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Nguyen Phuoc Minh
Tra Vinh University, Vietnam

Correspondence:
Nguyen Phuoc Minh
Tra Vinh University, Vietnam.

Investigation of Vegetable Soy Sauce Fermentation by *Bacillus* Sp.

Nguyen Phuoc Minh

Abstract
In addition to essential nutrients, soybean products, especially fermented soybean products, contain various functional components including peptides, isoflavonoids and more. Our research focuses on vegetable soy sauce fermentation with following results: bacteria inoculation 1%, salt 15%, temperature 45 ^{o}C in direct sunlight, fermentation in 10 days to get the best soy sauce quality with optimal acid amin content.

Keywords: soybean, bacteria, salt, temperature, fermentation, soy sauce

1. Introduction
Soy sauce is a traditional fermented food in Vietnam that has been practiced long time ago. Traditionally, soy sauce has been used in Japan and several oriental countries and is presently used as a liquid seasoning in cooking worldwide. Soy sauces are the main condiments for foods and table-top seasoning in the most of the Asian countries. Studies have shown that soy sauce has a promotive effect on human health, such as on iron absorption in human subjects (Baynes *et al*., 1990). Indeed, soy sauce has been produced for centuries under natural conditions. Soy sauce is one of the world's oldest condiments and has been used in China for more than 2,500 years. Soy sauce is a hydrolysis product of the soybean. Soy sauce is a dark brown liquid with a pleasant aroma, used primarily as flavoring agents for meat, poultry, fish, vegetables and rice. Its high salt content of about 18% and distinct flavor makes it a useful adjunct for many of the bland food products in which it is used.
The fermentation of the soy sauce is very complicated, including starch saccharification, sugar degradation, alcoholic fermentation, proteolysis, aroma formation, pantothenic acid reaction and Maillard reaction etc. The conventional batch process of brewing soy sauce starts with a solid-state fermentation of *Aspergillus* species on a mixture of soybeans and wheat with a certain ratio (Van der Sluis *et al.*, 2001). During an aerobic fermentation for 2-3 days at 30 °C, *Aspergillus* produces extracellular enzymes. After that, the moulded raw materials are mixed with saline water. The brine solution is kept around 40-50°C. In the brine solution the *Aspergillus* enzymes continue to hydrolyze the soybeans and wheat and as a result, the soy sauce was obtained (Yong and Wood, 1977).
Soy sauce in Vietnam is naturally brewed by two step fermentation processes, namely, kojifermentation and moromi fermentations (Ta Yeong Wu *et al.*, 2010). The koji fermentation process involves the mixtures of soybean andwheat flour with the inoculation of *Aspergillus oryzae*. During koji fermentation, the addition of *A. oryzae* excretes protease, amylase and other enzymes. These enzymes will hydrolyze the raw materials into simplerforms. Proteolytic enzymes will convert soy beans pro-teins into peptides and amino acids while amylase enzymes will hydrolyze starch into simple sugars. The hydrolyzed nutrients will be utilized by the yeast and bacteria in moromi stage.
In Vietnam, the moromi fermentation process is usually carried out in closed tanks that are kept under the sun for3 - 4 months. In this process, there are three important types of microorganisms, which play crucial roles for a good soy sauce production. The simpler sugars from koji fermentation are mainly metabolized into lactic acid and acetic acid by *Pediococcus halophilus* (Iwasaki *et al.*, 1993).
The qualitative and quantitative composition of soybean components is dramatically changed by physical and enzymatic processes during the preparation of soy-based foods (L.M. Baek *et al.*, 2008; Garcia *et al.*, 1997; Jang *et al.*, 2008; Y.W. Lee *et al.*, 2007; Nakajima *et al.*, 2005; J.S. Park et al., 1994; Yamabe *et al.*, 2007). Fermentation is an excellent processing method for improving nutritional and functional properties of soybeans due to the increased content of small bioactive compounds.

The conformation of soy protein (glycinin) is easily changed by heat (steaming) and salt (K.S. Kim *et al.*, 2004). The large protein, lipid, and carbohydrate molecules in raw soybean are broken down by enzymatic hydrolysis during fermentation to small molecules such as peptides, amino acids, fatty acids, and sugars, which are responsible for the unique sensory and functional properties of the final products. Short-term fermented soy foods such as chungkukjang, which are fermented with *B. subtilis*, for less than 72 hr have a much greater concentration of large molecules than do long-term fermented foods including meju and doenjang, which are fermented for more than 6 months with *Bacillus* and *Aspergilus* species from rice straw and koji, respectively (Jang *et al.*, 2008; Y.W. Lee *et al.*, 2007; J.S. Park *et al.*, 1994; Yamabe et al., 2007). Proteomic analysis for soluble proteins from chungkukjang at different fermentation periods suggested that most of the soluble soy proteins were degraded into smaller forms within 20 hr, and many microbial proteins, such as mucilage proteins which, are assimilated into the bacterial biomass, dominated by the soluble protein fraction. The proteomic profile of chungkukjang was very different from that of natto, in terms of the 2-D gel protein profile (Santos et al., 2007).

Shin-ichi Sugiyama (1984) select micro-organisms for use in the fermentation of soy sauce. Micro-organisms related to soy sauce production are mainly koji mold (*Aspergillus oryzae* or *Aspergillus sojae*), lactic acid bacteria (*Pediococcus halophilus*) and osmophilic yeasts (*Saccharomyces rouxii*, *Candida (Torulopsis) versatilis*, *Candida (Torulopsis) etchellsii*). B. S. Luh (1995) covered the method for production of fermented soy sauce, and that for acid-hydrolysis of defatted soy bean proteins. The microorganisms involved in soy sauce production, and biochemical and chemical changes in soy bean and wheat during fermentation influence greatly the sensory attributes and quality of soy sauce. Recent progress in industrialization of soy sauce manufacture is discussed. Tin Mar Lynn *et al.*, (2013) studied on the production of fermented soybean sauce by using *Aspergillus oryzae* and *Aspergillus flavus*.

Purpose of our research is to investigate the effect of different factors influencing to vegetable sauce fermentation including bacteria inoculum, salt concentration, temperature, fermentation time.

2. Material & Method

2.1 Material

Soy bean, salt are purchased in Tra Vinh province, Vietnam. *Bacillus* sp is supplied by Pasteur Institute HCM City, Vietnam. Fermented sauce flavour is supported from Swiss.

2.2 Research method

Survey the effect of salt concentration, bacteria inoculum and temperature to the protein hydrolization, soy sauce quality and sensory. Experiments are randomly designed with 3 factors with 3 different levels.

Table 1: Three experimental factors and three levels

Factor	Symbol	Level 1st	Level 2nd	Level 3rd
Bacteria	V	1%	1.5%	2%
Temperature	T	Sunlight	Normal	
Salt concentration	M	5%	10%	15%

2.3 Statiscal analysis

All data are processed by ANOVA (Stagraphics) for three factors to check the significant difference via LSD.

3. Result & Discussion

3.1 Effect of bacteria inoculum, salt concentration, and temperature to the protein hydrolization to acid amin formation

Table 2: Acid amin formation by different fermentation times

Sample	Fermentation time (N amin g/l)		
	7th day	10th day	13th day
M1V1T1	4.597	5.618	4.529
M1V2T1	4.057	4.545	3.696
M1V3T1	2.241	5.665	3.934
M2V1T1	3.429	7.295	1.996
M2V2T1	2.370	10.536	4.359
M2V3T1	3.076	9.274	6.056
M3V1T1	3.656	8.210	5.886
M3V2T1	2.818	8.924	2.764
M3V3T1	2.102	9.467	4.359
M1V1T2	2.010	8.381	4.765
M1V2T2	3.711	5.377	3.340
M1V3T2	4.637	6.158	3.409
M2V1T2	3.118	8.398	3.714
M2V2T2	3.231	10.638	9.042
M2V3T2	4.016	10.535	2.713
M3V1T2	3.981	11.181	4.036

From table 2, we see the acid amin content increases from 7th to 10th day of fermentation. The faster growth of bacteria is, the more acid amin accumulates until the 13th day. This phenomenon happens owing to bacteria consuming acid amin during fermentation.

3.2 Effect of bacteria inoculum, salt concentration, and temperature to the protein hydrolization to ammoniac formation

Table 3: Amomniac formation by different fermentation times

Sample	Fermentation time (N amoniac g/l)		
	7th day	**10th day**	**13th day**
M1V1T1	1.003	1.326	1.258
M1V2T1	1.207	1.615	1.258
M1V3T1	1.343	1.615	1.479
M2V1T1	1.275	1.105	1.377
M2V2T1	1.326	1.224	1.054
M2V3T1	1.292	1.926	1.224
M3V1T1	1.275	1.870	1.207
M3V2T1	1.326	1.156	1.343
M3V3T1	1.258	1.173	1.241
M1V1T2	1.462	1.139	1.768
M1V2T2	1.105	1.343	1.513
M1V3T2	1.411	1.122	1.258
M2V1T2	2.482	1.122	1.326
M2V2T2	1.921	1.122	1.411
M2V3T2	1.360	1.105	1.394
M3V1T2	1.955	1.139	1.564
M3V2T2	1.258	1.224	1.479
M3V3T2	1.224	1.258	1.377

From table 3, ammoniac content changes not significantly during fermentation.

Table 4: Effect of bacteria to the protein hydrolization into acid amin

Bacteria inoculum (%)	Fermentation time, N amin (g/l)		
	7th day	**10th day**	**13th day**
1.0	3.47^{a}	8.55^{a}	4.21^{a}
1.5	3.25^{a}	8.03^{a}	4.63^{a}
2.0	3.37^{a}	8.50^{a}	3.90^{a}

From table 4, there is not significant difference regarding to the effect of bacteria to the protein hydrolization to acid amin formation.

Table 5: Effect of salt concentration to acid amin formation

Salt concentration (%)	Fermentation time, N amin (g/l)		
	7th day	**10th day**	**13th day**
5	3.54^{a}	5.96^{a}	3.99^{a}
10	3.21^{a}	9.45^{b}	4.71^{a}
15	3.34^{a}	9.69^{b}	4.11^{a}

At 7th day of fermentation, there is not statistically significant difference. At 10th day with salt concentration 5 – 10% and 5 – 15%, there is statistically significant difference. At 13 th day, there is not statistically significant difference. 5% salt concentration is not enough to inhibit bacteria so ammoniac formation is too much and acid amin is too low.

Table 6: Effect of temperature to the protein hydrolization to acid amin formation

Temperature (ºC)	Fermentatio time, N amin g/l		
	7th day	**10th day**	**13th day**
30^{0}C	3.15^{a}	7.73^{a}	4.25^{a}
45^{0}C	3.58^{a}	9.00^{b}	4.29^{a}

Regarding to effect of temperature to fermentation, the statistically significant difference is only noticed at 10th day of fermentation From table 7, there is not statistically significant difference regarding to bacteria during fermentation.

Table 7: Effect of bacteria to ammoniac formation

Bacteria	Fermentation time, N amoniac g/l		
	7th day	**10th day**	**13th day**
1.0	1.57^{a}	1.28^{a}	1.42^{a}
1.5	1.36^{a}	1.28^{a}	1.34^{a}
2.0	1.32^{a}	1.37^{a}	1.39^{a}

Table 8: Effect of salt concentration to ammoniac formation

Salt concentration (%)	Fermentation time, N amoniac g/l		
	7th day	10th day	13th day
5	1.26[a]	1.36[a]	1.42[a]
10	1.61[a]	1.27[a]	1.30[a]
15	1.38[a]	1.30[a]	1.37[a]

From table 8, there is not statistically significant difference regarding to salt concentration during fermentation.

Table 9: Effect of temperature to ammoniac formation

Temperature (ºC)	Fermentation time, N amoniac (g/l)		
	7th day	10th day	13th day
30	1.26[a]	1.45[a]	1.27[a]
45	1.57[b]	1.18[b]	1.45[b]

During fermentation, there is statistically significant difference regarding to temperature to ammoniac formation. Soy sauce fermented under sunlight has more ammoniac than in normal room temperature at 7th day and 13th day.

Figure 1, 2 and 3 show more clearly about the effect of salt concentration, bacteria and temperature to the protein hydrolization to create acid amin.

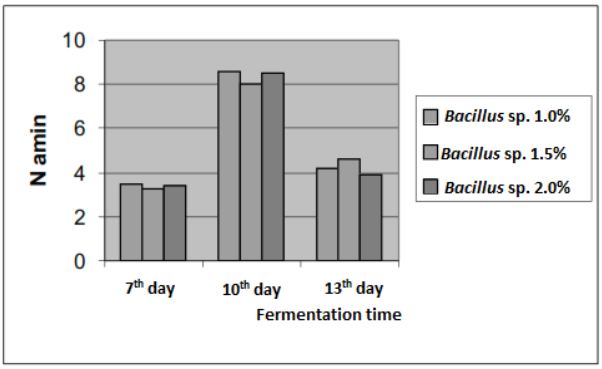

Fig 1: Effect of bacteria to the protein hydrolization to acid amin accumulation during fermentation

From figure 1, in the same day at 7th and 10th day, bacteria inoculum 1% creates the highest acid amin.

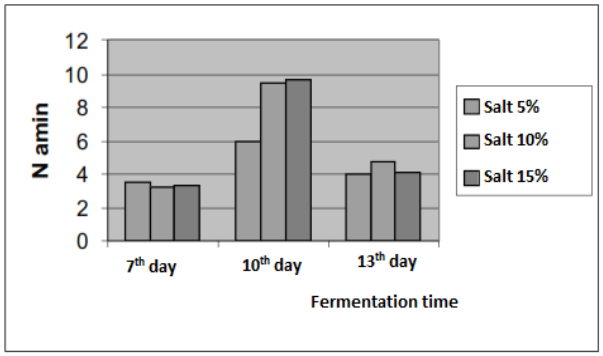

Fig 2: Effect of salt concentration to the protein hydrolization to acid amin accumulation

At 7th day of fermentation, 5% salt concentration shows the highest acid amin accumulation.

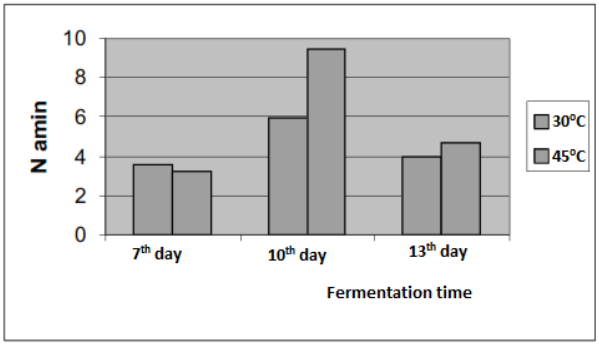

Fig 3: Effect of temperature to the protein hydrolozation to acid amin accumulation

From figure 3, 7th day fermentation in normal room temperature has more acid amin than sunlight. At normal room temperature 30 °C, *Bacillus* can grow effectively to produce more acid amin. At 10th and 13th day of fermentation, sunlight will create more acid amin.

Figures 4, 5 and 6 will show clearly about the effect of salt concentration, bacteria, and temperature to ammoniac accumulation.

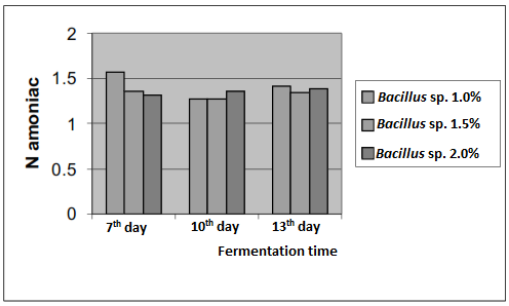

Fig 4: Effect of bacteria to ammoniac accumulation
At 7th day, bacteria 1% will show the highest ammoniac content.

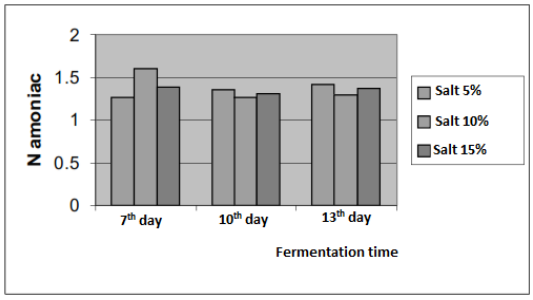

Fig 5: Effect of salt concentration to ammoniac accumulation
At 7th day of fermentation, 10% salt concentration shows the highest ammoniac content. Meanwhile 5% salt concentration shows the lowest ammoniac concentration.

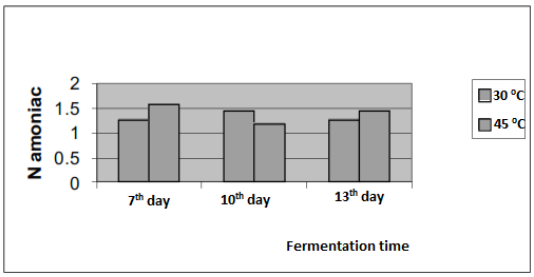

Fig 6: Effect of temperature to ammoniac accumulation
At 7th day of fermentation under sunlight, ammoniac accumulation is highest.

Table 10: Compostion in the fermented vegetable soy sauce

Sample	Protein (g/l)				
M3V1T2	Salt	N amoniac	N amin	Total N	N formol
	100	0	12	25	12

M: salt 15%; V: *Bacillus* sp. 1%; Temperature 45 ^{0}C

Fig 7: Vegetable fermented soy sauce
After fermentation, we see quite clearly the effect of temperature to ammoniac accumulation in direct sunlight at 7th day and 13th day.

4. Conclusion

Soy sauce has been made for centuries by traditional methods, and consumed as the source of protein and vitamins. Soy sauce contains essential amino acids such as Valine, Tryptophan, Lysine, and Histidine and also contains vitamins (especially vitamin B6) and antioxidants (isoflavones). Fermentation increases protein content, eliminates trypsin inhibitors, and reduces the peptide size in soybean meals. These effects of fermentation might make soy foods more useful in human diets as a functional food and benefit livestock as a novel feed ingredient. We recommend further studies relating to screening and selecting bacteria strains as well as preservation condition.

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