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Changes in Biochemical and Nutritional Qualities of Aerobic and Vacuum-packaged Thua Nao During Shelf-life Storage

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Abstract: Thai traditionally fermented soybean (Thua Nao) has been suggested as a good source of available amino acids and aglycone isoflavones. The objective of this research was to investigate the changes of biochemical and nutritional qualities in aerobic- and vacuum-packed thua Nao during the storage at 4 and 40°C for 60 days. Three Thua Nao samples including *Bacillus subtilis* TN51-fermented Thua Nao (TNB51), spontaneously fermented Thua Nao (TNMX) and commercial product (MH) were used in this study. It was found that the storage of packed Thua Nao at 4°C could prolong the product shelf-life up to 40 days. The moisture contents, pH values and colour L a* b* of these products were not different in both aerobic and vacuum-packaged products and remained stable throughout the experiment. The Thiobarbituric Acid (TBA) values of all storage Thua Nao were increased during storage; this is in particular for the aerobic packages of the TNMX and TNB51 products, indicating high oxidation of lipids. There was a slight decrease in DPPH radical scavenging effect (18%) and phenolic compounds (6%) of the vacuum-packaged product when stored at 4°C. In contrast, the great losses in total phenolic content (44%), inhibitory activity of DPPH radicals (83%) and total antioxidant (41%) were observed when the products were stored aerobically at 40°C. A reduction in total free amino acids was also found with the highest decrease of Arg in both aerobic- (69%) and vacuum-packages (68%).

Key words: Soybean, fermented soybean, Thua Nao, storage, shelf-life

INTRODUCTION

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 food product which is mainly used as an ingredient and/or a flavour enhancer in various local dishes. Many investigators have showed that Thua Nao's quality can be improved by inoculation of soybean cultivars with selected starter organisms in combination with fermentation under controlled conditions. For example, Chantawannakul *et al.* (2002) and Visessanguan *et al.* (2005) have demonstrated the fermentations of soybeans can be accelerated by using a pure starter culture of *B. subtilis* exhibiting strong proteolytic activity. Besides, Tangitjaroenkun *et al.* (2004) reported that, using a mixed culture between *Bacillus* sp. B4 and *Klebsiella* sp. KB2 isolated from the

local product, an increase of vitamin B12 could be detected. Recently, present study also showed that *Bacillus subtilis* TN51 previously isolated by Dajanta *et al.* (2009a) could be used as an inoculum in improving the quality of Thua Nao's product (Dajanta *et al.*, 2009a, b). However, the shelf-life of Thua Nao product is rather limited with only 2 days at room temperature when stored in its native form.

Food deterioration occurs as a result of biochemical changes in food during storage; this event can also be accelerated by a growth of undesirable microorganisms. Such changes lead to consumer's unacceptability which can be seen by the changes of the product's appearance including dark colour, off-flavour and rancid or putrid smells. In addition, nutrition loss is also affected when the food storage is inappropriately carried out (Garcia-Alonso *et al.*, 2009; Murcia *et al.*, 2009). Therefore, this study aims to investigate the quality of

Thua Nao product under aerobic- and vacuum-storage. The product shelf-life was also determined based on biochemical and nutritional qualities changes during storage at 4°C.

MATERIALS AND METHODS

Preparation of Thua Nao: Thua Nao was prepared in accordance with the procedures described by Dajanta *et al.* (2009a, b). Briefly, soybeans were washed and soaked in tap water for 16 h, decanted and cooked by autoclaving (121°C, 40 min). *Bacillus*-fermented Thua Nao (TNB51) was subsequently prepared by inoculating the steamed soybeans with 10⁴ CFU g⁻¹ of *B. subtilis* strain TN51 and incubating at 42°C for 72 h. For control product (TNMX), soybeans were boiled for 4 h and allowed to spontaneously ferment by naturally occurring microbes under the same condition.

Storage of Thua Nao products: Three thua Nao samples including TNB51, TNMX and commercial product (MH) which purchased from Mae Hia Market (Chiang Mai, Thailand), were used in this study. Prior to storage, all samples were steamed for 30 min. After cooling, 100 g of sample was packed in Polyethylene (PE) plastic bags for aerobic-package or in PETNPP plastic bags for vacuum-package. The PETNPP film is a three-layered laminate consisting of the outer layer (Polyethylene Terphthalate (PET) with a thickness of 12 µm), the 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.

The packaged products were then divided into two groups and stored at 4 and 40°C for 60 days or until they were spoiled as indicated by strong dark brown colour, swelling package, putrid smell and other deterioration characteristics. During the storage, three packages of these products were randomly selected on day 0, 5, 10, 20, 40 and 60 to determine their physicochemical, nutritional and microbiological qualities.

Determination of physicochemical quality: Moisture content of the product was determined using the standard AOAC methods (AOAC, 2000). For pH value, approximately 5 g of fermented soybeans were homogenised in a blender with 50 mL of distilled water for 15 sec 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 L a* b* system by colourimeter Minolta Data Processor DP-301 (Chroma Meter CR-300 Series, Japan). The lipid oxidation rancidity of Thua Nao products was determined by Thiobarbituric Acid (TBA) value using the method of Kirk and Sawyer (1991). The TBA value was calculated as milligrams of malondialdehyde per kilogram of dry sample (mg MDA kg⁻¹).

Determination of microbiological quality: A number of total viable count, spore-forming bacteria and total yeast and mould count were examined. Fermented soybeans (5 g) were homogenised with 45 mL of sterile peptone water (1 g L⁻¹) by stomaching for 2 min. Serial dilutions were prepared in peptone water (1 g L⁻¹) and 1 mL of appropriate dilutions were poured in duplicate plates of plate count agar for viable counts of aerobic mesophilic bacteria and yeast malt extract agar (pH 3.5) for yeasts and moulds. Spore counts were also determined with plate count agar using the suspensions heated at 85°C for 20 min. Cultures were then incubated at 37°C for 2 days (plate count agar for bacteria), 25°C for 3 - 5 days (yeast malt extract agar for yeasts and fungi). The colonies were counted and expressed as logarithmic colony forming units per gram (log CFU g⁻¹) of sample.

Analysis of antioxidant quality: Based on the procedure of Lee *et al.* (2007), 30 g of Thua Nao samples were ground to powder and extracted with 300 mL of 80% methanol for 24 h at room temperature with continuous shaking. The extracts were filtered through Whatman No. 1 paper, concentrated under vacuum (Büchi Rotavapor R-200, Switzerland) at 40°C and freeze-dried (LABCONCO, FREEZONE 4.5, USA).

Total phenolics were then analysed using the protocol of Lin *et al.* (2006). The methanolic extract solution (0.1 mL) was added to a mixture of 1.9 mL of deionised water and 1 mL of Folin-Ciocalteu phenol reagent (Sigma-Aldrich Co., St. Louis, MO, USA). After 8 min incubation, 5 mL of sodium carbonate (20 g 100 mL⁻¹ (w/v)) were added and this mixture was then heated for 1 min. The absorbance was measured at 750 nm by a spectrophotometer (Perkin Elmer UV WINLAB, USA). Quantification of the total phenolics was performed using the linear regression equation of the gallic acid (Sigma-Aldrich Co., St. Louis, MO, USA) standard curve and expressed as gallic acid equivalents (GAE).

Free radical scavenging activity was also determined using the stable free radical 2,2-diphenyl-picrylhydrazyl (DPPH) (Fluka Biochemica, Buchs, Switzerland) method (Yun, 2005). One milliliter of methanolic lyophilised

extracts was added to 2 mL of methanolic solution of DPPH (75 $\mu\text{mol L}^{-1}$). The mixture was shaken and allowed to stand in the dark at room temperature for 5 min. The decrease in absorbance at 517 nm (Perkin Elmer UV WINLAB, USA) was then measured against methanol. Butylated hydroxytoluene (BHT) (Sigma, St. Louis, MO) was used as a standard with a correlation coefficient (R^2) of 0.99 in a standard curve of the scavenging activity. The results of inhibitory effect of DPPH-radicals were expressed as butylated hydroxytoluene equivalent per gram of dried sample extracts (mg BHT g^{-1} extract).

The total antioxidant activity of Thua Nao extracts was determined by using the β -carotene linoleic acid model system (Shon *et al.*, 2007). A solution of β -carotene was prepared by dissolving 2 mg of β -carotene (Fluka, Spain) in 10 mL of chloroform (Labscan, Dublin, Ireland). Two milliliters of this solution were transferred into a 100 mL round-bottom flask. After the chloroform was removed under vacuum, 40 mg of purified linoleic acid (Sigma-Aldrich Co., St. Louis, MO, USA), 400 mg of Tween 40 emulsifier (Fluka, Spain) and 100 mL of aerated distilled water were added to the flask with vigorous shaking. This emulsion (4.8 mL) was added into 0.2 mL of 10 mg mL^{-1} sample extract. For control, 80% methanol was used in the reaction instead of the sample extracts. The mixture was then shaken and stored at 50°C for 2 h. The absorbance of the samples was measured at 470 nm (Perkin Elmer UV WINLAB, USA) against emulsion without β -carotene (blank) at the beginning (0 min) and at the end of the experiment (120 min). Antioxidant activity was then calculated using the following equation:

$$\% \text{ Total antioxidant activity} = \frac{1 - A_{s_0} - A_{s_{120}}}{A_{c_0} - A_{c_{120}}} \times 100$$

where, A_{s_0} and $A_{s_{120}}$ were absorbance of the sample at 0 and 120 min and A_{c_0} and $A_{c_{120}}$ were absorbance of the control at 0 and 120 min.

Analysis of free amino acids: The contents of free amino acids were assessed by reversed-phase high performance liquid chromatography (RP-HPLC) after pre-column derivitisation with 9-fluorenylmethyl chloroformate (Fmoc-Cl) according to the procedures described by Dajanta *et al.* (2011a, b).

Statistical analysis: Data were expressed as Means \pm SD 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 $p < 0.05$.

RESULTS AND DISCUSSION

Physicochemical quality of storage Thua Nao: In this study, three Thua Nao samples: (i) TNB51 prepared by a pure starter of *B. subtilis* TN51, (ii) TNMX prepared by natural fermentation and (iii) MH (a commercial Thua Nao from local market), were studied for changes of physicochemical and microbiological qualities during storage under aerobic and vacuum-packaging at 4 and 40°C. In general, we define the spoilage of Thua Nao products by using criterion of deteriorated characteristics such as strong dark brown or undesirable colour of the beans, swelling of package, accompanied the foreign pigment slimy material on the beans (Fig. 1) and liberated putrid smell when the package is opened.

The storage temperature was shown to be the major factor affecting the shelf-life quality of the products. Present data revealed that the storage of packed Thua Nao products at 4°C could prolong their shelf-life up to 40 days (Fig. 2). In contrast, all Thua Nao packages were spoiled with visible deteriorated characteristics when stored at 40°C for just 5 days; these samples were therefore not taken into an account for further analysis. As a result, only the data of the packages stored at 4°C were presented.

Changes of moisture, pH and the 2-thiobarbituric acid (TBA) value of the Thua Nao products stored at 4°C for 60 days are shown in Table 1. The moisture contents and pH values of Thua Nao products were similar for both aerobic- and vacuum-packaged treatments and remained stable throughout the trial. It should be noted that the TNB51 and MH products had neutral pH (7.35-8.07), while the alkaline value was observed in the TNMX product (8.23-8.69). This might be due to the effect of large microbial population involved in the product.

The TBA value used to evaluate the degree of lipid oxidation is widely used for the assessment of the secondary phases of the lipid oxidation in food. In this study, the TBA value appears to be well-correlated with a characteristic of unpalatable odour and flavour of the product and is considered to be the subjective organoleptic judgment of off-flavour quality of food. According to Table 1, the TBA values of all samples tended to increase during storage at 4°C for 60 days; this is in particular for the aerobic-packages of TNMX (from 3.42 to 129.37 mg MDA kg^{-1}) and TNB51 (from 2.94 to 67.66 mg MDA kg^{-1}). In contrast, the TBA values of the vacuum-packages reached their peak on day 10 or 20 (12.54, 21.78 and 57.46 mg MDA kg^{-1} for the TNB51, TNMX and MH samples, respectively) and then became stable. These variations can be explained as the result of the different phases of peroxides decomposition,



Fig. 1(a-f): Appearance deterioration characteristics were considered as spoiled Thua Nao: 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) Swelling of package and (f)

Table 1: Changes in physicochemical quality of Thua Nao under aerobic- and vacuum-packed conditions during storage at 4°C

Quality	Storage time (day)	Aerobic-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 ^{abB}
	5	58.93±0.65 ^{bc}	69.98±0.20 ^{aA}	63.48±1.50 ^b	58.72±0.77 ^{abc}	68.52±0.67 ^{abA}	61.70±0.38 ^{abB}
	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 ^{abB}
	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 ^{aA}	61.56±0.37 ^{abB}
	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 ^{aA}	61.80±0.70 ^{abB}
	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 ^{ab}	8.49±0.09 ^{abA}	7.47±0.01 ^{bc}	7.72±0.04 ^{bcB}	8.29±0.06 ^{aA}	7.41±0.01 ^{cC}
	10	7.67±0.00 ^b	8.28±0.07 ^{bcA}	7.44±0.03 ^{bc}	7.75±0.09 ^{abB}	8.25±0.09 ^{abA}	7.39±0.00 ^{cC}
	20	7.72±0.04 ^{cb}	8.35±0.08 ^{bcA}	7.46±0.02 ^{bc}	7.79±0.00 ^{ab}	8.33±0.04 ^{aA}	7.45±0.00 ^{cC}
	40	7.61±0.04 ^{db}	8.23±0.11 ^{cA}	7.36±0.02 ^c	7.70±0.04 ^{bcB}	8.35±0.03 ^{aA}	7.35±0.01 ^{bc}
	60	7.49±0.01 ^e	-	-	7.63±0.04 ^f	-	-
TBA test (mg MDA kg ⁻¹)	0	2.94±0.09 ^B	3.42±0.08 ^B	5.04±0.61 ^{ba}	2.94±0.09 ^B	3.42±0.08 ^B	5.04±0.61 ^{aA}
	5	9.46±4.64 ^{dB}	33.36±7.21 ^{cA}	9.80±2.18 ^B	3.05±0.24 ^B	7.77±0.37 ^{bcAB}	10.63±2.81 ^{aA}
	10	32.62±13.14 ^{EB}	74.12±8.60 ^{baA}	43.52±2.52 ^B	12.54±2.95 ^{ab}	18.26±0.53 ^{ab}	55.49±3.97 ^{aA}
	20	55.05±6.66 ^{cb}	98.36±14.92 ^{abA}	37.35±7.78 ^B	12.52±1.79 ^{ab}	21.78±5.07 ^B	57.46±2.01 ^{aA}
	40	66.44±3.96 ^{dB}	129.37±21.34 ^{aA}	36.45±6.56 ^B	12.29±3.27 ^B	10.23±0.51 ^{bb}	35.49±6.56 ^{aA}
	60	67.66±1.73 ^a	-	-	12.15±3.59 ^a	-	-

Data are Mean±SD (n = 3), Means within same column with different superscripts of small letter are significantly different (p<0.05) in interval samples and that with different superscripts of capital letter are significantly different (p<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



Fig. 2(a-b): Appearance of steamed Thua Nao packed in, ARO: Aerobic and (VAC) Vacuum conditions at the (a) Beginning of the storage and (b) at 40-days storage at 4°C MH, commercial Thua Nao; 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

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 (Fernandez *et al.*, 1997; Chouliara *et al.*, 2004). These observations are in agreement with the 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 of Thua Nao when stored at 4°C, suggesting the most critical

factor being the presence of oxygen influencing 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 (Rossel, 1994).

Table 2 shows the values of L (lightness), a* (redness) and b* (yellowness) of Thua Nao prepared in aerobic and vacuum-packaging during storage at 4°C.

Table 2: Changes in colour of Thua Nao under aerobic and vacuum-packed conditions during storage at 4°C

Quality	Storage time (day)	Aerobic-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 ^{ab}	52.98±0.40 ^{aA}	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 ^{aaA}	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 ^{ab}
	10	7.70±0.49 ^{aa}	6.90±0.08 ^{aaB}	6.69±0.15 ^{bb}	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 ^{ab}
	40	8.00±0.54 ^{aa}	6.77±0.17 ^{bb}	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 ^{bc}	21.50±0.84 ^{aa}	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 ^{bb}
	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 ^{aa}	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 ^{bc}	21.58±0.21 ^{ab}	16.78±0.37 ^{bb}
	40	13.15±1.33 ^{cdB}	22.45±0.90 ^{aa}	14.01±0.67 ^{bb}	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±SD (n = 3), Means within same column with different superscripts of small letter are significantly different (p<0.05) in interval samples and that with different superscripts of capital letter are significantly different (p<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

Although, there was a slight change in colour with storage time, the differences between both treatments were not significant (Table 2). The initial brightness and yellowness values of the surface colour of the TNMX sample were higher than those of the TNB51 and MH samples throughout the experimental period for both aerobic and vacuum-conditions, whereas the reddest appearance was identified in 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.

Microbiological quality: Changes in microbial flora of packaged Thua Nao during the storage at 4°C are shown in Table 3. In general, Total Viable Count (TVC) and Spore Count (SPC) were not different during the storage of all thua Nao products under aerobic and vacuum-conditions. This indicates that the endospore-forming bacteria are predominant in the Thua Nao products. Among these products, significantly high contents of TVC and SPC (p<0.05) were observed in the naturally fermented samples (both TNMX and MH) throughout the trial. It might be due to contamination with a variety of 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 the 4°C storage 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 were not a major part of the

widespread micro-flora during storage of both aerobic- and vacuum-conditions (<1.4 log CFU g⁻¹).

Antioxidant quality of storage Thua Nao: After storage at 4°C for 40 days, all packagings of the TNMX and MH products were spoiled and discarded from further analysis due to visual deteriorated characteristics. Therefore, only the TNB51 packages were evaluated for the nutritive values (i.e., antioxidant activity, phenolic compounds and free amino acids) at the initial (Day 0) and terminal day (Day 60) of shelf-life storage.

Table 4 shows the changes in the total phenolic compounds, anti-DPPH radical power and total antioxidant activity of the TNB51 products on Day 0 and 60 at 4°C. The results clearly showed that vacuum-packaging was effective in maintaining 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%, from 7.17 to 5.87 mg BHTE g⁻¹ extract) and phenolic compounds (6%, from 38.98 to 36.8 mg GAE g⁻¹ extract) were found in refrigerated vacuum-packaged product; besides, the activity against linoleic acid oxidation was improved by 47% (from 23.45 to 34.44%) under this condition. In contrast, the great losses of 41% (from 38.98 to 22.90 mg GAE g⁻¹ extract) of total phenolic content, 83% (from 7.17 to 1.19 mg BHTE g⁻¹ extract) of anti-DPPH radicals and 41% of total antioxidant were observed in Thua Nao product prepared aerobic-package. This result could be explained by a high phenolic content (Table 4) and might be the result of exerted antioxidant capacity from other antioxidant components apart from phenolics present in

Table 3: Changes in microorganisms of Thua Nao under aerobic- and vacuum-packed conditions during storage at 4°C

Quality	Storage time (day)	Aerobic-packed			Vacuum-packed		
		TNB51	TNMX	MH	TNB51	TNMX	MH
Total viable count (log CFU g ⁻¹)	0	8.36±0.42 ^{ab}	9.42±0.04 ^{aA}	9.28±0.04 ^{ba}	8.36±0.42 ^{ab}	9.42±0.04 ^{aA}	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 ^B	9.52±0.03 ^{ba}	9.45±0.73 ^{aA}
	10	7.95±0.07 ^{abc}	9.39±0.15 ^{aA}	8.93±0.06 ^{cb}	7.94±0.26 ^B	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 ^B	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 ^B	9.66±0.00 ^{aA}	9.45±0.09 ^{aA}
	60	7.95±0.90 ^{ab}	-	-	8.07±0.27 ^B	-	-
Spore count (log CFU g ⁻¹)	0	8.12±0.58 ^{ab}	9.38±0.08 ^{aA}	9.26±0.02 ^{abA}	8.12±0.26 ^B	9.38±0.08 ^{aA}	9.26±0.02 ^{bcA}
	5	7.89±0.34 ^{bc}	9.65±0.00 ^{aA}	8.85±0.21 ^{cb}	7.88±0.54 ^C	9.65±0.02 ^{aA}	9.09±0.01 ^{ab}
	10	7.75±0.81 ^{ab}	9.62±0.17 ^{aA}	9.12±0.03 ^{baB}	7.74±0.24 ^B	9.59±0.08 ^{baA}	9.14±0.11 ^{caA}
	20	7.06±0.73 ^{ab}	9.52±0.12 ^{aA}	9.26±0.06 ^{abA}	7.31±0.15 ^B	9.60±0.18 ^{baA}	9.38±0.04 ^{aA}
	40	8.15±0.57 ^{ab}	9.63±0.10 ^{aA}	9.42±0.03 ^{aA}	7.74±0.80 ^B	9.68±0.04 ^{aA}	9.58±0.06 ^{aA}
	60	7.71±0.94 ^a	-	-	7.75±0.83 ^a	-	-

Data are Mean±SD (n = 3), Means within same column with different superscripts of small letter are significantly different (p<0.05) in interval samples and mean within same column with different superscripts of capital letter are significantly different (p<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* TNB51, TNMX: Thua Nao prepared by fermentation of boiled soybeans with naturally occurring microbes, MH: Commercial Thua Nao purchased from Mae Hia market

Table 4: Changes in antioxidant activities and phenolic contents of *Bacillus subtilis* TN51-fermented Thua Nao under aerobic and vacuum-packed conditions storage at 4°C

Quality	Before storage	After storage for 60 days	
		ARO	VAC
Anti-DPPH radical effect (mg BHTE g ⁻¹ extract)	7.17±0.41 ^a	1.190±0.24 ^f	5.87±0.39 ^g
Total antioxidant activity (%)	23.45±0.06 ^{ab}	13.04±0.23 ^b	34.44±6.18 ^e
Total phenolics (mg GAE g ⁻¹ extract)	38.98±2.56 ^a	22.90±4.26 ^b	36.80±3.31 ^a

Data are Mean±SD (n = 3), Means in the same row with different small letters are significantly different (p<0.05). Total antioxidant activity measured at 10 mg mL⁻¹ of dried sample extract, ARO: Aerobic-package of the product; VAC: Vacuum-package of the product

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 when stored cucumber and zucchini at 4°C for 7 days.

Free amino acid profiles of storage Thua Nao: According to Table 5, Phe, Leu and Glu were the most dominant amino acids in Thua Nao at Day 0 with a proportion of 20, 13 and 10%, respectively and this observation was still present 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 stored products. After shelf-life storage, 31% of total FAA were reduced with the highest loss of Arg in both aerobic- (69%, from 0.35 to 0.11 g kg⁻¹) and vacuum-packages (69%, from 0.35 to 0.11 g kg⁻¹); this is possibly due to the activity of *B. subtilis* to utilise these amino acids as a nitrogen source for growth (Teng *et al.*, 2004). The packaging condition seemed not to affect the free amino acids content in present study; a similar content of each amino acid was also found in both packaged types.

Table 5: Changes in free amino acids of *B. subtilis* TN51-fermented Thua Nao under aerobic- and vacuum-packed conditions storage at 4°C

FAA	Before storage	After storage for 60 days	
		ARO	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±0.23 ^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±SD (n = 3) and expressed in the unit of g kg⁻¹ dry basis. Means in the same row with different small letters are significantly different (p<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

CONCLUSION

This study clearly indicated that storage temperature strongly affected the visible deteriorated characteristics of Thua Nao. Storage at high temperature did not maintain a desirable quality of the product. On the other

hand, based on visual quality, the shelf-life of Thua Nao when stored at 4°C can be improved for both aerobic and vacuum-packages. In addition, based on the TBA value, the shelf-life of the vacuum-packed TNB51 could be extended by 400% (with the TBA value not more than 5 mg MDA kg⁻¹). Vacuum packaging of thua Nao was more effective to stabilise the TBA value and also assisted in preserving the nutritional values of the product based on antioxidant quality and free amino acids content during shelf-life storage at 4°C. Apart from storage conditions, the fermentation process of Thua Nao also affected the shelf-life storage of the product. Thua Nao prepared by starter *B. subtilis* TN51 showed longer shelf-life than the naturally fermented products. This study suggested that vacuum-packaging and refrigerated temperature are optimum to help maintain the quality and bioactive compounds of Thua Nao products.

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