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OPTIMIZATION OF BLACK SOYBEAN (*GLYCINE SOJA* (L) MERRIT) AS SOY SAUCE INGREDIENTS THROUGH *RHIZOPUS OLIGOSPORUS* FERMENTATION, *ASPERGILLUS SOJAE* AND ITS CONSORTIUM

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ABSTRACT

The use of black soybeans is limited to the manufacture of soy sauce, which is the case with yellow soybeans which were already popular earlier, therefore this study aims to optimize the analysis of protein and amino acids fermented by R. oligosporus, A. sojae, and their consortium. The research method used was Completely Randomized Design (CRD) and factorial pattern. The data obtained were collected and processed by analysis of variance (ANOVA). If there is a significant difference in the controls, then continue with Duncan's multiple range test. The results showed that the best mushroom that could increase crude protein was the R oligosporus + A sojae consortium, with an increase of 58.11%, an inoculum dose of 2 grams and a fermentation time of 96 hours. The best research results were then analyzed for amino acids, eighteen types of amino acids were obtained, which consisted of essential and non-essential amino acids, the highest essential amino acid was leucine (3.17%) which gave a savory and delicious taste to soy sauce.

KEYWORDS: Analysis, Black soybean, R oligosporus, A sojae and consortium

INTRODUCTION

The most widely used soybean in Indonesia is yellow soybean. This soybean can be used as side dishes and snacks, especially for the lower middle class. As a source of protein, soybeans can be processed into various foods and beverages such as tempeh, tofu, soy sauce, fried soybeans, soy flour, soy milk, and others. Black soybeans originate from China and then spread to various countries such as countries in Latin America, South America, and Asian countries. In Indonesia, this soybean is widely grown in Java Island, Lampung Province, West Nusa Tenggara Province, and Bali Province. Black soybean contains anthocyanins which function as antioxidants whose activity is higher than vitamins E and C. Black

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Vol. 4, Issue.4, July-Aug 2023, page no. 60-69

soybeans are also a source of vegetable protein with an average protein content of 37% with the highest amino acid content, namely tyrosine (Nurrahman 2015) [14]. Soybean is generally used as an ingredient in making soy sauce, a mixture of peanut brittle and tempeh. As the main ingredient for making soy sauce, black soybean has a high nutritional content, especially protein and carbohydrates. The amino acids leucine and lysine in black soybeans produce a delicious and savory taste that is unique to soy sauce. Black soybeans are preferred because they can give a natural black color, are shiny, and have a delicious and tasty taste (Ginting, et al. 2015) [7]. Food can be processed in various ways such as fermentation, non-fermentation and fortification. Examples of foods that go through the fermentation process are soy sauce, tauco, miso, natto, tofu and soy milk. While non-fermented foods include fresh soybeans, tofu, soy milk, ice cream, burgers, synthetic meat and others. Fortification materials derived from soybean flour are rich in nutrients (Burssens, et al. 2011) [5]. Soy sauce is a fermented food that is used as a flavoring and coloring in food. In Indonesia there is sweet and salty soy sauce. Sweet soy sauce has a thick texture; whereas soy sauce has a thinner texture because it contains more salt (Suprapti 2005) [18]. In Japan, there are five types of soy sauce based on their quality, namely koikuchi-shoyu (plain soy sauce), usukuchi-shoyu (lightly colored soy sauce), tamari-shoyu, soshikomi-shoyu, and shiro-shoyu. These types of soy sauce are classified into three classes, namely (1) based on sensory characteristics, such as taste & aroma; (2) analytical values for nitrogen & alcohol; and (3) dissolved solids content (Schueller 1996) [17].

In Indonesia, black soybean is widely used as a functional food. Therefore, a new and effective strategy is needed to increase the nutritional value of black soybeans, one of which is by fermentation using R oligosporus, A sojae and a consortium of both.

MATERIALS AND METHODS

Material

The materials used in this study were black soybean (Glycine soja (L) Merrit), R. oligosporus and A. sojae microbes.

Research methods

This study used a 3x3x3 factorial Completely Randomized Design (CRD) with three replications. The first factor is the type of microbe (m), namely m1 = R oligosporus, m2 = A. sojae and m3 = m1 + m2; the second factor is the fermentation time (w) consisting of w1 = 48 hours, m2 = 96 hours and m3 = 144 hours; and the third factor is the dose (d) consisting of d1 = 2 grams, d2 = 3 grams and d3 = 4 grams.

Data analysis

Data were analyzed using ANOVA (Analysis of Variance). If there is a significant difference, continue with Duncan's multiple range test.

Protein Analysis (Kjeldahl Method)

Determination of protein content was measured by the Kjeldahl method according to AOAC (1995) [3]. This method is based on the determination of total nitrogen in the sample. This analysis consists of three stages, namely digestion, neutralization & distillation, and titration. The percentage of nitrogen in the sample is calculated using the following formula:

$$\%N = \frac{(ml\ HCL\ Sample - Blang)\ \times\ Normality \times\ 14.007\ \times\ 100}{sample}$$

Protein content is determined using a conversion factor (F). The change in nitrogen content to protein content is calculated by the following formula:

$$\%\text{Protein} = \%\text{N} \times \text{F}$$

Amino Acid Analysis

In this study, the analysis of amino acids from fermented black soybeans used the HPLC method (AOAC 1995) [3]. Protein samples were washed, rinsed, and dried in the oven. After that, 2-5 g of protein sample was put into an 18x150 nm Pyrex tube. Next, 1 ml of 6 N HCl was added to the tube. Then, the tube is frozen in acetone ice. After being frozen, the air is removed so that the condition is vacuumed, then it is frozen and vacuumed again. This procedure is repeated one more time. If there are air bubbles, 1-2 ml of n-octyl is added to the tube followed by a vacuum process for 20 minutes and closed tightly. After reaching room temperature, the tubes were put into the oven at 110°C for 22-24 hours. This mixture is cooled, then transferred to the evaporator flask. The tube was washed with 2 ml of 0.01 N HCl and transferred to the evaporator flask (This work was repeated 2-3 times). The contents of the evaporator flask are drained and the evaporator is vacuumed. In the dry state, 10-20 ml of deionized distilled water is added, and allowed to dry. After drying, 10 ml of 0.01 N HCl was added, and the solution was ready to be injected into the HPLC.

$$\%Amino\ Acid = \frac{Mikcomoles\ of\ Amino\ Acid\ \times\ Amino\ Acid\ Molecular\ Weight \times 100}{mg\ sample}$$

RESULTS AND DISCUSSION

Proteins

Protein is a source of essential amino acids needed for the growth and formation of proteins in serum, hemoglobin, enzymes, hormones and antibodies. In addition, protein also plays a role in replacing damaged body cells, maintaining the acid-base balance of body fluids and as a source of energy. Protein is a nutrient needed by living things to carry out its functions. Protein can be obtained from animals (animal protein) and plants (vegetable protein). One source of protein that is cheap but no less nutritious than vegetable protein is soy. Soybeans can be processed into various types of food, including tofu, tempeh, tauco, soy sauce and others. Black soybeans are used less frequently than yellow soybeans. One of its uses is to make soy sauce. To process black soybeans into soy sauce, several stages are carried out, including fermentation. Table 1 presents the effect of microbial type on protein content in black soybeans.

Table 1. Average crude protein (%) which is affected by the type of microbe

Types of Microbes	Average (%)	Signification
m1	34.96	a
m2	42.68	b
m3	47.37	c

Note: - Lowercase letters (a,b,c) indicate a significant difference according to Duncan's range test at the 5% level.
- m1 = *R oligosporus* ; m2 = *A sojae* ; m3 = *R oligosporus* + *A. sojae*

Based on the results in Table 1, the three types of microbes showed very significant differences in crude protein content. The highest protein content is found in m3 of 47.37%; followed by m2 of 42.68%; and the lowest was on m1 of 34.96%. Rhizopus sp is a fungus that produces various enzymes such as amylase, protease and lipase. This fungal community has expanded the use of these enzymes to ferment soybeans thereby increasing their nutritional value which is beneficial to health (Bujang A. 2014) [4]. Rhizopus sp is one of the mushrooms used to make tempeh. Tempe extract shows antimicrobial properties against Bacillus substilis and Staphilococcus aureus (Mambang, Rosidah and Suryanto 2014) [13]. The occurrence of antimicrobial activity in tempeh is during the fermentation process. The advantages of fermenting using Rhizopus sp are reducing anti-nutrients and increasing taste & aroma. In addition, this fungus also functions as an antibacterial, antioxidant, anti-aflatoxin, and heavy metal reducer (Roubos-van den Hil, et al. 2010) [15]. The fungus Rhizopus sp produces protease enzymes (Hsiao, et al. 2014) [8]. Proteases are also called peptidases or proteinases, which catalyze the hydrolysis of peptide bonds into short oligopeptides and free amino acids (López-Otín and Bond 2008) [12]. In Japan, A. sojae is used

ISSN: 2582-6271

Vol. 4, Issue.4, July-Aug 2023, page no. 60-69

to ferment soy sauce, miso, mirin, and other fermented condiments such as tsukemono (Leboffe and Piere 2006) [11]. According to Saono and Basuki (1978) in Abdul Choliq (2008) [6], A. sojae also produces protease enzymes. In line with the results of the research above, the mushrooms Rhizopus sp and A. sojae produced protease enzymes. The more enzymes produced from the mushroom consortium in the fermentation, the higher the protein produced. The increase in protein is influenced by the length of fermentation. The time required for black soybean fermentation is presented in Table 2.

Table 2 Average increase in crude protein (%) which is affected by time

Times	Average (%)	Signification
w3	39.42	a
w1	41.73	b
w2	43.17	c

Note: - Lowercase letters (a,b,c) indicate a significant difference according to Duncan's range test at the 5% level.
- w1 = 48 hours; w2 = 96 hours; w3 = 144 hours

Based on the results of the study, the highest time needed to produce protein was 96 hours (w2) at 43.17%, then w1 at 41.73% at 48 hours, and the lowest was 39.42% (w3) at 144 hours. This is in line with Mien et al. (1996) [10] who stated that R oligosporus in Potato Dextrose Agar (PDA) media at 280°C produced a protease that could hydrolyze casein with maximum activity at pH 3.0 – 5.5 and fermentation time of 72 – 96 hours. The microbial doses used for black soybean fermentation can be seen in Table 3.

Table 3. Average Crude Protein (%) Affected by Microbial Doses

Dose	Average (%)	Signification
d3	40.78	a
d2	42.06	b
d1	42.17	b

Note : - Lowercase letters (a,b,c) indicate a significant difference according to Duncan's range test at the 5% level.
- d1 = 2 grams; d2 = 3 grams; d3 = 4 grams

ISSN: 2582-6271

Vol. 4, Issue.4, July-Aug 2023, page no. 60-69

The results showed that d1 and d2 were not significantly different; however, d3 differs significantly from the others. The dose of d1 resulted in a protein content of 42.17%, followed by d2 of 42.06% and the lowest was d3 of 40.78%. Based on the opinion of Gusti, et al 2020 [9], states that if the number of microbes is too large while the nutrients are depleted in the substrate it can cause the accumulation of toxic metabolites such as ethanol which is produced by lactic acid bacteria. In line with the opinion of Sastramihardja (1989) [16] which states that doses that are too high produce too much biomass, causing a lack of nutrients needed for product formation. When viewed from the dose, d2 and d1 do not provide a significant difference but produce different protein levels, namely d1 (2 grams) produces higher protein levels (42.17%) compared to d2 (3 grams) of 42.06%, so the optimal dose is used is 2 grams (d1). The effect of the interaction of microbial types and fermentation time is presented in Table 4.

Table 4. Microbial Interactions and Fermentation Time on Crude Protein (%)

Times	Type Microbial		
	m1	m2	m3
w1	34.83 a	44.90 c	45.43 c
w2	35.71 a	44.16 c	51.75 d
w3	34.34 a	38.98 b	44.91 c

Note: - Lowercase letters (a,b,c) indicate a significant difference according to Duncan's range test at the 5% level.
- w1 = 48 hours; w2= 96 hours; w3 = 144 hours
- m1 = R oligosporus ; m2 = A sojae ; m3 = R oligosporus + A sojae

Table 4 shows that the highest average value of crude protein content (51.75%) was found in m3 w2, followed by m3 w1 (45.43%) then m3 w3 (44.91%) , m2 w1 (44.90 %). Long fermentation times will produce too many microbes resulting in a lack of nutrients needed for microbial growth, which results in low protein formation. The time is too short, the microbes have not produced enzymes to produce proteins. Research conducted by Sastramihardja (1989) [16] stated that doses that were too high produced too much biomass, causing a lack of nutrients needed for product formation. So the best at this interaction is w2m3. The effects of microbial and dose interactions are presented in Table 5.

ISSN: 2582-6271

Vol. 4, Issue.4, July-Aug 2023, page no. 60-69

Table 5. Interaction of Microbial Types and Doses of Crude Protein (%)

Times	Type Microbial		
	m1	m2	m3
d1	35.09 a	42.33 a,b	48.14 c,d
d2	34.09 a	43.83 a,b, c	48.58 d
d3	35.09 a	41.88 b	45.37 b,c,d

Note: - Lowercase letters (a,b,c) indicate a significant difference according to Duncan's range test at the 5% level.
- d1 = 2 grams; d2 = 3 grams; d3 = 4 grams
- m1 = R oligosporus ; m2 = A sojae ; m3 = R oligosporus + A sojae

The results in Table 5 show that the highest average value of crude protein content (48.58%) is found in m3 d2, followed by m3 d3 (45.37%) then m3 d1 (48.14%) and m2 d2 (43.83%). The effect of dose on m1 did not give a significant difference, but on m2 and m3 the dose gave a significant difference in protein. The highest protein m2 microbe (43.83%) had no significant effect. The highest protein m3 microbe (48.58%) had no significant effect. The results of the study are in line with Sastramihardja (1989) [16] which states that doses that are too high produce too much biomass, causing a lack of nutrients needed for product formation. Thus, the optimal dose to produce the best protein is micro m3 (48.58%) with a dose of d2 (3) gram (d2m3).

Amino acid

Amino acids consist of non-essential amino acids and essential amino acids. Non-essential amino acids are amino acids that can be produced by the body and do not require an outside source. Essential amino acids are amino acids that cannot be produced by the body. Therefore, foods enriched with essential amino acids need to be consumed (Anonymous, 2020) [1]. Results of research on black soybeans fermented by a consortium of R oligosporus and A sojae. The material was analyzed at the Animal Feed Quality Testing and Certification Center, Bekasi. The results are presented in Table 6.

ISSN: 2582-6271

Vol. 4, Issue.4, July-Aug 2023, page no. 60-69

Table 6. Average of Essential and Non-Essential Amino Acids

Essential Amino Acids	Average (%)	Non-Essential Amino Acids	Average(%)
Histidin	1.04	Alanin	1.59
Isoleusin	1.83	Arginin	2.86
Leusin	3.17	Sistin	0.53
Lisin	2.09	Glutamic Acid	4.17
Metionin	0.44	Glutamin	7.10
Fenilalanin	2.33	Glisin	1.78
Treonin	1.62	Prolin	2.08
Triptofan	0.22	Serin	2.18
Valin	1.82	Tirosin	0.97

The results of the research in Table 6 are the amino acids resulting from the breakdown of the protease enzyme produced by the mold Rhizopus sp. During the fermentation the enzymes produced, namely α amylase, lipase and protease, increased the amount of nitrogen and water-soluble solids. This increase in water soluble protein is due to the activity of protease enzymes which decompose proteins into fragments that are more water soluble. Water soluble nitrogen increased from 32.73% to 51.75% after 96 hours of fermentation. The increase in the number of water-soluble solids and nitrogen is caused by an increase in the number of free amino acids. The research results of Leucine (3.17) % and Lysine (2.09) % of these two amino acids which provide a delicious and tasty taste are in line with research (Nurrahman, 2015) [14]. Leucine and Lysine are also very beneficial for health. Leucine is important for protein synthesis and muscle healing, helps regulate blood sugar levels, stimulates wounds and produces growth hormone while Lysine is involved in protein synthesis, hormone and enzyme production as well as calcium absorption, energy production, immune function and collagen & elastin production (Anonymous, 2020) [2].

CONCLUSION

From the results of research in general it can be concluded as follows:

1. Black soybean fermentation is a potential ingredient for food processing such as soy sauce. Black soybean fermentation causes natural color, distinctive aroma, delicious and savory taste with an increase in protein content of 58.11%.

   $$\frac{(51.75 - 32.73)\ )\times 100\%}{32.73}$$

2. The highest protein content was obtained from the consortium fermentation (*R oligosporus* and *A*

sojae) of 51.75%, with a dose of 2 grams and a fermentation time of 96 hours.

3. Overall the best dose, type of microbe and fermentation time was d2m3w2.
4. Fermented black soybeans contain 18 amino acids consisting of essential and non-essential amino acids, which are very beneficial for health.
5. Leucine and Lysine give soy sauce a delicious and savory taste.

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