Chemical changes associated with lotus and water lily *natto* production

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Abstract. *Natto* is a traditional Japanese food made by fermenting whole soybean seeds with pure culture of *Bacillus subtilis* subsp. *natto*. The purpose of this study was to investigate the suitability of lotus (*Nelumbo nucifera*) and water lily (*Nymphaea stellata*) seeds as the raw materials for *natto* production. Chemical (proximate, amino acids and minerals) changes were observed on raw, steamed and fermented seeds. Proximate compositions of all samples were calculated in both wet basis and dry basis. In wet basis calculation, steaming and fermentation tended to lower the carbohydrates, ashes, fats and protein content which were attributed to the increase of moisture. The total amino acid, iron and magnesium contents of raw lotus seeds were 24.29%, 5.08 mg 100g⁻¹ and 174.23 mg 100g⁻¹ dry matter, respectively. After a 24h-fermentation at 40°C, the total amino acids decreased while iron and magnesium contents increased significantly reaching, in respective order, 9.9 mg 100g⁻¹ and 411.36 mg 100g⁻¹ dry matter. Changes in chemical composition after fermentation were more pronounced in lotus seeds than water lily seeds indicating that their nutrient composition were more suitable to support *Bacillus subtilis* growth.

1. Introduction

*Natto* is a traditional Japanese fermented soybean prepared commercially using a pure starter culture of a spore-forming Gram-positive bacterium, *B. subtilis*. There are some other unsalted fermented soybean foods resembling *natto*, for instance Himalayan *kinema* [1], Korean *chongkukjang* [2] and Thai *thua nao* [3]. *Kinema* is traditionally made by wrapping lightly crushed boiled soybeans in local fern leaves inside a bamboo basket to provide a source of *Bacillus subtilis* bacteria and let to ferment at warm temperature for 1-3 days. The same steps applied to *natto* and *chongkukjang* beside the use of rice straws instead of fern leaves. As for *thua nao*, the boiled soybean is wrapped in bamboo baskets lined and covered with banana leaves. All of these products do have similar properties for instance distinct flavor and stickiness. The nutritional properties of the raw materials used are improved while the texture and wide variety of flavors develops during fermentation. *Natto* fermentation also adds the functional properties of soybean by the presence of nattokinase, polyglutamic acid and dipicolinic acid [4]. Nattokinase is a fibrinolytic enzyme shows ability to break the cross-linking of fibrin in *vitro* [5]. Research conducted by [6] indicated that the enzyme has optimal pH value and temperature of 8.0 and 40 °C, respectively and shows high thermostability at 30 to 50 °C.

Other raw materials have been used to replace soybean, for instance chickpeas (*Cicer arietinum*) [7] [8] and pigeon pea [9] based on their nutritional value and acceptability as a food. Lotus (*Nelumbo nucifera*) and white lily (*Nymphaea stellata*) are underutilized water plants which seeds have been found...
to be highly nutritious [10] [11]. The seeds of N. nucifera are rich in protein, unsaturated fatty acids, minerals and starch [12]. Several bioactive compounds have also been derived from lotus, including alkaloids, flavonoids, glycosides, triterpenoids, and vitamins which all have therapeutic impact [13]. Isoflavone, tannins, saponins and lignan are also detected in both plants [14], adding their benefits to human’s health. Based on their richness in nutritious and functional components, lotus and water lily seeds stand as promising raw materials for the preparation of fermented food products, including natto. However, their suitability as raw materials of solid-state natto fermentation has not been reported. Therefore, the current research focused on bringing to light their potentials as natto raw materials by evaluating the amino acids profile, proximate and mineral changes of lotus and water lily seeds during natto production.

2. Experimental detail

2.1. Starter preparation
The overnight culture of natto starter culture (Bacillus subtilis) on NB medium was streaked steriley into NA medium and incubated at 37 °C for 24h. Actively growing colonies on NA were diluted to 0.85% saline water in order to create turbidity equaled to Mc Farland standards 1.

2.2. Seeds preparation
Mature lotus (Nelumbo nucifera) and water lily (Nymphaea stellata) fruits were collected from several locations of swamp waters in Banyusain and Ogan Ilir districts, South Sumatera. Ripen lotus seeds with black color were separated from the fruit’s pods. Water lily fruits sinking below the water were collected, cut, slightly crushed and washed thoroughly to separate the small dark green ripen seeds. Both types of seeds were then sun-dried and subjected to hard shell removal leaving brown-colored seeds which were used as natto raw materials.

2.3. Natto preparation
The method used to prepare natto was similar to the current common practice in Japanese households in respect to the relative proportions of seeds and water as well as the fermentation time and conditions. Lotus and water lily seeds were soaked in tap water for 24h at 29±1 °C with a ratio of 1:3 w/v and during the process the soaked water was changed once. At the end of soaking period, the water was discarded. After dehulling, the lotus seeds were rinsed and cut in two pieces in order to remove the bitter green embryos inside. Both type of seeds were washed thoroughly, drained and weighed. Steaming was done for 10 minutes at 121 °C in an autoclave by placing the seeds inside the stainless steel baskets. After steaming, both seeds were placed in a sterile polypropylene container, cooled down until the temperature reached 50 °C and directly inoculated with 4mL of Bacillus subtilis in saline solution (0.85% NaCl) per 100 g steamed seeds. The cell density of the inoculums was approximately 3 x 10^8 CFU/mL, attained by adjusting the turbidity of the cells with McFarland standards 1 (Himedia). After mixed thoroughly, the containers were covered with sterile cheese clothes and their lids were put just on top of cheese clothes for air circulation. Samples were incubated in the water bath (Memmert) for 24h at 40 °C and 99% relative humidity. After fermentation, samples were stored in a refrigerator at a temperature of 5±1 °C for 12h.

2.4. Analytical methods
The nutritional compositions (ash, fat, moisture and protein) of the fermented lotus and water lily seeds were evaluated according to the Indonesian National Standard (SNI) 01-2891-1992 method. The carbohydrate content was estimated using by-difference calculation. The nutritionally essential elements including Fe and Mg were determined using Atomic Absorption Spectrophotometer (AAS) and Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES). The energy contents of the samples were obtained by multiplying crude protein, crude fat and carbohydrate by factors of, in respective order, 4, 9 and 4. Amino acids analyses were performed by using Waters Acquity UPLC H
Class Amino Acid Analysis System following the method outlined in [15]. Amino acids were identified by comparing retention times with a standard mixture of 15 authentic amino acids. The content of each amino acid was calculated on the basis of the standard curve of each authentic amino acid standard.

3. Results and discussion

Chemical changes during the preparation of natto were assessed in relation to the raw materials. The chemical changes that took place during the preparation of natto are given in table 1.

| Parameters                  | Lotus seeds | Water lily seeds |
|-----------------------------|-------------|------------------|
|                             | Raw Steamed Fermented | Raw Steamed Fermented |
| Moisture (%)                | 11.18 73.8 74.92 | 13.58 54.25 71.67 |
| Dry matter (%)              | 88.82 26.2 25.08 | 86.42 45.75 28.33 |
| Ashes (% DM)                | 4.40 4.08 5.02 | 0.79 0.74 0.78 |
| Total Fat (% DM)            | 2.09 2.44 3.39 | 1.13 1.18 1.98 |
| Protein (% DM)              | 27.18 29.96 34.09 | 11.18 11.82 11.79 |
| Total Carbohydrates (% DM)  | 66.33 63.51 57.49 | 86.90 86.23 85.46 |
| Total energy (Kal)          | 392.86 395.88 396.85 | 402.52 402.84 406.78 |
| Energy from fat (Kal)       | 18.85 21.98 30.50 | 10.21 10.62 17.79 |
| Soluble dietary fiber (% DM)| 5.92 Nd Nd | 5.38 nd nd |
| Total sugar                 | 6.98 Nd Nd | 0.86 nd nd |
| Fe (mg/100g)                | 5.08 0.77 9.90 | 3.54 2.82 7.18 |
| Mg (mg/100g)                | 174.22 226.03 411.36 | 61.50 83.61 70.03 |
| Vitamin K (mcg/100g)        | <2 <2 <2 | <2 <2 <2 |

*nd* not determined

The initial proximate composition of lotus seeds used in this study was 11.18% moisture, 3.81% ashes, 1.86% fats, 24.14% proteins and 58.91% total carbohydrates (wet basis) with the total energy of 348.94 Kal/100g. The nutritional value of lotus seeds had also been evaluated by [10]. In 100g, lotus seeds contains 63-68% of carbohydrate which mostly in the form of starch, 17-18% of protein, 1.9-2.5% of fat, and the remainders are water (about 13%) and minerals (mainly sodium, potassium, calcium, and phosphorus) providing about 350 Kal of energy. The author also found out that lotus seeds were low in fiber and not a good source of vitamins.

Water lily seeds have lower protein and total sugar content than lotus seeds, accounted for only 11.18% and 0.86% dry basis compared to 27.18% and 6.98% dry basis respectively. Soybean, which is the common raw material for natto production, has 44.19% protein [16]. In Japan, small sized and white colored Nattoshoryu soybean cultivar with 42.6% protein and 30.4% carbohydrates (dry basis) was preferred for natto production [17].

During the fermentation of natto, the biochemical processes that cause typical changes in chemical and physicochemical characteristics of the raw ingredient take place. Those changes are induced by sugars, proteins and fats decomposing-enzymes which present in the tissue or secreted by microbes. Soluble sugars, especially sucrose, are the major source of energy for fermentation [18]. The amount of sucrose in soybean ranges from 1.5 to 10.2% of total dry matter [19]. As the free sugars composition of soybean influences the effective fermentation, increased total sugar and sucrose content is important to achieve the proper rate of fermentation during the natto-making process and to improve the taste and flavor since sucrose is consumed faster than other types of sugars such as raffinose and stachyose on the fermentation process [17]. Enzymatic degradation of proteins is also a key process in natto fermentation. In general, the proteins are hydrolyzed into peptides, amino acids, and ammonia. All enzymatic activities in the process are expected to contribute to distinct attributes of natto including texture, appearance, flavor, and aroma.

Good quality natto products should be covered with a white colored mucous substance, has a slimy appearance, softer texture and are able to generate silky and sticky mass when mixed/stirred with a pair.
of chopsticks [20]. Based on those criteria, only lotus seeds natto gave satisfactory result. No formation of white-colored viscous substance was observed in water lily seeds after a 24h-fermentation at 40°C. The changes in sensory attributes were in line with its chemical properties. From Table 1, it could be noticed that in dry basis analysis, fermentation process lowered the total carbohydrates of lotus seeds, while the ashes, protein, and fats increased. The proximate value of soybean and soybean natto had been compared and it was revealed that fermentation increased total fat, slightly lowered the protein and ashes and considerably decreased the total carbohydrates [16]. Increase in fats, moisture and carbohydrates followed by decrease in proteins of cooked soybeans in the fermentation of chongkukjang for 2-4 days at 40-43°C were also reported [2].

Some of the carbohydrates were used up as carbon source by the fermenting organism and converted to microbial biomass. It contributed to the decrease in carbohydrate content of lotus seeds after fermentation. The increase in the protein content of lotus seeds after fermentation in this study might be due to the secretion of extracellular enzymes by Bacillus subtilis which consequently increased the protein content of the fermented product, as well as the microbial biomass. The protein values of raw, steamed and fermented lotus seeds samples which determined based on the total N were, in respective order, 27.18%, 29.96% and 34.09%. The amino acids profiles of those three products are presented in Table 2. The quantities of total amino acids analyzed were found to be 24.294%, as dry basis, in raw lotus seeds. Steaming slightly increase the total amount of fifteen amino acids to 25.799% while fermentation decreased them to 22.237%. The gaps between total protein and total amino acids were accounted for other amino acids, such as methionine, tryptophan, sulfurous amino acids and probably other non protein nitrogen compounds.

### Table 2. Changes in amino acids composition of lotus and water lily seeds during natto preparation

| Seeds type  | Lotus          | Water Lily | Soybean [16] | Thua Nao [16] | Natto [16] |
|-------------|----------------|------------|--------------|----------------|------------|
| Processing step | Raw | Steamed | Fermented | Raw | Steamed | Fermented | Raw | Steamed | Fermented | Raw | Steamed | Fermented |
| Amino acids | | | | | | | | | | | | |
| L-Asp | 2.337 | 2.626 | 1.876 | 0.648 | 0.665 | 0.619 | 4.842 | 5.006 | 4.335 |
| L-Glu | 4.958 | 5.674 | 5.403 | 1.760 | 1.868 | 1.790 | 8.255 | 8.740 | 9.363 |
| L-Ser | 1.917 | 1.876 | 1.173 | 0.822 | 0.885 | 0.879 | 2.304 | 2.353 | 1.967 |
| Gly | 1.312 | 1.292 | 1.209 | 0.346 | 0.380 | 0.364 | 2.025 | 2.134 | 1.804 |
| L-Ala | 1.052 | 1.288 | 1.308 | 0.565 | 0.609 | 0.592 | 1.920 | 2.220 | 1.668 |
| L-Pro | 0.786 | 0.882 | 0.697 | 0.259 | 0.269 | 0.241 | 2.047 | 2.297 | 1.990 |
| L-Tyr | 0.664 | 0.655 | 0.792 | 0.390 | 0.402 | 0.331 | 1.441 | 1.894 | 1.903 |
| L-His* | 0.705 | 0.651 | 0.658 | 0.366 | 0.375 | 0.315 | 1.012 | 1.026 | 1.055 |
| L-Arg* | 2.508 | 2.125 | 1.539 | 1.391 | 1.398 | 1.203 | 3.533 | 3.297 | 2.601 |
| L-Ile* | 1.008 | 1.092 | 0.924 | 0.496 | 0.505 | 0.467 | 2.099 | 2.186 | 1.879 |
| L-Leu* | 1.746 | 1.878 | 1.561 | 1.000 | 1.011 | 0.933 | 3.564 | 3.680 | 3.300 |
| L-Lys* | 1.892 | 2.342 | 1.722 | 0.272 | 0.270 | 0.285 | 2.751 | 2.628 | 2.699 |
| L-Val* | 1.180 | 1.293 | 1.119 | 0.556 | 0.570 | 0.533 | 2.054 | 2.136 | 1.971 |
| L-Phe* | 1.117 | 0.978 | 1.202 | 0.647 | 0.626 | 0.456 | 2.455 | 2.384 | 2.213 |
| L-Thr* | 1.112 | 1.146 | 1.039 | 0.494 | 0.497 | 0.471 | 1.742 | 1.849 | 1.593 |
| Total | 24.294 | 25.799 | 22.237 | 10.013 | 10.331 | 9.480 |

*essential amino acids
Data expressed as % sample dry matter

Amino acid analyses are usually feasible for the expression of total amino acids in various foods. Their amino acid content and pattern are important from nutritional point of view. Changes of fifteen amino acids composition were analyzed in every step of natto processing. Predominant amino acids in lotus natto were glutamic acid, followed by aspartic acid, lysine, leucine, arginine, alanine, glycine and phenylalanine. The predominant amino acids compared favorably with those of soybean thua nao and natto [16] despite a slight difference in pattern was noted. In both soybean products, the order was
glutamic acid, aspartic acid, leucine, arginine, lysine and serin. Some hydrophobic amino acids, such as alanine and phenylalanine increased after fermentation, while histidine remained at the same level in all processing steps. The same trend was also observed by [16]. The decrease in the rest of amino acids suggests that they were consumed or transformed somehow at a greater rate than they were formed by proteolytic activity.

Amino acids, peptides and proteins have direct contribution to the flavor of the foods. L-glutamic acid is known for its flavor-enhancing properties. According to [21], pure amino acids have their own taste properties. L-tryptophan, L-phenylalanine, L-tyrosine and L-leucine are amino acids which contribute to product’s bitterness. Two out of these were presented in lotus natto in considerable amounts and constitute natto’s prominent amino acids. Lotus natto was also rich in amino acids which tastes are barely perceptible, for instance L lysine and L arginine. The fermentation process of lotus natto contributed to the increase of some sweet-tasted amino acids such as L-alanine and glysine.

4. Conclusions
Based on chemical assessment on both lotus and water lily natto, this research confirmed the suitability of lotus seeds as promising natto raw material. Decomposition of carbohydrates took place during the fermentation of lotus seeds, the same trend was also observed on soybeans by other authors. Very low total sugar content in water lily seeds compared to lotus seeds and soybeans was probably the reason of their unfitness as natto raw material.

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