

Thua nao: Alkali Fermented Soybean from *Bacillus subtilis*

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Abstract

Thua nao was a Thai alkali fermented product from soybean by *Bacillus* and it had a special flavour and aroma. The microorganisms involved in traditional thua nao were mixed natural flora of *Bacillus*, *B. subtilis*, *B. megaterium* and *B. cereus*, contaminated from cooked soybeans. The product had a very strong smell of ammonia. Ammonia content in dried product was lower than in the wet product. The increase of ammonia was controlled by fermentation under the atmosphere of CO₂ or adding phosphate buffer (0.1 mol kg⁻¹ wet wt KH₂PO₄, pH 6.5), which had no effect on the growth of *Bacillus*, proteolytic activity or amino acid formation. The major volatile compounds in thua nao were different from those found in natto, Japanese alkali fermented soybean.

What is alkali fermented legume from *Bacillus* food?

Alkaline, proteolytic fermented foods from legume are found in various parts of the world, Japanese natto, Nigerian dawadawa or iru, Nepalese kinema and Thai thua nao. Soybean seeds are cooked and fermented by pure cultures of *Bacillus subtilis*, as for natto in northern Japan (Ohta, 1986), or fermented using contaminant natural *Bacillus* species, as for dawadawa/iru in west and central Africa (Campbell-Platt, 1980; Odunfa, 1985; Odunfa, 1986; Campbell-Platt, 1987), kinema in Nepal, Sikkim and Darjeeling (Tamang et al., 1988; Sarkar et al., 1993; Sarkar et al., 1994),

thua nao in northern Thailand (Sundhagul et al., 1972) and in- si (tou-si) in China (Hesseltine, 1989). The fermentation of legumes changes the texture and organoleptic properties of the original legume seeds. The unpalatable flavour of the unfermented legume is eliminated (Reddy et al., 1986). The fermented product has a different flavour and aroma, requires less cooking time.

Most of these products are prepared by solid-state fermentation in which substrate is allowed to ferment by spontaneous or by adding *Bacillus* inoculum. The fermented products have a distinct odor. Some products, such as natto or dawadawa became commercialized in the market. Dawadawa is sold in cubed packages, as are Nestle Maggi soup cubes in the African markets (Steinkraus, 1996).

In Asia, thua nao and natto are very similar in term of proteolytic *Bacillus* fermentation but these products have different aroma. Natto is a Japanese fermented whole soybean product which is eaten uncooked as a relish with rice, whereas Thai thua nao is generally sold as dried disks of ground material and is used as a flavouring agent in soups and curries.

This article focuses on Thai fermented soybean, thua nao, which is produced in the north of Thailand. It is consumed as a food and used as a condiment for enhancing flavour in soups, curries, or as a substitute for shrimp paste (Leejeerajumnean, 2000). Thua nao production, bacteria involves in fermentation, strong smell of ammonia development, and volatile compounds found in thua nao are discussed.

What are the differences between thua nao and the other alkali fermented products?

Bacillus fermented food products are classified according to the substrates used. Dawadawa is made from African locust beans (*Parkia biglobosa*) whereas kinema, natto and thua nao are made from soybeans (*Glycine max* (L) Merrill). The *Bacillus* fermented foods are used in different ways. Generally they are used as meat substitutes or as a flavouring agent by adding to soups, as dawadawa,

kinema or thua nao, or eaten directly with rice in the case of natto (Steinkraus, 1983; Reddy et al., 1986; Sarkar et al., 1996). The fermented legumes is consumed as a fresh product without further processing, as with Japanese natto, while some products are fried, as with Nepalese kinema (Tamang et al., 1988; Sarkar et al., 1994). Thua nao is sold as paste or as dried disks, dark brown in colour with a quite different aroma from natto, often with a strong proteolytic and ammonia smell.

The finished product of *Bacillus* fermented soybeans is greyish and covered with a sticky polymer produced by the bacterial cells. The slime on the beans is a mixture of α -polyglutamic acid and levan, and most of viscous materials are polyglutamic acids containing D- and L- glutamates (Ogawa et al., 1991; Kunioka, 1995; Yamaguchi et al., 1996). The fermented soybeans give a distinct odour, always accompanying with an ammoniacol odour (Campbell-Platt, 1980; Steinkraus, 1983; Odunfa, 1986; Hesseltine, 1989; Sarkar et al., 1993).

Traditional thua nao production

Thua nao prepared from dry soybean seeds (*Glycine Max* (L) Merrill) variety SJ 2 or SJ 4. The process began with washing the seeds with water, at least twice. The washed seeds were then boiled in aluminum pots over wood fires for about 7 h, or more, to soften the seeds. After boiling, the water was drained off and the seeds were spread in bamboo baskets lined with fresh leaves from a specific kind of fern (*Thelypteris subelata* (Bak.) K.Iwats.). The fern leaves were made into a cylinder-shaped bar and put in the centre of the basket. The seeds were put in the basket while they were hot. Then the baskets were wrapped with one layer of polypropylene mesh and covered with a thick plastic bag (polyethylene) to provide warmth and a humid atmosphere (Fig. 1 and Fig. 2). The basket was not completely sealed and air was able to enter through the base. Each bamboo basket contained 3-5 kg wet wt of boiled seeds. The baskets were placed outside the building or in the house if it rained. The fermentation was complete within 3 d.



Fig.1 Bamboo basket lined with fern leaves and a single layer of polypropylene mesh. The basket in the background is covered with a plastic bag.
(Photo by S. Leejeerajumnean from Fang District, Chiangmai)

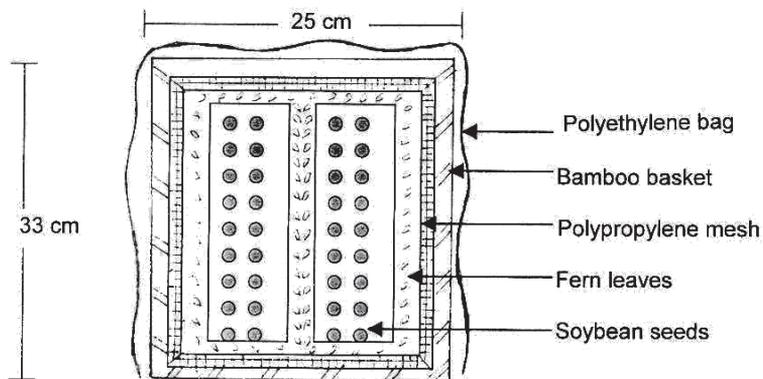


Fig. 2 Diagram of bamboo basket used in Thua nao fermentation.

The fermented seeds appeared brownish in colour, with a slightly sticky covering and had a strong smell of ammonia. The fermented seeds were removed from the basket and ground into a paste using a grinding machine. The paste was moulded by hand into circular-shaped balls, around 3 cm in diameter. These were then pressed between a plastic sheet and a glass sheet to produce circular, flat disks, around 10 cm diameter and 2 mm thick. The disks were then sundried for a day (Fig. 3).

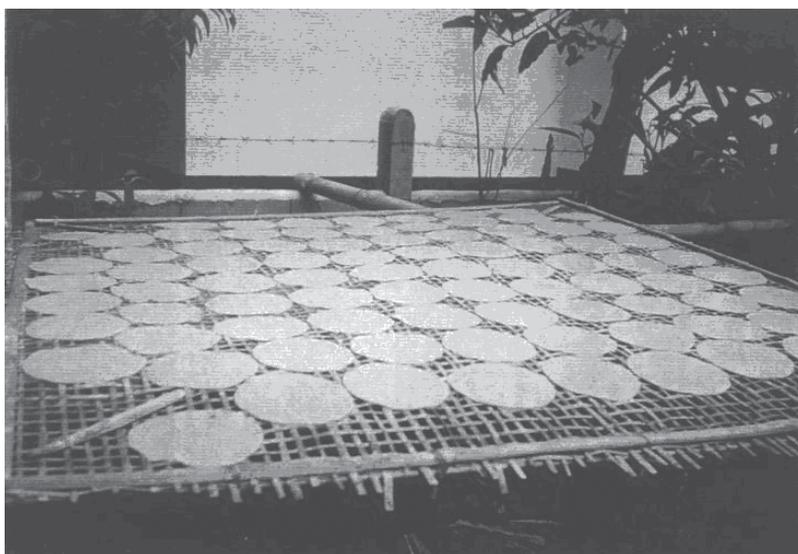


Fig. 3 Thua nao disks are dried in the sun for one day. (Photo by S. Leejeerajumnean from Fang District, Chiangmai)

Sometimes, thua nao was mashed to produce a paste. Salt and sometimes garlic, onion, and red pepper were added. The paste (wet product) was then wrapped in small portions in banana leaves and cooked either by steaming or over an open fire (Sundhagul et al., 1972). The bamboo baskets were lined with teak leaves or banana leaves instead of fresh fern leaves. The dry product could be kept for up to 6 months. The dry product produced during the rainy season sometimes had a problem with fungal growth, presumably due to high moisture content. After drying for one day, the dry product had less smell of ammonia than the fresh one.

Bacteria involved in fermentation

The fermentation process was similar to that of Japanese natto, whereas natto was now usually fermented by a pure culture of *Bacillus subtilis*, thua nao was still made by a traditional method with a mixed natural microflora. Thua nao fermentation was caused by mixed culture of *B. subtilis* and *B. megaterium*, but the predominant was *B. subtilis*. Natto has only pure culture of *B. subtilis* (Table 1). Isolation and identification was clearly that *B. subtilis* was the predominant species from the beginning to the final products (Leejeerajumnean et al., 1997a).

Table 1 *Bacillus* sp. found in thua nao fermentation.

Time of fermentation	<i>Bacillus</i> sp. found
Thua nao 0 h	<i>B. subtilis</i> , <i>B. megaterium</i>
Thua nao 24 h	<i>B. subtilis</i> , <i>B. megaterium</i>
Thua nao 36 h	<i>B. subtilis</i> , <i>B. megaterium</i> ,
Thua nao 72 h	<i>B. subtilis</i> , <i>B. megaterium</i> , <i>B. cereus</i>
Finished product Thua nao	<i>B. subtilis</i> , <i>B. megaterium</i>
Natto (Aji)	<i>B. subtilis</i>
Natto (Mito)	<i>B. subtilis</i>

(Leejeerajumnean et al., 1997a)

Most of microorganisms involved in the fermentation were bacteria (Leejeerajumnean, 2000). Colonies on plate count agar were spreading, and each colony appeared to have an irregular edge. The colonies were flat and dull. These were tentatively identified as *Bacillus* species. The total viable count rose to 10^{10} cfu g⁻¹ wet wt at the end of fermentation. The colonies on the plate count agar for total viable counts appeared identical to those on spore counts plates, and the number of total viable counts and spore counts were nearly the same, after 48 h of the fermentation (Fig. 4), suggesting that the same *Bacillus* species constituted most of the microbial population. Lactic acid bacteria was found after 24 h of fermentation, and

The production of ammonia was a consequence of the utilization of amino acids as an energy source (Allagheny et al., 1996). When the pH reached 8 to 8.3, ammonia (pK_a 9.25) was present as volatile ammonia, which gave the product a strong ammoniacol odour (Njoku and Okemadu, 1989; Sarkar and Tamang, 1995).

Leejeerajumnean (2000) analysed the concentrations of ammonia in thua nao and Japanese natto by an enzymatic method (Boehringer kit 1112 732; Boehringer Mannheim UK Ltd., Lewes, BN7 1LG, UK) and found that the ammonia content in fermented thua nao at 72 h (wet product) was 5.0 g kg^{-1} dry wt, whereas only 1.9 g kg^{-1} dry wt was found in dry thua nao (Table 2), which suggested that the ammonia content was lost during drying. Commercial natto showed a lower ammonia concentration than that in fermented thua nao at 72 h, probably was due to limitation of the ammonia production in natto. Normally, the fermented beans were kept at the low temperature after fermentation process (Ohta, 1986).

Table 2 Ammonia concentrations in thua nao and natto. (Leejeerajumnean, 2000)

Sample	NH_3 concentration* (g kg^{-1} dry wt) □
Experimental thua nao	
Fermented thua nao at 0h	0.5
Fermented thua nao at 24h	0.5
Fermented thua nao at 48h	0.6
Fermented thua nao at 72h	5.0
Dry thua nao	1.9
Commercial thua nao	
Mae Sao market □	2.5
Loung market □	0.9
Commercial natto	
Mito §	4.3
Aji §	4.0

*, Ammonia was assayed enzymatically.

□, Mean of duplicate analyses of ammonia concentrations in one extract; \pm deviation from mean.

□, Dry samples

§, Wet samples

How to control the increasing of ammonia?

In efforts to overcome this strong ammoniacal odour, Thai traditional fermented soybeans were sundried for a day (Sundhagul et al., 1972), while Nepalese kinema was fried prior to consumption (Sarkar et al., 1993). Previously, attempts were made to control ammonia formation by the addition of humectants, such as NaCl or glycerol (Allagheny et al., 1996), or by limiting the amount of initial O₂ by doing the fermentation in sealed containers (Allagheny et al., 1996). The addition of humectants reduced water activity and proteolytic activity. Limiting the initial amount of O₂ led to reduce growth and less increase in pH, and no ammonia was generated.

The buffering system was developed to control the increase of pH and reduce the strong smell of ammonia. The fermentation was done under the atmosphere of CO₂ or adding phosphate buffer in the fermented soybeans. The results found that CO₂, up to 40 % v/v and phosphate buffer (0.1 mol kg⁻¹ wet wt KH₂PO₄, pH 6.5) had no effect on the growth, proteolytic activity, amino acid production and ammonia production during soybean fermentation by *B. subtilis*. Carbon dioxide and phosphate buffer reduced the increase in pH, without affecting ammonia formation. However, to avoid a complicated CO₂ buffer system, a phosphate buffering system should be used (Leejeerajumnean and Owens, 2002).

In the case of natto, ammonia formation is limited by storage at low temperature (5-10 °C) as an expedient to restrict bacterial growth while the proteolytic enzymes continue to work (Ohta, 1986).

Volatile compounds production

In general, finished product of natto was covered by a light brown growth of bacteria which had sticky material. Natto had a fruity or nutty aroma without strong ammonia odour. While thua nao was a dried disk with dark brown in colour, strong smell and noticeable ammonia.

Many researchers had studied the volatile compounds found in natto (Kanno et al., 1984; Kanno and Takamatsu, 1987 and Tanaka

et al., 1998). Pyrazines and sulphur-containing compounds were reported to be the main contributors to the characteristic natto odour (Kanno et al., 1984). Sugawara et al. (1985) identified pyrazines in natto and reported that the main pyrazines in natto were tetramethyl, 2,3,5-trimethyl and 2,5-dimethyl pyrazines. These following pyrazines have been identified from the cultures of *B. subtilis*: methyl, 2,3-dimethyl; 2,5-dimethyl; 2,6-dimethyl; trimethyl; tetramethyl; ethyl; 2-ethyl-6-methyl; 3-ethyl; 2, 5-dimethyl; 2,6- diethyl-3-methyl and 2-methyl-5-propyl derivatives (Kosuge et al., 1961; Sugawara et al., 1985; Yamaguchi et al., 1993).

Volatile compounds of thua nao have been extracted by steam distillation-solvent extraction (Nickerson and Likens apparatus) and identified by GC and GC-MS (Chairote and Kobayashi, 1987). Esters, pyrazines, carbonyl compounds, and various other compounds contributed to the aroma of thua nao. Pyrazines, including 2,5-dimethyl; 2,6-dimethyl; 2-ethyl-5-methyl; 2,3,5-trimethyl and 3-ethyl-2, 5-dimethyl derivatives were identified (Chairote and Kobayashi, 1987).

The volatile compounds found in thua nao and natto had different chromatographic profiles (Leejeerajumnean et al., 2000). Significant difference in the composition and the pattern of chromatogram might be used to identify the specific flavour characteristics of thua nao (Leejeerajumnean et al., 1997b). The similar characteristics of volatile compounds in these products were 3-hydroxy- 2 butanone (acetoin), dimethyl pyrazine, trimethyl pyrazine and tetramethyl pyrazine. Some pyrazines and derivative compounds found in natto and thua nao were shown in Fig. 5.

The volatile compounds found only in thua nao not in natto were several alcohols and several aldehydes, acids and esters and sulfur containing compound. Natto had a higher concentration of ketones and pyrazines than thua nao.

The volatile compounds of octen-3-ol, pentanal, hexanal and 2- pentyl furan found in thua nao and natto were caused by *Bacillus* fermentation except some compounds were originated from cooked soybean such as 1- octen-3-ol, pentanal, hexanal and 2- pentyl furan (Leejeerajumnean et al., 1997b).

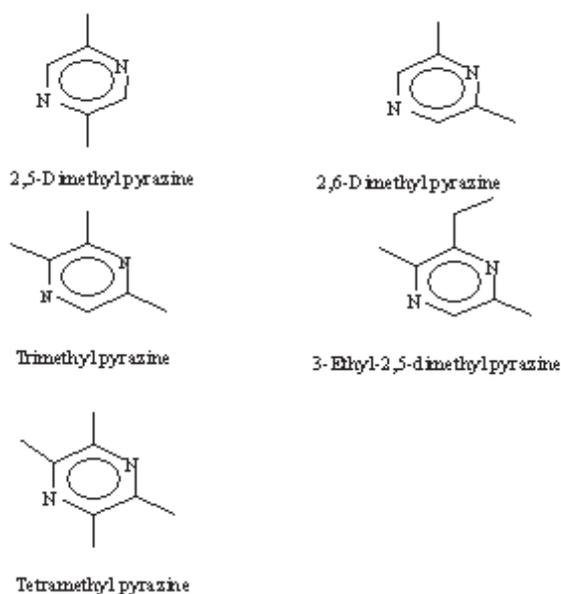


Fig. 5 Some pyrazines and derivative compounds found in thua nao and natto.

Conclusions

In conclusion, thua nao is a proteolytic fermentation of soybeans which is similar to Japanese natto, whereas natto is now fermented by pure culture of *B. subtilis*, thua nao is still made by traditional method with a mixed natural microflora. Natto consists of whole beans covered with sticky bacterial growth and has a fruity or nutty aroma without ammonia odour. Thoa nao is sold as paste or dried disks and has a quite different aroma, often with a strong ammonia smell. The buffering system, fermentation under CO_2 , up to 40 % v/v or adding phosphate buffer (0.1 mol kg^{-1} wet wt KH_2PO_4 , pH 6.5) is developed to control the increase of pH and reduce the strong smell of ammonia.

The development of thua nao in Thailand has to focus on small-scale productions. First of all, it is important to make the product more acceptable with its smell. The development of small-scale manufactures should emphasize good hygienic practice,

processing technology, plant sanitation, reduction in the strong smell of ammonia, extension of shelf-life and improvement of the packaging. The packaging form of the products should be changed to gain a higher price, possibly from disks to cubes, similar to African dawadawa cubes, which are pulverized fermented beans and ready for addition to soups (Stainkraus, 1991). The promotion of large-scale manufacture, similar to Japanese natto factories should be considered after the products are widely accepted.

References

- Allagheny, N., Obanu, Z.A., Campbell-Platt, G. and Owens, J.D. (1996), Control of ammonia formation during *Bacillus subtilis* fermentation of legume. *International Journal of Food Microbiology* 29: pp. 321-333.
- Campbell-Platt, G. (1980), African locust bean (*Parkia* species) and its West African fermented food product, dawadawa. *Ecology Food Nutrition* 9 : pp. 123-132.
- Campbell-Platt, G. (1987), *Fermented Foods of The World- A Dictionary and Guide*. (London: Butterworths)
- Chairote, G. and Kobayashi, A. (1987), Volatile Constituents of 'Toa-nao' (Fermented Soybean). Open File Report ISSN 0857-0051. Faculty of Science, Chiang Mai University, Chiangmai, Thailand.
- Hesseltine, C.W. (1989), Fermented products. In *Legumed : Chemistry, Technology and Human Nutrition* ed. Matthews, R.H. pp.161-185. (New York: Marcel Dekker)
- Kanno, A., Takamatsu, H. and Takano, N. (1984), Determination of several volatile components produced by *Bacillus natto* in commercial "Natto". *Nippon Shokuhin Kogyo Gakkaishi* 31: pp. 587-595.
- Kanno, A. and Takamatsu, H. (1987), Changes in the volatile components of "Natto" during manufacturing and storage. *Nippon Shokuhin Kogyo Gakkaishi* 34: pp. 330-335.
- Kosuge, T. Kamiya, H. and Adachi, T. (1961), An odorous component of natto, fermented soybeans. *Journal of the Pharmaceutical Society (Japan)* 82: pp. 190.
- Kunioka, M. (1995), Biosynthesis of poly (γ -glutamic acid) from L-glutamine, citric acid and ammonium-sulfate in *Bacillus subtilis* IFO3335. *Applied Microbiology and Biotechnology* 44: pp. 501-506.
- Leejeerajumnean, A., Duckham, S.C., Campbell-Platt, G., Ames, J.M., and Owens, J.D. (1997a), *Bacillus* fermentation of soybean. SAB Summer'97 Conference, Symposium: Toxins, 14-17 July 1997, University of East Anglia, Norwich, UK.

- Leejeerajumnean, A., Duckham, S.C., Ames, J.M., Campbell-Platt, G. and Owens, J.D. (1997b), Changes in volatile compounds produced during the fermentation of soybean by a mixed *Bacillus* culture. UK Universities Flavour Consortium Postgraduate Symposium, 4-6 September 1997, Queen's Elms Halls of Residence, The Queen's University of Belfast, Northern Ireland, UK.
- Leejeerajumnean, A. (2000), *Bacillus* fermentation of soybeans: characterization of traditional thua nao manufacture. PhD. Thesis, The University of Reading, UK.
- Leejeerajumnean, A., Ames, J.M. and Owens, J.D. (2000), The volatile compounds produced during the fermentation of soya beans by *Bacillus* species. *Journal of the Science of Food and Agriculture* 18 : pp. 525-529.
- Leejeerajumnean, A. and Owens, J.D. (2002), Development CO₂ buffer system to control the increase of pH in alkali fermented soybeans. The 4th Agro-Industrial Conference. Thaifex-Thaimex 2002, 31 June- 1 July Bitech Bangna, Bangkok, Thailand.
- Njoku, H.O. and Okemadu, C.P. (1989), Biochemical changes during the natural fermentation of the African oil bean (*Pentaclethra macrophylla*) for the production of Ugba. *Journal of the Science of Food and Agriculture* 64 : pp. 457-465.
- Ogunfa, S.A. (1985), Biochemical changes in fermenting African locust bean (*Parkia biglobosa*) during 'iru' fermentation. *Journal of Food Technology* 20 : pp. 295-303.
- Ogunfa, S.A. (1986), Dawadawa. In *Legume-based Fermented Foods* ed. Reddy, N.R., Pierson, M.D. and Salunkhe, D.K. pp.173-189. (Boca Raton : CRC Press)
- Ogawa, Y., Hosoyawa, H., Hamano, M. and Motai, H. (1991), Purification and properties of γ -glutamyltranspeptidase from *Bacillus subtilis* (natto). *Agricultural and Biological Chemistry* 55 : pp. 2971-2977.

- Ohta, T. (1986), Natto. In *Legume-based Fermented Foods* ed. Reddy, N.R., Pierson, M.D. and Salunkhe, D.K. pp.85-93. (Boca Raton: CRC Press)
- Reddy, N.R., Pierson, M.D. and Salunkhe, D.K. (1986), Introduction. In *Legume-Based Fermented Foods*. ed. Steinkraus, K.H. pp. 1- 3. (Boca Raton : CRC Press)
- Sarkar, P.K., Cook, P.E. and Owens, J.D. (1993), *Bacillus* fermentation of soybeans. *World Journal of Microbiology and Biotechnology* 9: pp. 295-299.
- Sarkar, P.K., Tamang, J.P., Cook, P.E. and Owens, J.D. (1994), Kinema - a traditional soybean fermented food: proximate composition and microflora. *Food Microbiology* 11: pp. 47-55.
- Sarkar, P.K. and Tamang, J.P. (1995), Changes in the microbial profile and proximate composition during natural and controlled fermentations of soybeans to produce kinema. *Food Microbiology* 12: pp. 317-325.
- Sarkar, P.K., Jones, L.J., Gore, W., Craven, G.S. and Somerset, S.M. (1996), Changes in soya bean liquid profiles during kinema production. *Journal of the Science of Food and Agriculture* 71: pp. 321-328.
- Steinkraus, K.H. (1983), Miscellaneous oriental fermentations, Chinese soy sauce, pastes and related fermented foods. In *Handbook of Indigenous Fermented Foods* ed. Steinkraus, K.H. pp. 530-571. (New York: Marcel Dekker)
- Steinkraus, K.H. (1991), African alkali fermented foods and their relation to similar foods in other parts of the world. In *Traditional African Foods: Quality and Nutrition* ed. Westby, A. and Reilly, P.J.A. pp. 87-92. (Stockholm: International Foundation for Science)
- Steinkraus, K.H. (1996), Indigenous fermented foods involving alkali fermentation. In *Handbook of Indigenous Fermented Foods*. 2 nd edn, ed. Steinkraus, K.H. pp. 349-362. (New York: Marcel Dekker)

- Sugawara, E., Ito, T., Odagiri, S., Kubota, K. and Kobayashi, A. (1985), Comparison of compositions of odor components of natto and cooked soybeans. *Agricultural and Biological Chemistry* 49: pp. 311-317.
- Sundhagul, M., Smanmathuroj, P. and Bhodacharoen, W. (1972), Thua-nao: a fermented soybean food of northern Thailand. I. Traditional processing method. *Thai Journal of Agricultural Science* 5: pp. 43-56.
- Tamang, J.P., Sarkar, P.K. and Hesseltine, C.W. (1988), Traditional fermented food beverages of Darjeeling and Sikkim - a review. *Journal of the Science Food and Agriculture* 44: pp. 375-385.
- Tanaka, T., Muramatsu, K., Kim, H., Watanabe, T., Takeyasu, M., Kanai, Y. and Kiuchi, K. (1998), Comparison of volatile compounds from Chungkuk-Jang and Itohiki-Natto. *Bioscience, Biotechnology and Biochemistry* 62: pp.1440-1444.
- Yamaguchi, N., Toda, T., Teramoto, T., Okuhira, T. Sugawara, E. and Ito, T. (1993), Effect of sugars on microbiological pyrazine formation by *Bacillus natto* in synthetic liquid medium. *Nippon Shokuhin Kogyo Gakkaishi* 40: pp. 841-848.
- Yamaguchi, F., Ogawa, Y., Kikuchi, M., Yuasa, K., and Motai, H. (1996), Detection of γ - polyglutamic acid (γ -PGA) by SDS-PAGE. *Bioscience, Biotechnology and Biochemistry* 60: pp. 255-258.