Figure 9.2). But at 40°C, all *Thua Nao* packages were spoiled with the visible

deterioration characteristics as described above after first 5 days of storage and thus did not include in further analysis. For this reason, only data of the refrigerated storage of samples were presented.

Figure 9.1 Appearance deterioration characteristics were considered as spoiled *Thua Nao*. *Thua Nao* spoilage as indicated by strong dark brown colour (A), appearance foreign slimy substance (B), appearance exudates, soft texture and slight swelling of package (C, D), appearance of soft texture and discolouration into green colour (E), and swelling of package (F).

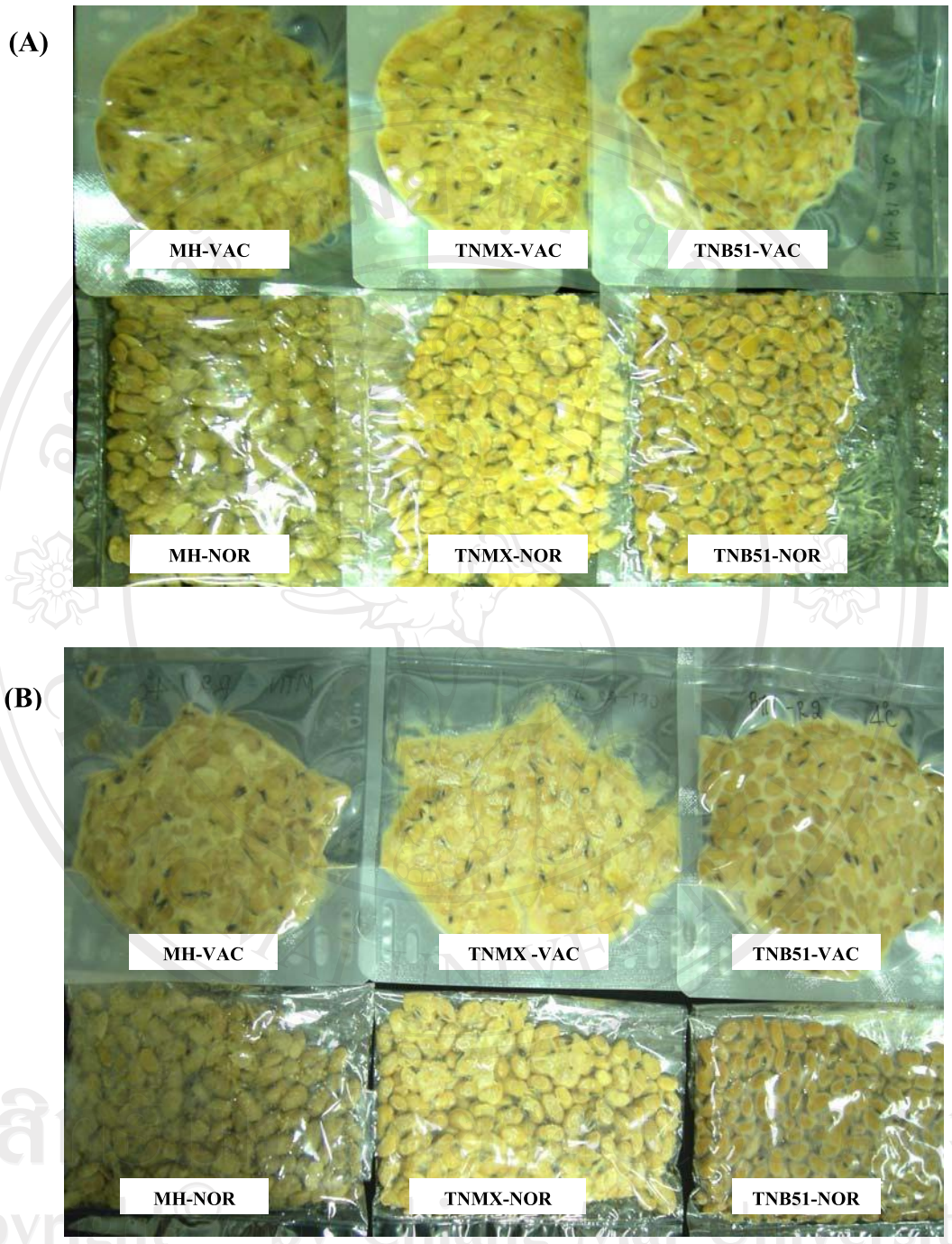

Figure 9.2 Appearance of steamed *Thua Nao* packed in normal (NOR) and vacuum (VAC) conditions at the beginning of the storage (A) and at 40-days storage at 4°C (B). MH, commercial *Thua Nao* purchased from Mae Hia market; TNMX, *Thua Nao* prepared by fermentation of naturally occurring microbes of boiled soybeans; TNB51, *Thua Nao* prepared by fermentation of autoclaved soybeans with pure starter culture of *B. subtilis* TN51.

Changes of moisture, pH and the 2-thiobarbituric acid (TBA) rancidity of the packaged *Thua Nao* products during storage at 4°C for 60 days are shown in Table 9.1. The moisture contents and pH values of *Thua Nao* products were not different in both normal- and vacuum packaged versions of each treatment and remained substantially stable throughout the storage trial. It is noticed that TNB51 and MH products had a close to neutral pH, while the alkaline value was observed in TNMX natural fermented *Thua Nao*. This might be due to the effect of large microbial population involved in the product.

TBA rancidity was used to evaluate the degree of lipid oxidation during storage of *Thua Nao*. This analytical technique is widely used for the assessment of the secondary phases of the lipid oxidation in food. The reaction between thiobarbituric acid and malondialdehyde (MDA), a final product of lipid oxidation, bring about a red chromogen which results from the condensation of two molecules of TBA with one molecule of malondialdehyde, and elimination of two molecules of water (Sinnhuber *et al*., 1958). TBA rancidity is correlated with a characteristic, unpalatable odour and flavour of the product and is considered to be the subjective organoleptic judgment of off-flavour quality of food. Lipid oxidation reactions including oxidation rancidity (interaction between lipid and atmospheric oxygen), hydrolytic rancidity (a hydrolysis of triglycerides in the presence of moisture), and ketonic rancidity (catalytic of enzyme release from food contaminated microorganisms, in the presence of limited account of oxygen and water) each play an important role to produce rancid substances in lipid food. According to Table 9.1, TBA rancidities of all *Thua Nao* were increased during storage at 4°C for 60 days, particularly normal packages of TNMX and TNB51 *Thua Nao*. Whereas in vacuum packages, there was a trend towards an increase in TBA values followed by either a decrease or stable amount. These variations can be explained as the result of the different phases of peroxides decomposition, formation of carbonyl compounds and interaction of malonyldialdehyde with nucleophilic molecules (e.g. proteins and amino acids) resulting in fluctuation or lower amount of free MDA in the product (Fernández *et al*., 1997; Chouliara *et al*., 2004; Cakli *et al*., 2006; Mbarki *et al*., 2009). These observations are in agreement with results reported in stored hot smoked

rainbow trout (Cakli *et al*., 2006) and chub mackerel (Mbarki *et al*., 2009) under vacuum packaging.

Our study clearly indicated that vacuum packaging is more efficient in suppressing lipid oxidation in *Thua Nao* during refrigerated storage than normal packaging, suggesting the most critical factor being the presence of oxygen influencing lipid oxidation. Besides, the greater availability of water (Table 9.1) and microbial activity (Table 9.5) in the packaged samples might be related to the acceleration of lipid oxidation. The effects of these factors have also been suggested in the refrigerated storage of chub mackerel (Mbarki *et al*., 2009) and hake (Ruiz-Capillas and Moral, 2001). Although, under cold storage, optimum packaging and sterilisation could limit the rancidity due to hydrolytic reactions, lipid oxidation is not completely inhibited because autoxidation is a low activation energy reaction (Rossell, 1994). For these reasons, increasing of TBA rancidity still presented in refrigerated *Thua Nao*.

The value of TBA rancidity indicated the quality of food, a good product should not contain TBA rancidity more than 5 mg MDA/kg and consumption limit for TBA value is between 7 and 8 mg MDA/kg (Cakli *et al*., 2006). In this result, shelf-life storage of *Thua Nao* based on the value of TBA rancidity, only TNB51 packed under vacuum conditions could be extended shelf-life for 10 days at 4°C.

Table 9.2 shows the values of L (lightness), a* (redness) and b* (yellowness) for *Thua Nao* in normal and vacuum packaging during storage at 4°C. Although there was a slight change in colour with storage time, the differences between normal and vacuum packaging of each product were mostly not significant. TNMX *Thua Nao* displayed the most initial brightness and yellowness of surface colour until the end storage trial for both normal and vacuum conditions, whereas the reddest was identified as the TNB51 product although this was not significant ($P > 0.05$). This study may suggest that the production process is the most influential factor leading to a darker brown colour of the product.

Table 9.1 Changes in physicochemical quality of *Thua Nao* under normal and vacuum packed conditions during storage at 4°C

Quality	Storage time (day)	Normal-packed			Vacuum-packed		
		TNB51	**TNMX**	**MH**	**TNB51**	**TNMX**	**MH**
Moisture (%)	0	58.57 ± 0.13^{bC}	69.01 ± 0.26^{aA}	61.73 ± 0.38^{aB}	58.57 ± 0.13^{abC}	69.01 ± 0.26^{aA}	61.73 ± 0.38^{aAB}
	5	58.93 ± 0.65^{bC}	69.98 ± 0.20^{aA}	63.48 ± 1.50^{aB}	58.72 ± 0.77^{abC}	68.52 ± 0.67^{abA}	61.70 ± 0.38^{aB}
	10	59.04 ± 0.49^{bC}	69.46 ± 1.56^{aA}	61.85 ± 0.28^{aB}	58.54 ± 0.15^{abC}	68.18 ± 0.25^{abA}	62.21 ± 0.24^{aB}
	20	58.86 ± 0.41^{bC}	68.68 ± 0.33^{aA}	61.97 ± 0.49^{aB}	57.63 ± 0.25^{bC}	67.77 ± 0.32^{cA}	61.56 ± 0.37^{aAB}
	40	59.16 ± 0.47^{abC}	68.62 ± 0.16^{aA}	61.50 ± 0.66^{aB}	58.29 ± 0.53^{abC}	67.87 ± 0.40^{cA}	61.80 ± 0.70^{aB}
	60	60.21 ± 0.2^{a}	-	-	59.00 ± 0.60^{a}	-	-
pH	0	8.07 ± 0.01^{aB}	8.69 ± 0.05^{aA}	7.74 ± 0.01^{aC}	8.07 ± 0.01^{aB}	8.69 ± 0.05^{aA}	7.74 ± 0.01^{cC}
	5	7.80 ± 0.00^{bB}	8.49 ± 0.09^{abA}	7.47 ± 0.01^{bC}	7.72 ± 0.04^{bcB}	8.29 ± 0.06^{bA}	7.41 ± 0.01^{cC}
	10	7.67 ± 0.00^{cB}	8.28 ± 0.07^{bcA}	7.44 ± 0.03^{bC}	7.75 ± 0.09^{bB}	8.25 ± 0.09^{bA}	7.39 ± 0.00^{aC}
	20	7.72 ± 0.04^{cB}	8.35 ± 0.08^{bcA}	7.46 ± 0.02^{bC}	7.79 ± 0.00^{bB}	8.33 ± 0.04^{bA}	7.45 ± 0.00^{aC}
	40	7.61 ± 0.04^{dB}	8.23 ± 0.11^{cA}	7.36 ± 0.02^{cC}	7.70 ± 0.04^{bcB}	8.35 ± 0.03^{bA}	7.35 ± 0.01^{bC}
	60	7.49 ± 0.01^{e}	-	-	7.63 ± 0.04^{c}	-	-
TBA test	0	2.94 ± 0.09^{eB}	3.42 ± 0.08^{cB}	5.04 ± 0.61^{bA}	2.94 ± 0.09^{bB}	3.42 ± 0.08^{cB}	5.04 ± 0.61^{cA}
(mg MDA/kg)	5	9.46 ± 4.64^{dB}	33.36 ± 7.21^{cA}	9.80 ± 2.18^{bB}	3.05 ± 0.24^{bB}	7.77 ± 0.37^{bcAB}	10.63 ± 2.81^{cA}
	10	32.62 ± 13.14^{cB}	74.12 ± 8.60^{bA}	43.52 ± 2.52^{aB}	12.54 ± 2.95^{aB}	18.26 ± 0.53^{aB}	55.49 ± 3.97^{aA}
	20	55.05 ± 6.66^{bB}	98.36 ± 14.92^{abA}	37.35 ± 7.78^{aB}	12.52 ± 1.79^{aB}	21.78 ± 5.07^{aB}	57.46 ± 2.01^{aA}
	40	66.44 ± 3.96^{aB}	129.37 ± 21.34^{aA}	36.45 ± 6.56^{aB}	12.29 ± 3.27^{aB}	10.23 ± 0.51^{bB}	35.49 ± 6.56^{bA}
	60	67.66 ± 1.73^{a}	-	-	12.15 ± 3.59^{a}	-	-

Data are mean ± standard deviation (n = 3). Means within same column with different superscripts of small letter are significantly different ($P \leq 0.05$) in interval samples and that with different superscripts of capital letter are significantly different ($P \leq 0.05$) in type of products. (-), not determined due to spoilage; TNB51, *Thua Nao* prepared by fermentation of autoclaved soybeans with pure starter culture of *B. subtilis* TN51; TNMX, *Thua Nao* prepared by fermentation of boiled soybeans with naturally occurring microbes; MH, commercial *Thua Nao* purchased from Mae Hia market.

Table 9.2 Changes of colour of *Thua Nao* under normal and vacuum packed conditions during storage at 4°C

Quality	Storage time (day)	Normal-packed			Vacuum-packed		
		TNB51	**TNMX**	**MH**	**TNB51**	**TNMX**	**MH**
Colour L	0	43.65 ± 1.86^{aB}	52.98 ± 0.40^{bA}	50.53 ± 4.35^{aAB}	43.65 ± 1.86^{bB}	52.98 ± 0.40^{cA}	50.53 ± 4.35^{aAB}
	5	44.30 ± 0.90^{aB}	55.93 ± 2.01^{abA}	45.73 ± 0.57^{abB}	45.91 ± 1.03^{abB}	56.91 ± 1.96^{aA}	48.27 ± 1.99^{aB}
	10	43.88 ± 1.15^{aB}	57.35 ± 1.31^{aA}	45.04 ± 0.01^{bB}	42.87 ± 1.47^{bC}	54.45 ± 0.27^{abA}	47.24 ± 1.29^{aB}
	20	43.63 ± 2.09^{aB}	55.56 ± 1.11^{abA}	45.81 ± 0.25^{abB}	45.57 ± 1.56^{abB}	53.70 ± 0.89^{cA}	48.43 ± 3.17^{aAB}
	40	45.84 ± 0.89^{aB}	56.00 ± 1.44^{abA}	45.34 ± 0.52^{abB}	47.05 ± 0.41^{aB}	52.09 ± 0.43^{cA}	47.09 ± 0.63^{aB}
	60	46.12 ± 0.84^{a}	-	-	44.69 ± 0.08^{ab}	-	-
Colour a*	0	8.03 ± 0.34^{aA}	6.97 ± 0.14^{aAB}	6.43 ± 0.75^{bB}	8.03 ± 0.34^{aA}	6.97 ± 0.14^{aAB}	6.43 ± 0.75^{bB}
	5	7.48 ± 0.40^{aA}	6.50 ± 0.44^{aAB}	6.10 ± 0.04^{bB}	6.92 ± 1.10^{aA}	5.76 ± 0.05^{bA}	6.69 ± 0.05^{abA}
	10	7.70 ± 0.49^{aA}	6.90 ± 0.08^{aAB}	6.69 ± 0.15^{abB}	8.00 ± 0.56^{aA}	6.93 ± 0.25^{aA}	7.85 ± 0.41^{aA}
	20	8.44 ± 0.05^{aA}	7.11 ± 0.94^{aA}	7.61 ± 0.51^{aA}	7.35 ± 0.81^{aA}	6.78 ± 0.21^{aA}	6.70 ± 0.79^{abA}
	40	8.00 ± 0.54^{aA}	6.77 ± 0.17^{aB}	6.31 ± 0.27^{bB}	6.89 ± 0.79^{aA}	6.52 ± 0.14^{aA}	6.07 ± 0.14^{bA}
	60	8.85 ± 1.04^{a}	-	-	7.71 ± 0.32^{a}	-	-
Colour b*	0	14.25 ± 0.59^{bcC}	21.50 ± 0.84^{aA}	16.89 ± 0.38^{aB}	14.25 ± 0.59^{aC}	21.50 ± 0.84^{abA}	16.89 ± 0.38^{aB}
	5	11.91 ± 0.14^{dB}	20.35 ± 3.98^{aA}	12.84 ± 0.22^{dB}	14.25 ± 1.91^{aB}	22.74 ± 1.51^{aA}	15.79 ± 1.86^{aB}
	10	13.41 ± 0.65^{bcdB}	22.04 ± 2.10^{aA}	12.65 ± 0.33^{dB}	13.19 ± 1.55^{aB}	20.70 ± 0.45^{abA}	18.56 ± 1.82^{aA}
	20	15.15 ± 0.71^{bB}	21.83 ± 2.96^{aA}	15.82 ± 0.19^{bB}	14.55 ± 0.35^{aC}	21.58 ± 0.21^{abA}	16.78 ± 0.37^{aB}
	40	13.15 ± 1.33^{cdB}	22.45 ± 0.90^{aA}	14.01 ± 0.67^{cB}	14.61 ± 3.54^{aA}	19.57 ± 0.53^{bA}	15.37 ± 2.04^{aA}
	60	17.10 ± 0.39^{a}	-	-	15.34 ± 0.89^{a}	-	-

Data are mean ± standard deviation (n = 3). Means within same column with different superscripts of small letter are significantly different ($P \leq 0.05$) in interval samples and that with different superscripts of capital letter are significantly different ($P \leq 0.05$) in type of products. (-), not determined due to spoilage; TNB51, *Thua Nao* prepared by fermentation of autoclaved soybeans with pure starter culture of *B. subtilis* TN51; TNMX, *Thua Nao* prepared by fermentation of boiled soybeans with naturally occurring microbes; MH, commercial *Thua Nao* purchased from Mae Hia market.

9.3.2 Phenolic content, antioxidant activity and free amino acid profiles

After shelf-life storage at 4°C for 40 days, all packaging of TNMX and MH products were spoiled and discarded from further analysis due to visual deterioration characteristics. Therefore, only refrigerated shelf-life storage of cooked TNB51 *Thua Nao* packages were evaluated for the nutritive values like antioxidant activity, phenolic compounds and free amino acids at the start and the completion of 60 days of shelf-life storage.

Table 9.3 shows the changes in the total phenolic compounds, inhibition DPPH radical power and total antioxidant activity of TNB51 *Thua Nao* on the initial and the final day of storage in the refrigerator. Steamed TNB51 *Thua Nao* extract showed improved content of phenolics (10%) and DPPH-radical scavenger effect (29%) while it presented great loss in inhibition of linoleic acid oxidation (62%) with respect to fresh product which has been reported in Table 5.2. This observation might be due to accelerated release of free phenolic compounds by the effect of the steaming process of cooking *Thua Nao*.

Table 9.3 Changes in antioxidant activities and phenolic contents of TNB51 *Thua Nao* under normal and vacuum packed conditions storage at 4°C

Quality	**Before storage**	**After storage for 60 days**	
		NOR	**VAC**
Anti-DPPH radical effect (mg BHTE/g extract)	7.17±0.41^{a}	1.19±0.24^{c}	5.87±0.39^{b}
Total antioxidant activity (%)	23.45±0.06^{ab}	13.04±0.23^{b}	34.44±6.18^{a}
Total phenolics (mg GAE/g extract)	38.98±2.56^{a}	22.90±4.26^{b}	36.80±3.31^{a}

Data are mean ± standard deviation (n = 3). Means in the same row with different small letters are significantly different ($P \leq 0.05$). Total antioxidant activity measured at 10 mg/ml of dried sample extract. NOR, normal package of the product; VAC, vacuum package of the product.

This study clearly indicated that vacuum packaging was more effective to maintain the antioxidant quality of *Thua Nao* during shelf-life storage at 4°C for 60 days. Only a slight decrease of DPPH radical scavenging effect (18%) and phenolic compounds (6%) were found in refrigerated vacuum-packaged product with respect to initially, besides the activity against linoleic acid oxidation was improved by 47% under this condition. Whereas in normal condition, the great losses of 44% of total phenolic content, 83% of inhibition DPPH radicals and 41% of total antioxidant were observed. This result could be explained by a potential for still higher phenolic content (as can be seen in Table 9.3) and might be the result of exerted antioxidant capacity from other antioxidant components apart from phenolics which existed in the vacuum-packed product. These results are in agreement with the finding of Murcia *et al*. (2009), who demonstrated that a loss of radical scavenging capacity after stored cucumber (24%) and zucchini (34%) for 7 days at refrigerated temperature. Besides this, a loss of total phenolic component with the affect of temperature and time of storage has been recorded in stored blueberry extract. The greater affect on phenolic compounds by storage factors such as temperature and atmosphere than other antioxidant phytochemicals has been suggested (Klimczak *et al*., 2007).

According to Table 9.4, at the start of shelf-life storage, Phe, Leu and Glu were the most dominant amino acids in cooked *Thua Nao* with a proportion of 20, 13 and 10%, respectively and this observation still remained in the final product after storage. The bitter tasting free amino acids (FAA) which related to the contents of hydrophobic and apolar FAA were a major component in all of stored products. After shelf-life storage, 31% of total FAA were reduced with the most loss being of Arg in both normal (69%) and vacuum (68%) packages, probably due to its facile utilization by *B. subtilis* as a nitrogen source for their growth (Teng *et al*., 2004). The packaging condition did not affect to free amino acids content in present study; a similar content of each amino acid was found in both types of package.

Table 9.4 Changes in free amino acids of TNB51 *Thua Nao* under normal and vacuum packed conditions storage at 4°C

FAA	Before storage	After storage for 60 days	
		NOR	VAC
Asp	0.26 ± 0.00[a]	0.18 ± 0.02[a]	0.21 ± 0.08[a]
Ala	0.66 ± 0.01[a]	0.47 ± 0.08[ab]	0.44 ± 0.06[b]
Arg	0.35 ± 0.05[a]	0.11 ± 0.04[b]	0.11 ± 0.05[b]
Asn	0.16 ± 0.01[a]	0.06 ± 0.03[b]	0.11 ± 0.01[b]
Cys	0.86 ± 0.04[a]	0.59 ± 0.14[ab]	0.49 ± 0.05[b]
Glu	1.57 ± 0.13[a]	1.22 ± 0.13[a]	1.27 ± 0.30[a]
Gly+His+Thr	2.12 ± 0.17[a]	1.36 ± 0.30[b]	1.46 ± 0.17[ab]
Ile	0.87 ± 0.08[a]	0.57 ± 0.04[b]	0.53 ± 0.04[b]
Leu	1.97 ± 0.06[a]	1.35 ± 0.15[b]	1.30 ± 0.07[b]
Phe	3.12 ± 0.19[a]	2.26 ± 0.02[b]	2.30 ± 0.13[b]
Pro+Tyr	2.66 ± 0.04[a]	2.01 ± 0.23[b]	1.89 ± 0.04[b]
Ser	0.26 ± 0.01[a]	0.14 ± 0.01[b]	0.18 ± 0.04[b]
Val	0.80 ± 0.07[a]	0.58 ± 0.02[b]	0.56 ± 0.00[b]
Total FAA	15.68 ± 0.75[a]	10.90 ± 0.75[b]	10.84 ± 023[b]
EAA	7.12 ± 0.46[a]	4.87 ± 0.15[b]	4.80 ± 0.03[b]
MSG-like FAA	1.84 ± 0.13[a]	1.40 ± 0.14[a]	1.48 ± 0.37[a]
Sweet FAA	0.92 ± 0.02[a]	0.61 ± 0.09[b]	0.62 ± 0.03[b]
Bitter FAA	7.12 ± 0.46[a]	4.87 ± 0.15[b]	4.80 ± 0.03[b]
Tasteless FAA	0.86 ± 0.04[a]	0.59 ± 0.14[ab]	0.49 ± 0.05[b]
Basic FAA	0.35 ± 0.05[a]	0.11 ± 0.04[b]	0.11 ± 0.05[b]
Acidic FAA	2.00 ± 0.14[a]	1.46 ± 0.17[a]	1.58 ± 0.38[a]
Total charge FAA	2.35 ± 0.19[a]	1.57 ± 0.21[a]	1.69 ± 0.33[a]
Hydrophilic	2.61 ± 0.18[a]	1.71 ± 0.20[b]	1.88 ± 0.30[ab]
Hydrophobic	6.77 ± 0.41[a]	4.77 ± 0.19[b]	4.68 ± 0.02[b]
Apolar	6.77 ± 0.41[a]	4.77 ± 0.19[b]	4.68 ± 0.02[b]

Data are mean ± standard deviation (n = 3) and expressed in the unit of g/kg dry basis. Means in the same row with different small letters are significantly different ($P \leq 0.05$). FAA, free amino acids; NOR, normal package of the product; VAC, vacuum package of the product. EAA, essential amino acid = Val+Ile+Leu+Phe+Arg; MSG-like FAA = Asp+Glu; Bitter FAA = Arg+Ile+Leu+Phe+Val; Tasteless FAA = Cys; Basic = Arg; Acidic = Asp+Glu; Total charge = basic+acidic; Hydrophilic = total charge+Ser; Hydrophobic = Val+Leu+Ile+Phe; Apolar = hydrophobic.

9.3.3 Microbiological quality during shelf-life storage

Changes in microbial flora of packaged *Thua Nao* during storage at 4°C are shown in Table 9.5. In general, total viable count (TVC) and spore count (SPC) did not give differences during storage of all *Thua Nao* products under normal and vacuum conditions. Among the *Thua Nao* products, significantly higher contents of TVC and SPC ($P \leq 0.05$) were observed in naturally fermented products (TNMX and MH) than found in TNB51 throughout the storage trial. It might be expected dueto contamination with of variety microorganisms other than the *Bacillus* strain. Some strains of spoilage and foodborne pathogenic bacteria such as *Clostridium botulinum*, *Pseudomonas*, *Staphylococcus aureus*, and coliform bacteria have been identified during prolonged refrigeration of vacuum packaged food products (Chouliara *et al.*, 2004; Mbarki *et al.*, 2009). Besides, it clearly shows that the predominant bacteria of *Thua Nao* products during storage are endospore-forming bacteria. Yeast and mould counts did not form a major part of the widespread micro-flora during storage both under normal and vacuum conditions (< 1.4 log CFU/g). With the storage, the limited oxygen supply through the packaging suppressed the growth of yeasts and moulds which require oxygen for viable activity (Abe and Kondoh, 1989).

Table 9.5 Changes in microorganisms of *Thua Nao* under normal and vacuum packed conditions during storage at 4°C

Quality	Storage time (day)	Normal-packed			Vacuum-packed		
		TNB51	TNMX	MH	TNB51	TNMX	MH
Total viable count (log CFU/g)	0	8.36 ± 0.42^{aB}	9.42 ± 0.04^{cA}	9.28 ± 0.04^{bA}	8.36 ± 0.42^{aB}	9.42 ± 0.04^{cA}	9.28 ± 0.04^{aA}
	5	7.00 ± 0.43^{bB}	9.66 ± 0.07^{abA}	9.39 ± 0.03^{abA}	7.36 ± 0.58^{aB}	9.52 ± 0.03^{bA}	9.45 ± 0.73^{aA}
	10	7.95 ± 0.07^{abC}	9.39 ± 0.15^{cA}	8.93 ± 0.06^{cB}	7.94 ± 0.26^{aB}	9.47 ± 0.03^{bcA}	9.07 ± 0.08^{aA}
	20	7.82 ± 0.19^{abB}	9.49 ± 0.01^{bcA}	9.35 ± 0.07^{abA}	7.98 ± 0.10^{aB}	9.48 ± 0.05^{bcA}	9.25 ± 0.27^{aA}
	40	7.98 ± 0.17^{abB}	9.71 ± 0.01^{aA}	9.49 ± 0.07^{aA}	8.02 ± 0.56^{aB}	9.66 ± 0.00^{aA}	9.45 ± 0.09^{aA}
	60	7.95 ± 0.90^{ab}	-	-	8.07 ± 0.27^{aB}	-	-
Spore count (log CFU/g)	0	8.12 ± 0.58^{aB}	9.38 ± 0.08^{aA}	9.26 ± 0.02^{abA}	8.12 ± 0.26^{aB}	9.38 ± 0.08^{bA}	9.26 ± 0.02^{bcA}
	5	7.89 ± 0.34^{aC}	9.65 ± 0.00^{aA}	8.85 ± 0.21^{cB}	7.88 ± 0.54^{aC}	9.65 ± 0.02^{aA}	9.09 ± 0.01^{dB}
	10	7.75 ± 0.81^{aB}	9.62 ± 0.17^{aA}	9.12 ± 0.03^{bAB}	7.74 ± 0.24^{aB}	9.59 ± 0.08^{abA}	9.14 ± 0.11^{cdA}
	20	7.06 ± 0.73^{aB}	9.52 ± 0.12^{aA}	9.26 ± 0.06^{abA}	7.31 ± 0.15^{aB}	9.60 ± 0.18^{abA}	9.38 ± 0.04^{bA}
	40	8.15 ± 0.57^{aB}	9.63 ± 0.10^{aA}	9.42 ± 0.03^{aA}	7.74 ± 0.80^{aB}	9.68 ± 0.04^{aA}	9.58 ± 0.06^{aA}
	60	7.71 ± 0.94^{a}	-	-	7.75 ± 0.83^{a}	-	-

Data are mean ± standard deviation (n = 3). Means within same column with different superscripts of small letter are significantly different ($P \leq 0.05$) in interval samples and mean within same column with different superscripts of capital letter are significantly different ($P \leq 0.05$) in products of each package condition. (-), not determined due to spoilage; TNB51, *Thua Nao* prepared by fermentation of autoclaved soybeans with pure starter culture of *B. subtilis* TN51; TNMX, *Thua Nao* prepared by fermentation of boiled soybeans with naturally occurring microbes; MH, commercial *Thua Nao* purchased from Mae Hia market.

9.4 Conclusion

This study clearly indicated that storage temperature strongly affected the visible deterioration characteristics of *Thua Nao*. High temperature storage conditions did not maintain a desirable quality of the product. At 4°C, based on visual quality, the shelf-life of *Thua Nao* are increased at least 1,900% for normal and vacuum packages when compared with the traditional storage method (2 days of shelf-life storage for wrapping banana leaves at ambient temperature) (Sundhagul *et al*., 1972). In addition, according to the limit of acceptability (5 mg MDA/kg) in terms of TBA rancidity, the shelf-life of vacuum-packed TNB51 could be extended by 400%. Vacuum packaging of *Thua Nao* was more effective to suppress TBA rancidity formation and distinctly preserved nutritional values based on antioxidant quality and free amino acids content during shelf-life storage at 4°C. Besides storage conditions, the fermentation process of *Thua Nao* also affected the shelf-life storage of the product. *Thua Nao* produced by starter *B. subtilis* TN51 had longer shelf-life with respect to naturally fermented products. This study suggested that vacuum packaging and refrigerated temperature are optimum to preserve the quality and bioactive compounds of *Thua Nao* products.