

ORIGINAL RESEARCH ARTICLE

Study on the Production of Fermented Soybean Sauce by Using *Aspergillus oryzae* and *Aspergillus flavus*Tin Mar Lynn ^{*1}, Kyaw Nyein Aye ², Khin Mar Khaing ²

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ABSTRACT

Soy sauce has been made for centuries by traditional methods, and consumed as the source of protein and vitamins. In this research work, the two fermenting microbes *Aspergillus oryzae* and *Aspergillus flavus* were mainly employed to produce soya sauce. *Aspergillus oryzae* was isolated from moldy soybeans. Also, *Aspergillus flavus* strain was received from the Department of Biotechnology, Yangon Technological University, Myanmar and recharacterized to confirm the correct strain. Four types of soy sauce products obtained from separated fermentation of each strain within two periods of different fermentation time (1.5 months and 3 months) and were analyzed for protein content, fat content, reducing sugar content, and alcohol content respectively after fermentation. Analysis of commercial soy sauce product from local market was also done as a comparative study for locally strain produced products. Finally, amino acid composition of each product was detected by the Thin Layer Chromatographic method as a part of research work. By comparing the physical and chemical analysis results, soy sauce produced by *A. oryzae*, brine ageing time 3 months had the best quality.

Keywords: Soy sauce, *Aspergillus oryzae*, *Aspergillus flavus*, Thin Layer Chromatography, Brain ageing.

1. INTRODUCTION

Nowadays, the world's population increases rapidly every day. Also, food demand is increasing as

every time. Protein is an important type in a balanced diet. Soybean product has a large amount of protein content, and so it was consumed as the protein source. Soy sauce contains essential amino acids such as Valine, Tryptophan, Lysine, and Histidine and also contains vitamins (especially vitamin B6) and antioxidants (isoflavones).¹

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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 preparation of soy sauce is known to involve the action of molds, yeasts, and bacteria such as *Aspergillus oryzae*, *Zygosaccharomyces soya*, and *Lactobacillus* species. Yokosuka and Sasaki² stated that *Aspergillus oryzae* and *A. soyae* are used by manufacturer. These fungal Aspergilli species are widely found in various sources. Some species are food grade species and others are pathogenic for human beings. *Aspergillus oryzae* has been used in soybean fermentation for several years and it was known as koji mold.

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.

In this research study, emphasis was made on the isolation and identification of *Aspergillus oryzae*

from the moldy soybean sauce. The strain characterization of receiving *Aspergillus flavus* strain was examined. The fermenting activities on the main substrate soybean were studied on two conditions: 1.5 months fermentation time and 3 month period, respectively. Microbial safety of the end products was also examined in this work. The presence of some amino acid in each produced soy sauce was also displayed by the Thin Layer Chromatography method.

The objectives of this research work are: to promote the role of soybean food consumption, to improve the traditional methods of soybean food production and to study and investigate the quality improvement of soy sauce by using *Aspergillus oryzae*.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Media and Chemicals

Culture media, media ingredients and other chemicals used were from "Australia Medical Diagnostics (AMD) Co., Ltd", "HiMedia Laboratories Limited, Mumbai, India", and Analytical grade chemicals available from local markets have also been used.

2.1.2. Microorganisms

Aspergillus oryzae were isolated from moldy soybeans. *Aspergillus flavus* were isolated from the

chili. These two microorganisms are used for the production of soy sauce. Strain recharacterization of *Aspergillus flavus*. The flavours were also done to confirm the correct strain.

2.1.3. Raw Materials for Soy Sauce Production

12 kg soybeans, 12 kg roasted wheat flour and 5 kg salt were used for soy sauce production. They were bought from the local market in Yangon.

2.2. Methods

2.2.1. Isolation of *Aspergillus oryzae*

The agar plate method was used to isolate *Aspergillus* colonies occurring in moldy soybeans. The moldy soybeans were placed on Czapek's Dox Agar, introduction 4 each in 9 cm Petri dishes. The plates were incubated at room temperature for 1 to 7 days. After incubation for 48 hours, the plates were observed in young fungus colonies.

2.2.2. Identification of *Aspergillus oryzae*

Aspergillus oryzae can be identified by colony morphology and microscopic characteristics. Hypo-septate, reproductive organs, the structure of fruiting organs, details of spores of the fungus were observed and matched with the synoptically keys of Thom and Church (1926), Thom and Raper (1945) and Raper and Fennell (1965).³⁻⁵ Measurements of vegetative and reproductive parts of the fungus were also taken from the state micrometer set.

To identify the colony morphology, *A. oryzae* were incubated on Czapek's Dox Agar, Sabouraud Dextrose Agar, Potato Dextrose Agar and Malt Agar. And then colonies were observed by colony size, color, reverse position and growth pattern.

The only one biochemical test for *Aspergillus oryzae* is Kojic acid test. Among the *Aspergillus* species, *A. albus*, *A. candidus*, *A. nidulans*, and *A. oryzae* produce Kojic acid.

2.2.3. Soy Sauce Production

An amount of 2 kg soybeans was soaked in water for overnight. Next, the soaked soybeans were cooked in autoclave at 121°C with 15 psi for 30 minutes. After that they were drained for 3-4 hours in a contamination free area. And then they were mixed with 2 kg of wheat flour. Finally, *Aspergillus oryzae* were added to the mixture of soybeans and wheat flour by spore count method. The inoculum size was 2.4×10^8 . The incubation period was 5 days. The same steps were made for the production of soy sauce by *Aspergillus flavus*.

After 5 days of fermentation by fungi, the mixtures were fermented by salt brine. This was called the brine fermentation stage. The brine fermentation was stirred at intervals of 2 days to supply more oxygen into the glass containers during brine ageing. After ageing for 1-3 months, the soy sauce was filtered and pasteurized at 70°C for 30 minutes. And then soy sauce was ready for consumption.

Four types of soy sauces were produced in this experiment for three replicates. They were soy sauce produced by *A. oryzae* (brine ageing time 1.5 months), soy sauce produced by *A. oryzae* (brine ageing time 3 months), soy sauce produced by *A. flavus* (brine ageing time 1.5 months), soy sauce produced by *A. flavus* (brine ageing time 3 months). Each of these soy sauces was measured by the appropriate parameters to determine the product qualities. One type of soy sauce from local market was also measured by the above parameters to compare the qualities.

Physical Analysis

- (1) pH measurements
- (2) Viscosity measurements

Chemical Analysis

- (1) Protein analysis
- (2) Fat analysis
- (3) Determination of reducing sugar by Lane and Eynon method
- (4) Alcohol analysis
- (5) Salt analysis

2.2.4. Microbial Safety for Soy Sauce Products

Each aliquot with 25 μ l of four types of soy sauce sample was spread on the nutrient agar before and after pasteurization. The plate was inverted and

incubated at 30°C for one day and the individual colonies developed on the nutrient agar were examined for microbial safety of the products.

2.2.5. Separation and Identification of Amino Acids in Soy Sauces by One Dimensional TLC

Preparation of solvent system

The solvent system was prepared by mixing n-butanol (40 ml), acetic acid (10 ml) and water (10 ml).

Preparation of standard amino acid solution

Standard solutions of nine different amino acids were prepared in 75% ethanol (v/v) with the addition.

Preparation of 0.2 % ninhydrin reagent

Ninhydrin powder (0.2 gram) was dissolved in 100 ml acetone.

Preparation of Sample

5 ml of sample was put into the separation funnel. 5ml of n-butanol was added into the funnel and shaken vigorously. The layers were separated by placing in standing position for 15 minutes. Then the upper layer was taken to use for TLC method.⁶⁻⁸

3. RESULTS

The isolation of *A. oryzae* was done by the agar plate method in Czapek's Dox agar, selective agar. The rate of growth was very rapid. The microscopic

morphology and cultural characteristics of *A. oryzae* and *A. flavus* was shown in Figure 1, 2, 3 and 4. The species was identified by comparing with the reference (Raper and Fennel, 1965).⁵

The colony morphology of *A. oryzae* was examined by plating on four different media and the results were shown in Table 1.

3.1. Biochemical Characteristics

Red blood color was obtained by the filtrates of *A. oryzae* and that of *Aspergillus flavus*. The flavors were not changed in Kojic acid test. On account of these character the specimen was identified tentatively as *Aspergillus oryzae*.

3.2. Soy Sauce Production

Four types of soy sauces were produced in this experiment. The yield of the finished products was 90% of the salt brine.

3.3. Physical Analysis

The pH and viscosity of soy sauces were measured and the results were described in Table 2.

3.4. Chemical Analysis

The protein%, fat%, reducing sugar% and alcohol% of soy sauces were measured in this experiment and the results were shown in Table 3.

The salt content of soy sauces was described in Table 4.

3.5. Microbial Safety of Soy Sauce Products

Some colony of Bacillus species were observed in before pasteurized sample. But, no microorganism was observed in after pasteurization sample.

3.6. Separation and Identification of Amino Acids in Protein Hydrolysate of Soy Sauces by TLC

By the comparison of R_f values of individual standard amino acids and sample hydrolyzate amino acids, the results were shown in Table 5.

Table 1: Colonial Morphology and Growth Characteristics of *A. oryzae* on Different Media

Types of media	Growth pattern	Size of colony (inch)	Surface colony color	Reverse colony color
Czapek's Dox agar	Growth rate rapidly	2	Brownish yellow	White
SDA	More rapidly of growth	3	Dark yellow	Brownish yellow
PDA	Growth rate moderately, (with other contaminants)	1.5	Pale yellow	Yellowish brown
Malt Extract agar	Growth rate slowly, present many contamination	1	yellow	Yellowish brown

Table 2: pH and Viscosity Measurements of Soy Sauces at Room Temperature

Types of Soy Sauces	pH	Viscosity (Centipoises)
S _{ory(1.5)}	5.14	1.6
S _{ory(3)}	4.84	1.739
S _{fla(1.5)}	5.21	1.6
S _{fla(3)}	4.99	1.922
S _(Wai Weng)	4.7	3.561

Table 3: Protein%, Fat%, Reducing Sugar%, Alcohol% of Soy Sauce

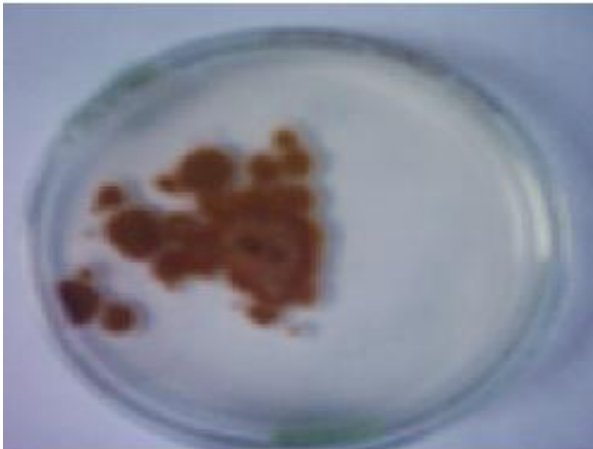
Types of Soy Sauces	Protein %	Fat %	Reducing Sugar %	Alcohol %
S _{ory(1.5)}	2.98±0.18	0.99±0.175	4.15±0.26	0.99
S _{ory(3)}	3.88±0.29	0.048±0.007	5.3±0.42	1.00
S _{fla(1.5)}	2.43±0.37	0.99±0.21	2.71±0.36	0.99
S _{fla(3)}	3.43±0.55	0.032±0.009	3.1±0.27	0.99
S _(Wai Weng)	2.05±0.0.14	0.077±0.008	9.56±0.85	0.33

Table 4: Salt Content of Soy Sauce Products

Types of Soy Sauces	Salt content (ppm)
S _{ory(1.5)}	140
S _{ory(3)}	155
S _{fla(1.5)}	140
S _{fla(3)}	150
S _(Wai Weng)	160

Table 5: R_f values of Individual Standard Amino Acids and Amino Acids in the Protein Hydrolyzate of Soy Sauces

No	Amino Acids	R _f values (standard)	R _f values of A. flavus products	R _f values of A.oryzae product	R _f values for Wai Weng
1	Valine	0.396	0.396	0.385	0.396
2	Threonine	0.231	0.233	-	-
3	Phenylalanine	0.462	-	-	-
4	Lysine	0.044	-	0.032	-
5	Isoleucine	0.495	-	-	-
6	Histidine	0.055	-	0.088	0.088
7	Leucine	0.593	-	-	-
8	Methionine	0.407	-	-	-
9	Tryptophan	0.516	0.516	0.516	-

**Figure 1:** Microscopic Morphology of *A. oryzae***Figure 2:** Microscopic Morphology of *A. flavus***Figure 3:** *A. oryzae* on Czapek's Dox agar**Figure 4:** *A. flavus* on Czapek's Dox Agar

4. DISCUSSION

To obtain the pure culture of *Aspergillus oryzae* was one of the main research works. Czapek's Dox medium is a selective medium and were continually employed in subculture preparation until pure culture was obtained. Morphological characteristics such as growth rate, colony size, color and conidiophore formation etc. were enough to

confirm the correct strain of *Aspergillus oryzae*. Moreover, according to Table 1, *A. oryzae* grow rapidly in Czapek's Dox agar and Sabouraud dextrose agar. But, potato dextrose agar and malt extract agar gave slower growth rate. This may be due to other contaminating microbes which inhibit the growth of *A. oryzae*. In the comparison of

colony size, the smallest colonial size was found in malt agar.

In the study of biochemical test with Kojic acid test, only *A. oryzae* changed into red blood color. *A. flavus* remained without any changes. From this point, we can distinguish the species identification of *A. oryzae* and *A. flavus* fungal strain.

According to the observed data in Table 2, the surest we say was that the smaller the pH value, the higher the viscosity in soy sauce products. But, all pH values existed in the specified range of soy sauce products (within 4.6-5.2). In viscosity measurements, S_{ory} (1.5) and S_{fla} (1.5) had the same value in each product.

In the study of protein contents, Table 3 indicated that S_{ory} (3) had better protein content than that of others. Moreover, its fat % and reducing sugar % were reliable for commercial production.

In the study of salt content described in Table 4, S_{ory} (1.5) and S_{fla} (1.5) had the same content of 140 ppm respectively.

As a part of research work, the presence of amino acids in soy sauce products was detected by Thin Layer Chromatographic technique. According to Table 5, only three amino acids (Valine, Threonine and Tryptophan) were present in *A. flavus* product. *Aspergillus oryzae* product contributes four amino acids (Valine, Lysine, Histidine and Tryptophan). However, commercial “Wai Weng” product

comprises only the two amino acids (Valine and Histidine). So, *A. oryzae* product had more suitable for commercial production as a human diet. Soy sauce produced by *A. oryzae* contains more amino acids than soy sauce produced by *A. flavus* because *A. oryzae* produced peptidase and proteinase than *A. flavus*.

By comparing the brine ageing time (1.5 months and 3 months), better results were obtained from 3 months. So, brine ageing time was also important for quality improvement of soy sauce.

5. CONCLUSION

The two fermented fungal strains *A. oryzae* and *A. flavus* were mainly employed throughout this study. Not only morphological, microscopical and biochemical test but also culture test on four different media were studied. Analysis of soy sauce product was performed by determining pH, viscosity, and protein %, fat %, reducing sugar %, alcohol %, salt content and finally amino acid composition. Produced soy sauces S_{ory} (1.5) and S_{fla} (1.5) had better viscosity activities (1.6 cp) and S_{ory} (3) had more protein content (3.88) than that of the other types. In *A. flavus* fermented product, only three amino acids (Valine, Threonine and Tryptophan) were found. *A. oryzae* fermented soy sauce had four amino acids composition (Valine, Lysine, Histidine and Tryptophan). But, in the analysis of commercial product (Wai Weng), only two amino acids (Valine and Histidine) were found.

Analysis of amino acid composition was performed by one dimensional Thin Layer Chromatographic method (TLC). In the overview study of this research work, *S_{ory}* (3) had better condition than that of the other types.

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