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ORIGINAL PAPER

Changes in Fermented Soybean Nutritional Content Generated Under the Different Fermentation Conditions by *Bacillus Subtilis*

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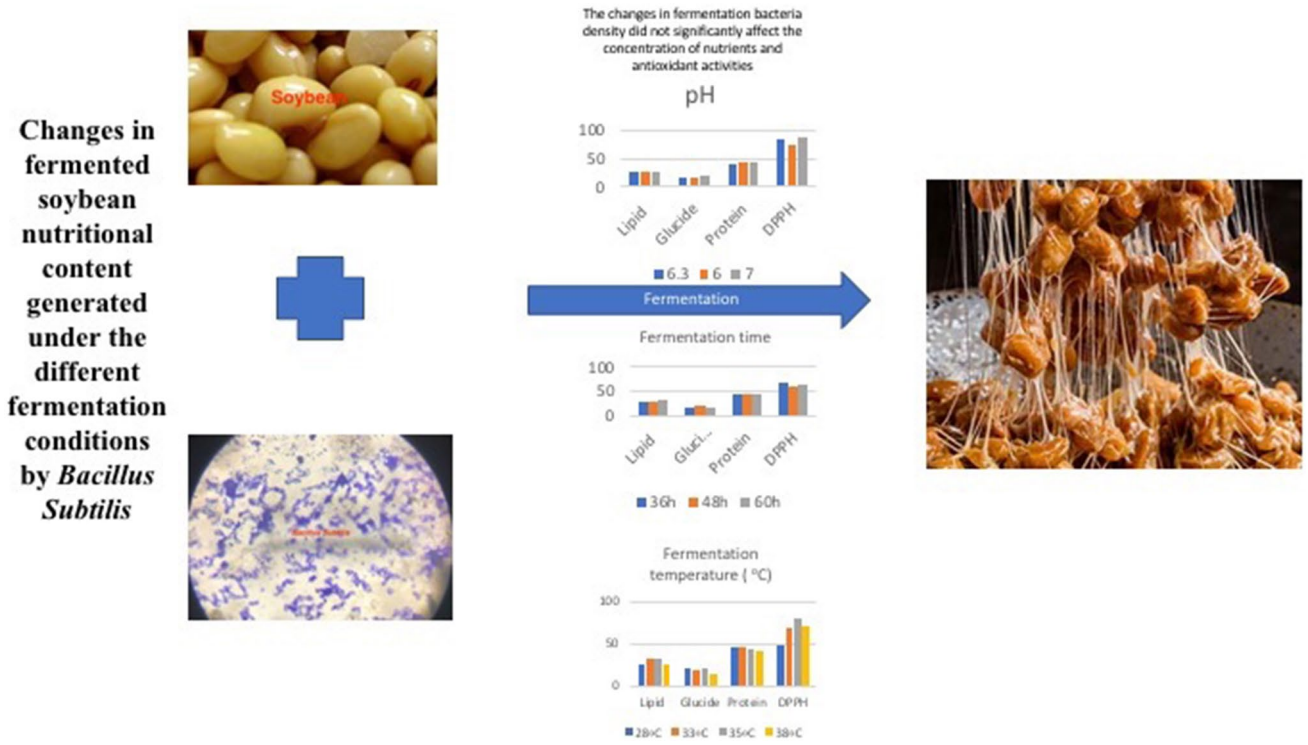
Abstract

The objective of the study was to determine the factors affecting post-fermentative nutritional content resulting from soybean fermentation by *Bacillus subtilis*. Conditions for the nutritionally optimal fermentation of soybeans by *B. Subtilis* were varied to analyze the overall nutrient content change. The conditions included: (1) pH, (2) fermentation temperature, (3) fermentation time, and (4) bacterial density. Experimental results show the highest concentration of lipids at a pH 6.0, temperature of 33 °C, and 60 h fermentation time. Carbohydrates were highest at pH 7, 28 °C and 48 h, and protein content yielded highest at pH 8, 28 °C, and 60 h of fermentation. Bacterial density had no statistically significant affect on nutritional content ($p > 0.05$). Optimization of fermentation conditions contributes to increased biomass during fermentation to increase reaction yield of the desired product. Therefore, this research is important at the industrial level to increase production efficiency and reduce energy consumption.

Graphic Abstract



Keywords Soy · Soybean · Protein · Lipid · Carbohydrates · *Bacillus subtilis*

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Statement of Novelty

This study determined the content of nutrients such as protein, lipids, carbohydrates, and DPPH antioxidant activity varied at different pH, fermentation temperatures, and fermentation times, while the bacterial densities had no significant affect on nutrient content and antioxidant activity. The results of the study make it easier to control the fermentation process of soybeans to produce desired products by changing the fermentation conditions. Besides controlling the fermentation process to increase biomass, collecting the desired product for this research will be able to connect with many other sciences to optimize production processes.

Introduction

Soybeans (SB) are a very popular ingredient for Vietnam and around the world in the food production sector. Soybeans are very nutritious and provide a healthy source of protein that is low in fat and cholesterol. All nine essential amino acids that the body cannot produce are found in soybeans. Increased interest in the nutritive properties of soy protein is well deserved. Soy protein products offer benefits to women in various life stages. Soy protein is attributable to improved diet and cardiovascular status, prevention of certain types of cancer, improved health following menopause, obesity prevention/control, and increased food variety [1]. On the other hand, a soybean processing facility, in which refined oil, soy protein concentrate and soy protein isolate are produced, generates residues that if undergo additional industrial operations may result in new products with commercial value [2].

Lipids represent one of the most important nutritional components of Soybeans. Economically, soybean oil (SBO) constitutes approximately 29% of the world's fat and oil production. Soy lipids are primarily in the soybean cotyledon and comprise about 20% of its weight. Physiologically, soybean lipids have a broad spectrum of functions, including being a part of membranes, acting as an energy reserve, and serving as the solvent medium for many lipid-soluble substances [3]. Soybean contains about 20% oil in seeds and it is therefore currently an important oilseed crop. Considering the fatty acid composition which constitutes the soybean lipid, the concentration of linolenic acid is less than 10% [4].

Soy protein has been shown to support lifelong health requirements and poses as a source of lean, cholesterol-free, and lactose-free protein. The field of nutrition science and exercise physiology supports the need for physical activity in combination with more nutritious choices—such as consuming sources of soy protein, to maintain a healthy quality of life [5]. Soy protein is a complete protein in that it meets all the essential amino acid requirements to support the normal growth and development of infants and children. Soy protein is also low in fat and free of saturated fat and cholesterol. It is an ideal protein source to boost the nutrient density of foods [6].

Soybean [*Glycine max* (L.) Merril] carbohydrates make up approximately 35% of soybean (SB) seed and 40% of soybean meal (SBM) dry matter (DM). Approximately half of these carbohydrates are nonstructural in nature, including low molecular weight sugars, oligosaccharides, and small amounts of starch, while the other half are structural polysaccharides, including a large number of pectic polysaccharides. The small amounts of free galactose, glucose, fructose, and sucrose make up the low molecular weight sugars. Galacto-oligosaccharides (raffinose, stachyose, and verbascose) comprise approximately 5% of the SB DM, while starch represents less than 1%. The structural carbohydrates can be divided into cotyledon meals and hull polysaccharides. The primary cotyledon meal polysaccharides are arabinogalactan and an acidic polysaccharide that is similar to pectin, whereas the hull contains pectin, hemicelluloses, and cellulose [7].

Although carbohydrate content and carbohydrate composition of SB and SBM have received limited attention compared to their protein and fat constituents, carbohydrates make up a significant part of the SB seed. Typically, SB contains approximately 30–35% carbohydrates. In SBM, carbohydrate content may be as high as 40%. Carbohydrates are usually divided into two main groups based on their physicochemical properties in plant material. The first group, the non-structural carbohydrates, includes low molecular weight sugars, oligosaccharides, and storage polysaccharides. The second group comprises the structural polysaccharides and includes dietary fiber components [8].

Therefore, soybeans are rich in essential nutrients for humans, but during storage and processing these nutrients can be modified or reduced. To minimize adverse changes, minimal washing, fermentation, reduction of hydrogenation temperature and thermal processing below 100 °C for short periods, are recommended [9].

Another study found that the rapid rise in the microbial population of fruit and vegetable discards (FVD) [without sodium metabisulfite (SMB)] resulted in biomass deterioration and substantial dry matter loss and sugar exhaustion [10]

In this study, soybeans were fermented in the laboratory under different fermentation conditions. The factors affecting the fermentation process and soybean nutritional content (proteins, lipids, and carbohydrates) included pH, fermentation temperature, fermentation time, and bacterial density.

At the ICEER 2019 conference presentation, this paper identified the change in the content of protease in the fermentation process, and the paper was published in Energy Reports. However, this paper mentions the change in the content of nutrients such as proteins, lipids, and carbohydrates during soybean fermentation.

Materials and Methods

Materials

Bacillus Subtilis strains were isolated from the biological laboratory of the Department of Agriculture and Aquaculture, Tra Vinh University. Soybeans (Average of 85–87 days after sowing, soybean seeds will be harvested and stored at – 18 °C as a source of raw materials for research [11]. In laboratory, Soybeans are stored in PE bags and sealed before storaged at – 18 °C in 6 months for research. At the experiments, they are put into a triangle bottle at a quantity of 50 g per bottle after steamed) were purchased from the Department of Plant Breeding, Department of Agriculture and Applied Biology, Can Tho University.

Chemicals (Meat Extract Powder, peptone, Glucose—made in India, analysed chemicals—made in Germany or India) were purchased from An Khanh Company and Dong Hanh Phat company.

The fermentation temperature was adjusted using a Memmert incubator, at the low fermentation temperature adjusted by put samples in a closed room with an air conditioner.

Research Methods

Bacterial isolation: *Bacillus* strains were isolated from commercial Natto by spreading on peptone (MPA) gypsum agar at 37 °C for 24 h. The colonies were purified. The colony morphology is then observed and noted, and biochemical reactions were tested in order to select specific strains of interest. The microorganisms were thereafter identified by the sequencing method and chosen strains were preserved in inclined agar at 4 °C.

Soybeans were soaked overnight to expand the outer shell and facilitate its separation. After separating the bean pods, they are rinsed several times to remove the overnight soaking water. Soybeans were put in a 45-min autoclave cycle until the beans were tender, then cooled. Afterward, 50 g quantities of soybeans/sample were placed into triangle bottles.

The previously isolated *Bacillus* spp. strains were then cultured in seeded soybeans at a rate of 1% of 2 cm environmental thickness and incubated at 37 °C. 24 h fermentation time elapsed before the protease and nattokinase enzymes were collected. Preliminary experiments are conducted in order to determine parameter-specific values maximizing protease activity. Each of the parameters (pH, fermentation temperature, bacterial density, and fermentation time, the amount of glucose added to the fermentation medium) are successively treated as variables while the remainder are attributed a fixed-value. At the same time, changes in the content of nutrients such as lipid, carbohydrates and protein were also noted under different fermentation conditions to determine the highest value of each indicator reached at which fermentation condition.

Physical and Chemical Analysis

Determination of reduced sugar content by Lane-Eynon method [12]. Weigh 3 g of the sample to be analyzed, add 50 mL of water, 5 mL of concentrated HCl, and proceed to water bath for 3 h. After hydrolysis, refrigerate immediately. Neutralize with NaOH (use phenolphthalein as color indicator). Reduce impurities with 7 mL Pb $(CH_3COO)_2$, filter the filtrate and dilute to a suitable factor for analysis. Then add 5 mL Fehling A + 5 mL Fehling B + 15 mL of the filtrate to becher. Burn on the stove and titrate. Add 1 mL of the sugar solution (sample solution used for analysis) to a brick red color with no blue sheen each time. Try again by dripping one drop of 1% methylene blue and the boiling solution turns blue back to brick red. Read the results and look up the sugar content spreadsheet.

Determination of total nitrogen content by Kjeldahl method [12]. Weigh 3 g of the sample into a triangle, add 0.5 g of the catalyst (a mixture of K_2SO_4, $CuSO_4$ and Selenium) and 10 mL of concentrated H_2SO_4 and heat until the solution in the triangle bottle is blue in color of $CuSO_4$, then stop. Add another 20 mL of 30% NaOH, close the lock quickly and proceed with nitrogen distillation. Prepare a 250 mL bottle containing 15 mL of 2% Boric acid to collect NH_3. Proceed to distill the solution until the solution in the receiver turns green, put the purple litmus in the NH_3 gas tube. If the purple does not change color, stop. Allow the obtained solution to titrate with 0.01 N H_2SO_4 until it turns pink.

Determination of amino protein content by OPA method [13]. Preparation OPA solution and Standard serine. Sample measurement:

200 μL standard serine + 1.5 mL OPA

200 μL water + 1.5 mL OPA

200 µL of diluted hydrolysis + 1.5 mL OPA, then leave for 2 min, and measure the sample at 340 nm.

Determination of the total lipid content by Soxhlet method [12]. Weigh 3 g of the moistened sample to constant weight, wrap it with filter paper (dried and weigh the weight of the filter paper), and place the sample package in

the extraction tube (Soxhlet). Then add solvent (ether 60–90) about 2/3 bottle and turn on Soxhlet system, fat extraction time is about 24 h. After fat distillation is completed, the packages are dried at 105 °C to constant mass and weighed.

The antioxidant capacity implemented by DPPH method [14]. Determination of antioxidant activity (%DPPH) and IC50 by DPPH free radical scavenging method. The DPPH free radical scavenging capacity of the sample was determined by Fu and Shieh [14]. with a few minor modifications. 3 g of sample were extracted overnight at – 18 °C with 27 mL of methanol. The mixtures were centrifuged by Hermle Labotechnik Z323K Centrifuge at 6000*g* for 10 min. Sucking 3 mL of the supernatants into vitro. Then add 1 mL of DPPH 0.1 mM solution (mixed in 99.5% methanol), mix well and let stand in the dark for 30 min. Samples are always kept away from direct sunlight during the analysis. Optical absorbance is measured at 517 nm. DPPH free radical scavenging ability is determined by the following formula: DPPH (%) = 100 × (ACT − ASP)/ACT (In which: ACT is the optical absorbance of the blank without extract; ASP is Optical absorbance of the sample containing the extract The result reported by the EC50 value is the extract concentration for the DPPH free radical scavenging ability of 50%. A linear correlation is developed, which determines the value of IC50 as a basis for comparing the antioxidant capacity of the samples.The sample with the lower IC50 value, the higher the antioxidant activity. (2,2-Diphenyl-1-picrylhydrazyl): determination of antioxidant activity (%DPPH) and IC50 by DPPH free radical scavenging method.

Table 1 Protein, carbohydrate, lipid, and DPPH contents at different pH levels in fermentation cultures

pH	Lipid content (% dry weight)	Carbohydrate content (% dry weight)	Protein content (% dry weight)	DPPH (%)
6.3	26.41 c	15.66 c	40.01 c	85.14 a
6	**27.04 a**	15.49 c	42.95 b	72.57 b
7	25.71 d	**18.68 a**	44.42 ab	86.81 a
8	26.71 b	17.43 b	**46.37 a**	**87.44 a**

Bold indicates high values in columns

Fixed parameters: fermentation temperature 37 °C, bacteria population 10^2 and 24 h of time fermentation. In the same column, the numbers with the same following letters are not different significant at 5%

Results and Discussion

The moisture content of the soybeans after soaking 61.85% and before fermentation 60.28%.

Nutritional components of soybean before fermentation: Lipid 21.54%, Glucid 17.33%, Protid 37.31% (% dry weight).

As presented in Table 1, carbohydrate content (18.68%) and protease activity (280 U/g) [15] were highest at a pH of 7, while lipid and protein content was low. This result is consistent with the research of Luong and Duong [16] and Slepecky and Hemphill [17], where the authors stated that *Bacillus Subtilis* is capable of biosynthesizing protease, amylase and Catalase enzymes at an appropriate pH of 7.0–7.4, with an optimum pH of 7 [15]. When fermentation produces natto it is necessary to create a product with a high nattokinase content. Therefore, the fermentation culture should be at pH = 7 where the antioxidant capacity is high. Besides, if we want to collect other substances with high levels, we can adjust the pH accordingly according to the research results in Table 1.

At 28 °C, the carbohydrate and protein content following fermentation was highest (21.95% and 47.35%, respectively), while the antioxidant activity was lowest (49.29%). At 33 °C, the carbohydrate, lipid, and protein content were all high with the lipid content at its highest and the DPPH content at a moderate level. At the remaining two fermentation temperatures (35 °C, 38 °C), the nutritional constituents were all at suboptimal quantities, especially at 38 °C, where there were lowest.Therefore, with the results shown in Table 2, depending on the purpose of the experiment, choose the appropriate fermentation temperature to create the desired product.

Fermentation times of 48 h and 60 h yielded the highest levels of nutrients and DPPH antioxidant activity. At 60 h of

Table 2 Protein, carbohydrate, lipid, and DPPH contents at different fermentation temperatures

Fermentation temperature (°C)	Lipid content (% dry weight)	Carbohydrate content (% dry weight)	Protein content (% dry weight)	DPPH (%)
28	26.71 c	**21.95 a**	**47.35 a**	49.29 c
33	**33.38 a**	19.84 a	46.37 a	69.59 b
35	32.92 b	21.31 a	44.42 b	**80.59 a**
38	26.12 d	16.35 b	42.95 b	70.71 b

Bold indicates high values in columns

Fixed parameters: pH = 7, bacteria population 10^2 and fermentation time 24 h. In the same column, the numbers with the same following letters are not different significant at 5%

fermentation, the lipid, protein and antioxidant activity were highest, while the carbohydrate content was decreased. This indicating that the sugar has been used by bacteria to ferment near the end of the duration. The amounts of carbohydrate are no longer synthesized so the bacteria use carbohydrate present in the fermentation environment to create products that further reduce the carbohydrate content.

Carbohydrates are involved in the fermentation process and their quantities are constantly changing, as well as the quantities of proteins and lipids during the fermentation process, which has many factors that are mentioned in some of the following studies. In this study, 860–3 soybeans [11] were used for fermentation with an initial protein ratio of 37.31%. Thus, through the fermentation process, the protein content of soybeans increased in all Tables 1, 2, 3 and 4 of research result (protein content > 40%), confirming the nutritional value of fermented soy products and this is consistent with the study of E. M. Morales about the solid-state fermentation [18].

Quantities of proteins, lipids and sugars are constantly changing as a result of the fermentation process. The factors affecting nutritional changes during fermentation are highlight by research conducted by Tien Man Weng and Ming Tsao Chen [19] on changes in protein concentrations. Fruthermore, research has shown that mixed culture, as opposed single bacterial culture, significantly reduces peroxide values and inhibits lipid oxidation [20].

Distribution of lipids morphology and evolution of lipids during soy sauce production were studied. It was found that oil bodies fused and migrated to the outside of soybean cells after steamed, and further fused to cystidiums. Linoleic acid (C18:2) decreased from 59.35 to 47.75% after 30 days of moromi fermentation. The contents of fatty acids from neutral lipids and free fatty acids increased to 20.98 and 13.47 mg/g, respectively, after moromi fermentation. Fatty acid of phospholipids increased to 8.34 mg/g during koji fermentation and reduced in the prior phase of moromi fermentation [21]. The total lipids from fermented rice bran (FB) decreased from 20.4 to 11.2% in the range between 0 and 120 h of fermentation while phospholipid contents were increased. In fermented bran, oleic, palmitic and linoleic acids prevailed, with a decrease in saturated fatty acids (20%) and increase in the unsaturated ones (5%) [22]. The presence of lipids in wort and beer are important due to their influence on yeast metabolism and beer quality. Lipids can also influence yeast protease activity as well as the production of ethanol [23].

These studies show that fermentation affects the lipids and changes the lipid content during fermentation. The nutritional composition of soybeans, including lipid, glucide, and protein, all increase after fermentation.

Table 4 Protein, carbohydrate, lipid, and DPPH contents at different fermentation time

Fermentation time (h)	Lipid content (% dry weight)	Glucide content (% dry weight)	Protein content (% dry weight)	DPPH (%)
36	29.49 b	19.28 b	46.18 ab	67.17 a
48	29.47 b	**22.08 a**	43.83 b	61.51 a
60	**31.77 a**	16.29 c	**46.37 a**	**64.03 a**

Bold indicates high values in columns

In the same column, the numbers with the same following letters are not different significant at 5%

Conclusion

After specific identification of the Bacterial strain (by align the sequencing to NCBI to find specific strain) was applied to soybean fermentation and valuable results were obtained beside the selection of *Bacillus Subtilis* strain. Results showed that the content of nutrients such as protein, lipid, carbohydrate, and DPPH antioxidant activity varied at different pH, fermentation temperatures and fermentation times, while the bacterial densities had no significant affect on nutrient content and antioxidant activity. Experimental results show the highest concentration of lipids at a pH 6.0, temperature of 33 °C, and 60 h fermentation time. Carbohydrates were highest at pH 7, 28 °C and 48 h, and protein content yielded highest at pH 8, 28 °C, and 60 h of fermentation. The results of the study make it easier to control

Table 3 Protein, carbohydrate, lipid, and DPPH contents at different bacteria densities

Bacteria density (CFU/mL)	Lipid content (% dry weight)	Glucide content (% dry weight)	Protein content (% dry weight)	DPPH (%)
10^2	29.89	19.33	45.56	65.65
10^3	30.56	18.43	45.56	62.26
10^4	30.48	18.75	44.74	64.08
10^5	30.35	19.89	45.72	66.15
10^6	29.93	19.68	45.72	61.05

Fixed parameters: pH = 7, fermentation temperature 33 °C. In the same column, the numbers with the same following letters are not different significant at 5%

the fermentation process of soybeans to produce desired products by changing the fermentation conditions. Besides controlling the fermentation process to increase biomass, collecting the desired product for this research will be able to connect with many other sciences to optimize production processes. All researches are in high-temperature conditions suitable for high humid tropical conditions.

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