Available online at www.rajournals.in

RA JOURNAL OF APPLIED RESEARCH
ISSN: 2394-6709
DOI:10.47191/rajar/v8i10.02
Volume: 08 Issue: 10 October-2022

International Open Access

Impact Factor- 7.108

Page no.- 718-724

Influence of Inocule Dose and Fermentation Time of *Lactobacillus acidophilus* on Characteristics and Antioxidants of Soyghurt Black Soybean (*Glycine soja* (L) Merrit)

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ARTICLE INFO

Published Online:
03 October 2022

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ABSTRACT

Black soybeans have been underutilized because of their black color. The purpose of this research is to make black soybean soyghurt containing antioxidants with a research design using a factorial completely randomized design (CRD). Existing data were collected and then processed by analysis of variance (Anava). If there is a significant difference with the control, then it is continued with Duncan's multiple distance test. The results showed that there was an effect between the inoculum dose and fermentation time on an increase in protein by 175%, a decrease in water content by 11.15% and an increase in antioxidants by 55% and a decrease in pH by 41%. The level of preference for black soybean soyghurt is on average to like it very much on taste, while in terms of texture, color, aroma and thickness, it is like to like it a bit.

KEYWORDS: Fermentation, *L acidophilus*, soyghurt, black soybean and antioxidants

INTRODUCTION

According to Salim (2013), soybean is one of the leguminous plants that has been cultivated since 3,500 years ago in East Asia. Soybean plants have long been cultivated in Indonesia since 1970. Soybeans are subtropical plants that are multipurpose and have long been used as a source to meet the needs of vegetable protein in various countries, including Indonesia and are a type of food plant that is commonly processed into legumes. various forms of processed food (Warisno and Dahana, 2010).

Various studies show that eating nuts is very beneficial for health, because these foods contain healthy fats, vitamins, minerals, fiber, and phytonutrients (natural chemicals in plants) that are antioxidants (Anonymous, 2021), the source of fiber does not always have to be vegetables, but also nuts. And among the types of beans, soybeans are the best source of fiber. Soybean is a high value commodity in Indonesia, soybean is the main source of vegetable protein nutrition. This is evident due to Indonesia's high demand for soybeans, so it has to import most of its soybean needs.

In general, soybeans are processed into foodstuffs such as tofu, tempeh, soy sauce, and soy milk, but yellow soybeans are used more. The use of black soybeans has received less attention and is not as popular as yellow soybeans. The lack of use of black soybeans is due to the unattractive color. So far, its use is only limited as a raw material for making soy sauce. Black soybeans contain isoflavones and anthocyanins which function as antioxidants for the body. With the development of technology in the food sector, soybeans can be made into yogurt which is known as soyghurt (soy yogurt). Yogurt is preferred over fresh milk (soy milk) because of its better flavor and texture. The fermentation process has increased the nutritional content of yogurt by breaking down some of the milk sugar (lactose) into simple components. These simple components are more easily absorbed by the body and do not cause diarrhea.

Tannins are generally defined as polyphenolic compounds and can form complexes with proteins to form water-insoluble copolymers. Tannins found in leaves, immature fruit are a group of plant active compounds including flavonoids, have astringent taste and have the ability to tan the skin (Robinson, 1995). Tannins have several properties, namely as astringent, antidiarrheal, antibacterial, anticancer or antitumor and antioxidant.

Yogurt is a fermented milk product using lactic acid bacteria. Yogurt can be made from several sources such as milk, yellow soy milk and black soy milk, peanuts or corn (Haryan, 2001).

In the process of making fermented milk, usually using the help of microorganisms such as *streptococci thermophillus*, *lactobacillus bulgaricus*, *lactobacclillus casei* and *lactobacillus acidophilus.* Yogurt has the potential to reduce blood cholesterol levels because the bacteria in the product produce organic acids such as glucuronic acid, propionic acid, folic acid and lactic acid which can act as agents for lowering low cholesterol levels. Black Soyghurt has several advantages compared to yogurt, namely it uses small amounts of culture, can be made at room temperature, is richer in taste, contains a higher protein content, and is more economical than yogurt (Winarno, 1984).

The manufacture of soyghurt needs to be introduced in Indonesia because soyghurt products have high nutritional value and are still difficult to obtain in the domestic market. In addition, the use of soy milk for yogurt will also help diversify processed soybean products as a source of quality protein. The addition of probiotic bacteria to soyghurt, such as *Lactobacillus acidophilus*, has the potential to improve product quality and health status. Soyghurt can provide ideal conditions for the growth of probiotic bacteria and for the human digestive system. In making soyghurt, it is possible to diversify the culture of Lactic Acid Bacteria (LAB) which are probiotics such as Lactobacillus acidophilus, with the aim of improving the balance of microflora in the intestine.

MATERIAL AND METHODS

The materials used in this study were black soybeans, Lactobacillus acidophilus, MRSA (DeMan Rogose Sharpe Agar), aquadest, Physiological NaCl, Barium chloride, Sulfuric acid.

The research design used a factorial 3x3 Completely Randomized Design (CRD) with 3 replications. The first factor consisted of the inoculum dose (D) with the following treatment levels: The first factor was the type of bacteria, namely Lactobacillus acidophilus. The second factor is the inoculum dose (D) consisting of d1 = 2 %, d2 = 3 %, d3 = 4 %. The third factor is the fermentation time (W) with the following levels: w1 = 4 hours, w2 = 8 hours and w3 = 12 hours.

Research Procedure

Making *Lactobacillus acidophilus* starter

L acidophilus aged 12 hours was harvested, then put into 10 ml of physiological NaCl, centrifuged at 600 rpm for 20 minutes, the precipitate + physiological NaCl was separated and the turbidity was equal to Mc Farlan standard 2. Make Mc Farlan 2 solution: 0.2 grams of barium chloride added 9.8 ml H2SO4.

Making black soy milk (Pricilia et al, 2015)

The black soybeans are sorted and then soaked in water for 12-24 hours, boiled for 15 minutes, then the soybeans are peeled and the husks removed. After that, 8000 ml of hot water with a temperature of 60-90 oC is added, then blended and then filtered. The filtered result obtained is soy milk.

Making Black Soy Soyghurt

The previously prepared soy milk was then fermented by *Lactobacillus acidophilus* with each dose of inoculum and then fermented according to the time of the research treatment.

Protein Analysis (Kjedahl Method) AOAC 2005

Determination of protein by the Kjedahl method is based on the determination of total nitrogen in the sample. The analysis consists of 3 stages, namely the destruction stage, the neutralization stage, the distillation stage and the titration stage. The percent nitrogen in the sample can be calculated using the following formula:

$$\%N = \frac{(\text{ml HCl Sampel} - \text{Blangko}) x\ Normalitas\ x\ 14{,}007\ x\ 100)}{\text{mg Sampel}}$$

Protein content was determined using a conversion factor (F). Nitrogen content conversion to protein content is calculated by the following formula:

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

Analysis of Water Content Sudarmadji (1989)

Moisture content was determined by weighing 2 samples. The sample was put into the oven at 105 oC for 3 hours, then removed from the oven and cooled in a desiccator for 30 minutes, then the weight of the sample was weighed. This treatment was carried out several times until the sample weight was constant.

$$\%\ \text{Kadar air} = \frac{berat\ awal - berat\ akhir}{berat\ awal} \times 100\%$$

Organoleptic Test

Organoleptic test or sensory test or sensory test is a method of testing using the human senses as the main tool for measuring product acceptance. The products tested consisted of texture, taste, aroma, color and viscosity. Respondents commented that they did not like it at all, did not like it, usually, liked it, liked it very much, did not like it very much.

Antioxidants from Tannins

Determination of Tanian content (Wardani, et al 2014)

Determination of Antioxidants (Kiay, 2011)

It was conducted at the Chemical Laboratory of the Bahakti Asih Analyst College, Bandung.

RESULTS

Protein

Protein is a source of amino acids that contain elements of C-H-O and N, all elements contained in these materials are counted as crude protein. Based on the results of the analysis of variance showed that there was an effect of dose and time of fermentation and the interaction of dose and time of fermentation on crude protein content. To find out the differences in each treatment, Duncan's multiple test was carried out which is presented in Tables 1, 2 and 3.

Table 1: The average effect of the inoculum dose on the crude protein content (%) in black soybean soyghurt

Inoculum dose	Average (%)	Signification
d1 (2 %)	6.37	a
d2 (3 %)	6.47	b
d3 (4 %)	8.33	c

Description :
Different lowercase letters in the vertical direction were significantly different according to Duncan's multiple spacing test at the 5% level.

Based on the results of the study in Table 1, it shows that the inoculum dose has a significant effect ($\alpha < 0.05$) on the protein content. The d3 dose gives the highest protein content of 8.33% compared to other doses which are d2 dose is 6.47% and d1 dose is 6.37%. With a high inoculum dose, a lot of material/substrate will be broken down into protein, as well as if the inoculum dose is low, then less material will be decomposed so that the protein content will be small as well. This research is in line with the research of Suliantari and Winiati (1990) in Yusmarini et al (2010) which stated that during the fermentation process the substrate underwent physical and chemical changes, in the presence of proteolytic enzymes.

Table 2: Average effect of time on crude protein content (%) in black soybean soyghurt

Fermentation Time	Average (%)	Signification
w1 (4 hours)	6.74	a
w2 (8 hours)	7.19	b
w3 (12 hours)	7.26	c

Description :
Different lowercase letters in the vertical direction were significantly different according to Duncan's multiple spacing test at the 5% level.

Based on the results of the study in Table 2, the fermentation time showed a very significant difference at the level of <0.05 where the highest protein content was found in w3 at 7.26%, followed by w2 at 7.19% and finally w1 at 6 74%. The longer the microbial fermentation time, the protein content increases as long as the substrate is still sufficient. In accordance with the opinion of Jeffry Tandrianto et al 2013 said that an increase in protein levels was obtained from the activity of protease enzymes produced by microbes in the fermentation process. The longer fermentation time makes the Lactobacillus population increase, thus making the dissolved protein content also increases. As the length of fermentation increases, the more time available for lactic acid bacteria to break down the nutrients contained in the substrate and can allow the accumulation of organic acids such as lactic acid in greater quantities.

Table 3: The average interaction effect of inoculum dose and fermentation time on crude protein content (%) in black soybean soyghurt

Inoculum dose /Fermentation Time	Average (%)	Signification
d2w3	5.36	a
d1w1	6.12	b
d1w3	6.50	c
d1w2	6.51	c
d2w1	6.53	c
d2w2	7.53	d
d3w2	7.53	d
d3w1	7.55	d
d3w3	9.90	e

Description :
Different lowercase letters in the vertical direction were significantly different according to Duncan's multiple spacing test at the 5% level.
d1 = 2 % d2 = 3 % d3 = 4 %.
w1 = 4 hours w2 = 8hours w3 = 12 hours

Based on the results from Table 3. It shows that d2w2, d3w2 and d3w1 do not show significant differences as well as d1w3, d1w2, d2w1 and d2w1. However, the d3w3, d1w1 and d2w3 show significant differences. The highest protein content is found in d3w3 which is 9.90%, while the lowest protein content is found in d2w3 at 6.12%. during the fermentation process, namely: lactose and casein. Lactose is used by microorganisms as a source of carbon and energy with the result of metabolism in the form of lactic acid and causes the pH of milk to drop to acid. Acid conditions cause the balance of casein to be disturbed and at the isoelectric point ±pH = 4.6. Casein will agglomerate to form a coagulant and semi-solid milk is formed.
Lulus Khafidhotul Khoiriyah and Fatchiyah, 2013 in Ramadzanti (2006) stated that chain fatty acids in goat's milk, such as: caproic, caprylic, and capric can cause a distinctive odor. The results of metabolism from LAB will

also form lactic acid in the form of diacetyl and acetoin compounds that give yogurt a distinctive smell and taste. The texture of yogurt is generally soft because of the breakdown of protein molecules into peptides so that the granules in milk will turn into smaller molecules due to the activity of LAB during fermentation.

The protein content in black soybean soyghurt was best at d3w3 with an increase of 175%.

$$Protein = \frac{(9.90 - 3.60)\ x\ 100\%}{3.60}$$

Water content

Food spoilage is generally a microbiological, chemical, enzymatic process or a combination of the three. The data from the research on water content are presented in Tables 4, 5 and 6.

Table 4: The average effect of the inoculum dose on the water content (%) in black soybean soyghurt

Inoculum dose	Average (%)	Signification
d3 (4 %)	85.10	a
d1 (2 %)	85.25	b
d2 (3 %)	85.41	c

Description :
Different lowercase letters in the vertical direction were significantly different according to Duncan's multiple spacing test at the 5% level.

Based on the results of the study in Table 4, it shows that the inoculum dose gave a very significant difference at the level of 5%. The highest water content is found in d2 of 85.41% followed by d1 of 85.25% and the lowest is achieved by d3 which is 85.10%. Water content that is too high can cause damage to food, so the best water content in this study is d3 = 85.10%. This research is in line with (FG Winarno, 2004) which states that the water content in food greatly affect the quality and shelf life of these foodstuffs. Determination of the water content of a food ingredient is very important so that in the processing and distribution process it gets proper handling. Because if there is improper handling in processing and determining the wrong water content, there will be damage to the food that can be harmful to health.

The total weight of ingredients in food, namely water, is about 60-95%, this component is the most dominant compared to other foods such as fats, oils, proteins, carbohydrates, minerals, salts, and acids. In foodstuffs, water can act as a continuous phase where other substances are dispersed in molecular form, colloids or as emulsions (Kumalasari et al., 2013). The presence of water in foods material is always associated with the quality of foodstuffs and as a measure of the dry matter or solids portion. Water in the ingredients can be used as an index of stability during storage as well as a determinant of organoleptic quality, especially taste and tenderness.

Table 5: The average effect of Fermentation time on water content (%) in black soybean soyghurt

Fermentation Time	Average (%)	Signification
w1 (4 hours)	85.10	a
w2 (8 hours)	85.25	b
w3 (12 hours)	85.41	c

Description :
Different lowercase letters in the vertical direction were significantly different according to Duncan's multiple spacing test at the 5% level.

Based on the results of the study in Table 5 shows that the fermentation time has a very significant effect. The highest water content was at time w3 of 85.41%, followed by w2 at 85.25% and the lowest at time of w3 was 85.10%. At the time of w1, the microbes were still in the adaptation stage, namely adjusting to the environment, so that they had not secreted many enzymes used to convert substrates into products, thus the levels were absorbed by the microbes a little.

Table 6: The average interaction effect of inoculum dose and fermentation time on water content (%) in black soybean soyghurt

Waktu Fermentasi/dosis inokulum	Rataan (%)	Signifikasi
d3w1	84.76	a
d1w3	85.03	b
d1w1	85.21	c
d2w1	85.21	c
d2w2	85.21	c,d
d3w2	85.26	d
d3w3	85.29	d
d1w2	85.50	e
d2w3	85.77	f

Description :
Different lowercase letters in the vertical direction were significantly different according to Duncan's multiple spacing test at the 5% level.

d1 = 2 % d2 = 3 % d3 = 4 %.
w1 = 4 hours w2 = 8hours w3 = 12 hours

Based on the results of the study in Table 6, it shows that the interaction effect of inoculum dose and fermentation time gives a very significant difference at the level of 5%. The interaction of d2w3 with water content of 85.77%, significantly different from d1w2 of 85.50%, d1w3 = 85.03% and d3w1 of 84.76%. As mentioned above,

microorganisms in w1 are still in the adaptation stage, namely environmental adjustments. Lactic acid bacteria utilize monosaccharides contained in the substrate during fermentation to form lactic acid which is the result of metabolites, the more lactic acid formed causes the pH to drop and the decrease in pH causes the formation of casein coagulants so that the texture becomes thick. In accordance with the research of Yulian Candra, 2014 stated that acidic pH conditions can reduce the solubility of casein, resulting in hydrophobic interactions between casein micelles forming the structure and consistency of yogurt which causes yogurt to become thicker so that viscosity increases. The best water content in black soybean soyghurt at d3w1 with a decrease of 11.15%.

$$\%N = \frac{(84.76 - 95.40)\ x\ 100\%}{95.40}$$

Organoleptic Test

Organoleptic testing was carried out on a panel of 15 people, consisting of color, aroma, taste, viscosity and texture. The results of the organoleptic tests are presented in Table 7.

Table 7: Organoleptic test on black soybean soyghurt.

Treat-ment	Texture	Flavor	Color	Smell	Viscosity
Soyghurt kedelai hitam	4.30	4.80	4.15	4.30	4.10

Description :

1. Don't like it at all
2. Do not like
3. Neutral
4. Like
5. Love it
6. amat suka

Texture

The hedonic scale shows the value with a numeric scale of 4.30. This means the level of likes (4) to like very much (5). Based on statistical analysis, it turned out that the treatments were not significantly different (P > 0.05). This means that each panelist has almost the same level of preference for the texture of the black soybean soyghurt product as a result of the study, which is between liking it very much.

Flavor

The hedonic scale shows the value with a numeric scale of 4.80. This means the level of likes (4) to like very much (5). Based on statistical analysis, it turned out that the treatments were not significantly different (P > 0.05). This means that each panelist has almost the same level of preference for the taste of the black soybean soyghurt product as a result of the study, which is between liking it very much.

Color

The hedonic scale shows the value with a numeric scale of 4.15. This means the level of likes (4) to like very much (5). Based on statistical analysis, it turned out that the treatments were not significantly different (P > 0.05). This means that each panelist has almost the same level of preference for the color of the black soybean soyghurt product, which is the result of the study, which is between liking it very much.

Smell

The hedonic scale shows the value with a numeric scale of 4.30. This means the level of likes (4) to like very much (5). Based on statistical analysis, it turned out that the treatments were not significantly different (P > 0.05). This means that each panelist has almost the same level of preference for the aroma of the black soybean soyghurt product as a result of the study, which is between liking it very much.

Viscosity

The hedonic scale shows the value with a numeric scale of 4.10. This means the level of likes (4) to like very much (5). Based on statistical analysis, it turned out that the treatments were not significantly different (P > 0.05). This means that each panelist has almost the same level of preference for the thickness of the black soybean soyghurt product as a result of the study, which is between liking it very much. Sugitha and Melia (2007) in Afriani et al, 2011, stated that the factors that affect the viscosity are the concentration and state of the protein, the concentration and state of fat, the temperature and the length of time the milk is stored. The increase in protein content can increase the viscosity.

Antioxidant

Antioxidants are all substances that can delay or prevent damage due to oxidation of the target molecule (Winarno, 1997). Therefore, if exposed to free radicals, antioxidants are needed in amounts sourced from the food consumed. The results of antioxidant research from black soybean tannins are presented in Table 8.

Table 8: The average interaction effect of inoculum dose and fermentation time on the antioxidant content (%) of black soybean tannins.

Waktu Fermentasi/dosis inokulum	Rataan (%)	Signifikasi
d3w1	1,85	a
d1w3	1,91	a
d3w2	2,07	b
d1w1	2,11	b
d2w3	2,65	c
d1w2	2,85	d
d2w2	2,87	d,e
d3w3	2,90	d,e
d2w1	2,94	e

Description :
Different lowercase letters in the vertical direction were significantly different according to Duncan's multiple spacing test at the 5% level.

d1 = 2 %	d2 = 3 %	d3 = 4 %.
w1 = 4 hours	w2 = 8hours	w3 = 12 hours

Based on the results of the study in Table 8, it shows that the highest antioxidant is found in d2w1 of 2.94%. d2w1, d3w3, d2w2 showed no significant difference then d3w3, d2w2, d1w2 also showed no significant difference, followed by d1w1, and d3w2. The lowest antioxidant content is found in d3w1. Microbial dose and fermentation time did not affect the antioxidant content of black soybean tannins. Thus black soybean soyghurt can be used as a beverage that contains antioxidants. This is in line with research (Hagerman, 2002): Tannins have complex biological roles ranging from protein precipitants to metal chelators, tannins have several properties including anti-diarrhea, anti-bacterial, astringent and antioxidant as well as active compounds secondary metabolites. can function as biological antioxidants. In fermented soybean processed foods such as miso and tempeh, free isoflavones (aglycones) are more dominant (Coward et al., 1998 in Sussy Astuti. 2010).

DISCUSSION

From the results of the study, the highest protein content was found in d3w3 (4 grams, 12 hours), the results of this study were in line with the results of research (Ida, 2014) on the growth curve of *L acidophilus*, the best age was 12 hours, where at that age the bacteria were producing lactase enzymes for break down lactose into lactic acid. During fermentation, *L acidophilus* also produces proteinase enzymes, the more the number of microorganisms, the more proteinases will be produced so that the dissolved protein increases, besides that the increase in protein levels is also caused by the increase in the number of microorganisms that act as single cell protein (SPC). produced by microorganisms. *L acidophilus* also utilizes free water on the substrate for its growth, so that with more microorganisms that grow, the water content will decrease, the longer the fermentation time, the more *L acidophilus* bacteria will grow so that the free water on the substrate will decrease because it is utilized by acid bacteria. lactate (LAB) *L acidophlus.*

CONCLUSION

The results showed that there was an effect of inoculum dose (d3) on the increase in protein by 8.33%, fermentation time (w3) by 7.25%, while the interaction of inoculum dose and fermentation time (d3w3) was 9.90%. The best protein increase is 175%. The effect of inoculum dose (d2) on water content is 85.41%, fermentation time (w3) is 85.41%, the effect of interaction between inoculum dose and fermentation time on water content (d3w1) is 85.77%, resulting in a decrease in water by 11.15%. The interaction effect of inoculum dose and fermentation time on the best antioxidant was at d2w1 of 2,94%. Based on the organoleptic test of soyghurt on texture, taste, color, aroma, and viscosity, the results were from liking to liking quite a bit.

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