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THE EFFECT OF FORTIFICATION OF BRANDS AND CHITOSAN ON TEMPEH ON FIBER LEVELS AND PROBIOTIC BACTERIA GROWTH

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Abstract.

Tempeh is a food made from soybean seeds processed through fermentation using *Rhizopus sp.* This processed food in the form of tempeh contains various nutrients that the body needs such as protein, fat, carbohydrates, and minerals but does not contain enough fiber. Therefore, fortification of rice bran and chitosan was carried out on tempeh. Rice bran is a by-product of rice milling which has a high fiber content. While chitosan is chitin that has removed the acetyl group. Chito-oligosaccharides (COS) contained in chitosan can act as natural prebiotics, preservatives, antimicrobials, lower cholesterol levels and are immunostimulants. The purpose of this study was to determine the fiber content and growth of the probiotic bacteria Lactobacillus acidophilus in soybean tempeh (A), rice bran (B), chitosan (C), the ratio of soybean tempeh: rice bran (6: 3) + 2% chitosan (D) and the ratio of soybean tempeh: bran (6: 2) + 2% chitosan (E). The method used for the analysis of fiber content was gravimetric, while the growth of probiotic bacteria Lactobacillus acidophilus was used to determine the potential of prebiotics contained in samples A, B, C, D, and E. The results obtained in this study were fortification of rice bran and chitosan in tempeh had high levels of fiber and the number of probiotic bacteria cells was higher than soybean tempeh. The higher the bran added, the higher the fiber content and the number of probiotic bacterial cells. while the growth of probiotic bacteria Lactobacillus acidophilus was used to determine the potential of prebiotics contained in samples A, B, C, D, and E. The results obtained in this study were bran and chitosan fortifications in tempeh had higher fiber content and cell counts of probiotic bacteria than soybean tempeh. The higher the bran added, the higher the fiber content and the number of probiotic bacterial cells. while the growth of probiotic bacteria Lactobacillus acidophilus was used to determine the potential of prebiotics contained in samples A, B, C, D, and E. The results obtained in this study were bran and chitosan fortifications in tempeh had higher fiber content and cell counts of probiotic bacteria than soybean tempeh. The higher the bran added, the higher the fiber content and the number of probiotic bacterial cells.

Keywords: rice bran, fiber content, chitosan, prebiotics, probiotics, tempeh.

1. INTRODUCTION

Tempeh is a compact, white, cake formed product, prepared from dehulled boiled soybeans through solid state fermentation using *Rhizopus sp.* **[1]**. Through this fermentation process, soybean seeds undergo a decomposition process into simple compounds that are easy to digest. This soybean processed food in the form of tempeh contains various nutrients needed by the body such as protein, fat, carbohydrates, and minerals.

Although it has good nutritional value, tempeh does not contain enough fiber and prebiotics for the body's needs. According to the fiber adequacy rate (AKS) in 2012, the need for human fiber per day is around 10-38 grams. Fiber needs will increase in women who are pregnant and breastfeeding **[2].** Therefore, in order to produce tempe products that have high fiber and prebiotic content, it is necessary to fortify tempe with the addition of rice bran and chitosan.

Rice bran is the outer layer of rice as a by-product of rice milling. The rice milling process usually produces up to 10% bran. Rice bran is a rice milling waste that is usually discarded and not consumed by humans. Rice bran has a high crude fiber content of 5,33-9,03 g/100 grams and can even reach 19,3 - 23% **[3,4].** It has bioactive components oryzanol, tocopherol, and ferulic acid which have the function to lowering cholesterol and improving defecation. The diet pattern of modern society often lacks fiber so it needs additional food sources, cheap fiber alternatives can be obtained from rice bran **[5]**.

Chitosan is chitin whose acetyl group has been removed leaving free amine groups, namely beta-(1,4)-N-acetyl-D-glucosamine and beta-(1,4)-D-glucosamine **[6]**. Chito-oligosaccharides (COS) contained in chitosan are able to act as natural prebiotics, preservatives, antimicrobials, lower cholesterol levels and are immunostimulants **[7][8][9]**.

Based on the nutritional content of rice bran in the form of dietary fiber and the ability of chitosan as a natural source of prebiotics, tempe fortification was carried out with the addition of rice bran and chitosan. Chitosan rice bran tempe can be used as a functional food by adding rice bran: soybean (2 : 1) and 2% w/w chitosan giving optimum organoleptic results in tempe fermentation **[10]**. Chito-oligosaccharides (COS) and rice bran are potential sources

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of natural prebiotics and synergistic effects of synbiotics (probiotics and prebiotics). This synbiotic is a safe biosupplement without causing residue, economical and multipurpose **[9]**. In this study, an analysis of the effect of fortification of rice bran and chitosan in tempeh on fiber content and growth of probiotic bacteria will be carried out to determine the role of prebiotics.

2. METHODS

This research was conducted at the Research Laboratory and Microbiology Laboratory, Department of Chemistry, FST Sunan Gunung Djati State Islamic University Bandung.

2.1. Tools and materials

The materials that will be used in this research are soybeans and rice bran from Ujungberung traditional market, chitosan, tempeh yeast, *Lactobacillus acidophilus* from SITH Bandung Institute of Technology, H_2SO_4 (Merck®), alcohol (Merck®), aquadest, media de Man Rogosa Sharpe (MRS) agar and broth, H_2SO_4 (Merck®), $BaCl_2$ (Merck®), n-hexane, Whatmann filter paper (no. 41).

The tools that will be used include glassware, reflux, heater, refrigerator, loop needle, centrifuge, autoclave, desiccator, and luminar air flow. To test the activity of the number of bacterial cells using a UV-Vis Spectrophotometer (Carry 60 Agilent).

2.2. Tempeh Making

Tempeh was made using 3 variations, namely soybean tempeh (A), the ratio of soybean tempeh: rice bran (6 : 3) + 2% chitosan (D) and the ratio of soybean tempeh: rice bran (6 : 2) + 2% chitosan (E) **[9]**. The tools used in making tempeh are plastic containers, pans, analytical balances, hot plates, measuring flasks. While the materials used in making tempeh are soybeans, Rhizopus sp, rice bran, chitosan, aquadest, and plastic.

Soybeans are cleaned and soaked for 12 hours, the husks removed. Steaming soybeans is done for 15 minutes then cooled. Soybeans were given Rhizopus sp 2 grams/1 kg of material. Stirring the soybeans and yeast is done until evenly distributed then put into a plastic that has been given a small hole. Meanwhile, for the manufacture of tempeh with the addition of rice bran and chitosan, steamed soybeans were added with bran solution (bran: distilled water = 2 : 1). The bran solution had previously been steamed for 15 minutes and chitosan was 2% w/w. Tempe with the addition of rice bran and chitosan was given Rhizopus sp 2 grams/1 kg of material and stirred until smooth. Then put in a plastic that has been given a small hole. Tempe was left at room temperature for 24-36 hours to form white micelles.

2.3. Fiber Content Analysis

Analysis of fiber content using the gravimetric method SNI 01-2891-1992 was carried out on the five samples, namely soybean tempeh (A), rice bran (B), chitosan (C), the ratio of soybean tempeh: rice bran (6 : 3) + 2% chitosan (D) and the ratio of soybean tempeh: rice bran. (6 : 2) + 2% chitosan (E). The tools used in the gravimetric method are analytical balances, sockets, hot plates, reflux equipment, beakers, volume pipettes, measuring cups, mortar and pestle, Buchner funnel, oven, furnace. While the materials needed in the analysis of fiber content are tempeh, NaOH 3,25%, H_2SO_4 1,25%, ethanol 96%, whatmann filter paper no.41.

Crude fiber analysis was carried out based on SNI 01-2891-1992. Extraction of samples with acids and bases to separate crude fiber from other chemicals. Tempe, rice bran and chitosan were weighed 2-4 grams. Fat extraction is carried out using a socket or by stirring, settling, pouring the sample in an organic solvent 3 times. The sample was dried, added 50 mL of 1,25% sulfuric acid and then boiled for 30 minutes using an upright cooler. After 30 minutes, 50 mL of 3,25% sodium hydroxide was added and boiled again for 30 minutes. The hot sample was filtered using a Buchner funnel containing Whatmann No. 41 ashless filter paper. The precipitate in the filter paper was washed successively using hot 1,25% sulfuric acid, hot water and 96% ethanol. The filter paper was dried at 105 ^oC for 1 hour, cooled and weighed until it was constant. If the crude fiber content is > 1% then the filter paper is ashed along with its contents.

2.4. Preparation of Lactic Acid Bacterial (LAB) Culture Stock on Oblique Agar Media

Lactobacillus sp. transferred to a slanted agar medium containing MRS using a needle loop, after which it was incubated at 37 ^oC for 2 days to obtain a culture stock. The culture stock was stored in the refrigerator at a temperature of 3-5 ^oC and regenerated every 3 weeks.

2.5. Bacterial Growth Curve Creation

Bacterial growth curves are made by measuring the density of sample cells at certain intervals. The growth medium was made with the same ingredients as 500 mL sloping agar medium without agar and then sterilized. After sterilization, the bacteria were inoculated into the Erlenmeyer. Then incubated using an incubator at room temperature. Every 1 hour interval, the absorbance and density of sample cells were measured for 24 hours.

2.6. Starter Making

The starter was made using MRS broth and LAB culture which had been prepared on an inclined MRS medium. 100 mL sterile MRS broth media in sterile Erlenmeyer was inoculated with two LAB culture loops. The culture medium was incubated at the time of the exponential phase of LAB at 37^oC.

2.7. Fermentation of Sari Tempe, Rice Bran and Chitosan

Samples A, B, C, D and E as much as 250 grams were steamed for 5 minutes at 80 ^oC, drained and milled by adding warm water in a ratio of 1: 3. After that it was filtered to produce juice and centrifuged at 3500 rpm for 10 minutes to obtain a clear liquid. The tempeh extract, rice bran and chitosan were pasteurized for 15-30 minutes at a temperature of 70-80 ^oC. Tempe, rice bran and chitosan juice were

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fermented using a starter as much as 3% (v/v) then incubated at room temperature for 3 days. Analysis of the number of bacterial cells in the sample was carried out at 0, 24, 48 and 72 hours of fermentation.

2.8. Bacterial Cell Count Analysis

The number of bacterial cells was calculated using a UV-Vis Spectrophotometer. A total of 2 ml of the sample was put into a cuvette, then the absorbance of the sample was measured using a UV-Vis Spectrophotometer with a wavelength of 610 nm. Count the number of bacterial cells using the Mc Farland standard. Mc Farland standard was prepared with 1% BaCl2 and 1% H_2SO_4 solution.

3. RESULTS AND DISCUSSION

3.1. Tempeh Making Results

Organoleptic analysis was carried out on soybean tempeh (A), soybean tempeh: bran (6 : 3) + 2% chitosan (D) and soybean tempeh: bran (6 : 2) + 2% chitosan (E) in the form of color, smell or aroma and texture. The results obtained tempe A has a yellow color, while tempe D and E have a brown color. The difference in color between tempe A and tempeh D and E was due to the addition of rice bran. Rice bran has phytochemical compounds that cause the brown color and distinctive aroma of the bran.

Tempe A has a typical tempeh aroma, while tempe D and E have a typical tempeh aroma and a slight aroma of rice bran. Rice bran has oil in the form of tocol, tocopherol and tocotrienol which causes the rice bran taste to appear. The oil in the bran can be reduced or even removed through a fat extraction process with a fat solvent and heating.

Judging from the texture, both tempeh A and D and E have a texture that is bound by mycellia. In addition, the fermentation time of tempe D and E was faster than tempe A. At the 24 hour fermentation time, tempe D and E were heavily bound by mycelium, while tempe A was only slightly bound by mycelia. Tempe A has been heavily bound by mycelia at 36 hours. The difference in fermentation time was caused by the addition of rice bran and chitosan.With the addition of rice bran in making tempeh, it can speed up the fermentation process, because in the bran there are high carbohydrates that can accelerate the fertility of mushrooms and increase the fiber content in tempe.

Figure 1 Soy Tempe (A), soybean tempeh : bran (6 : 3) + 2% chitosan (D) and soybean tempeh : bran (6 : 2) + 2% chitosan (E)

3.2. Fiber Content Analysis

Determination of crude fiber content was carried out using the gravimetric method SNI 01-2891-1992. Organic compounds in tempe, rice bran and chitosan will dissolve when boiled using 1,25% H_2SO_4 and 3,25% NaOH except crude fiber and ash. If the results of the hydrolysis are completely burned, the crude fiber will evaporate into gas and leave ash. The more rice bran added to tempeh, the higher the fiber content in tempeh.

Table 1 Fiber Content Analysis

Sample	Analysis of % Fiber Content		
	I	II	Average
A	3.9868	3.8289	3.9079
B	8,2012	9.2829	8.7421
C	25,3225	27.0182	26.1703
D	6.4593	7.1052	6.7823
E	6,4202	6.0321	6.2262

Information:
A = soybean tempeh,
B = rice bran,
C = chitosan,
D = soybean tempeh: rice bran (6: 3) + 2% chitosan,
E = soybean tempeh: rice bran (6: 2) + 2% chitosan.

Based on the data obtained, it can be seen that the fiber content in sample A 3.9079 %, sample B is 8.7421 %, sample C is 26.1703%, sample D is 6.7823% and sample E is 6,2262%. So, the fiber content of D higher than E. E fiber content higher than A.

The data show that Tempe fortified using rice bran and chitosan had a higher fiber content than tempe without fortification. The more bran added to tempe, the higher the crude fiber content. This is due to the dietary fiber content in rice bran 8.7421 % and 26.1703% chitosan will increase tempe fiber. Cempaka *et. al.* (2018) report that the tempeh sample with the addition of 20% (w/w) rice bran consisted of 57.23% of water content, 37.42% of protein content, 19.72% of fat content, and 83.98 mg GAE/100 g of TPC **[13].**

3.3. Lactic Acid Bacteria Growth Curve

Bacterial growth curve is an indication to determine the increase in the number of bacteria on a regular basis in order to obtain the optimum time for the growth of lactic acid bacteria *Lactobacillus acidophilus*. *L. acidophilus* was grown in Man Rogosa Sharpe (MRS) broth for 24 hours and the absorbance was read at a wavelength of 610 nm every hour.

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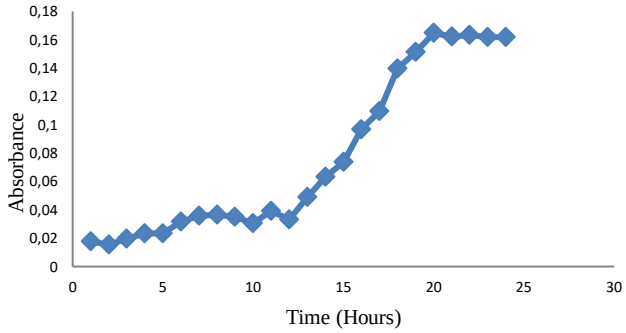

Figure 2 Bacterial growth curve to determine harvest time of *Lactobacillus acidophilus*

Lactic acid bacteria *L. acidophilus* has three phases, namely the lag phase, the exponential phase and the stationary phase. The lag phase occurs at the 1st hour to the 12th hour. In this phase, the lactic acid bacteria *L. acidophilus* undergoes adaptation or adjustment to the environment so that the division runs slowly. The next phase is the exponential phase, which occurs at the 13th hour to the 20th hour. In the exponential phase or log phase, bacterial cells divide and a balanced growth pattern occurs. After experiencing a balanced division, there is a decrease in bacterial growth which is called the stationary phase. This phase occurs at the 21st hour.

From the growth curve, the data obtained that the optimum time for bacterial growth *L. acidophilus* occurs in the exponential phase of the 20th hour. The starter was made using MRS broth media at 37 ^{o}C for 20 hours. The starter is then used to ferment the soy bean, bran, chitosan, tempeh extract from soybeans: rice bran = 6 : 3 + 2% chitosan w/w and tempeh from soybeans: bran = 6 : 2 + chitosan 2% w/w.

3.4. Fermentation by Lactobacillus acidophilus

Samples A, B, C, D and E were extracted using a water solvent in a ratio of 1 : 3. This water solvent can dissolve food juices. Pasteurization was carried out on samples A, B, C, D and E. This was done to kill pathogenic bacteria present in the sample.

Pasteurization should not be carried out at a temperature of less than 70 ^{o}C or more than 100 ^{o}C. At temperatures less than 70 ^{o}C, some bacteria can still live and contaminate the sample. Meanwhile, at a temperature of more than 100 ^{o}C, the nutrients in the sample will be damaged. So that the *L. acidophilus* bacteria that will be inoculated in the sample cannot live properly. Pasteurization is usually carried out at a temperature of 70-80 ^{o}C.

The pasteurized extracts of samples A, B, C, D and E were fermented by *L. acidophilus* at 0,24, 48 and 72 hours. The longer the fermentation time, the sour taste in all samples will increase. This is because the glucose from the five samples was utilized by lactic acid bacteria as an energy source and further metabolized into organic acids, especially lactic acid. The increased amount of lactic acid can make the taste sour because the more concentration of H^{+} ions.

3.5. Bacterial Cell Count Analysis

Analysis of the number of bacterial cells showed the number of bacterial cells per mL from 0 to 72 hours of fermentation of extracts A, B, C, D and E. The longer the fermentation time, the more the number of bacterial cells increased.

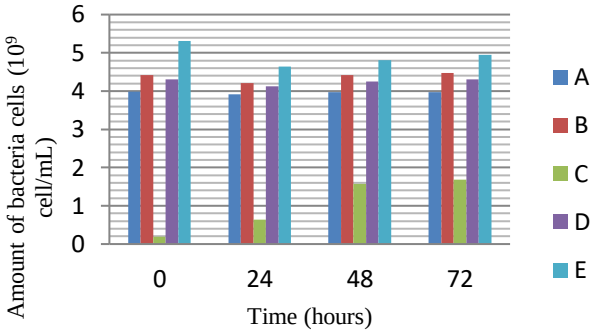

Figure 3 Result of Analysis of Lactic Acid Bacterial Cell Number of A: soybean tempeh, B: rice bran, C: chitosan, D: soybean tempeh: rice bran (6: 3) + 2% chitosan, E: soybean tempeh: rice bran (6: 2) + 2% chitosan.

Fermentation by *L. acidophilus* at A, B, C, D and E decreased from 0 to 24 hours. The decrease in the number of bacterial cells is caused by the process of adjustment or adaptation to the environment so that division runs slowly. At the 24th hour to the 72nd hour, almost all samples increased, except for soybean tempeh which decreased at 72 hours. The decrease in the number of bacterial cells in sample A at 72 hours is thought to have caused some of the *L. acidophilus* bacteria in soybean tempeh to die or can be referred to as the stationary phase.

The number of *L. acidophilus* bacterial cells in sample C always increased from 0 to 72 hours, namely 1.6799 x 109 cells/mL. The growth of microbial cells in the fermentation medium tends to increase as long as the nutrients needed for cell growth or division are met. When the nutrient content is insufficient or depleted, there will be very little or no cell division so that the number of cells is static.

The increase in the number of bacterial cells during the fermentation process was due to the condition of the substrate still allowing for bacterial metabolism to take place. The longer the metabolism of probiotic bacteria indicates that the availability of nutrients in the form of prebiotics is increasing. The sample fermentation curve shows that chitosan and rice bran contain prebiotics that can increase the number of probiotic bacteria cells from 0 to 72 hours.

Sample A had optimum growth at 48 hours. If bran and chitosan were added to sample A, it would increase the viability or viability of the probiotics. Probiotic bacteria can inhibit bacteria that are potentially pathogenic **[12]**. In addition, the prebiotic ability of chito-oligosaccharides

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(COS) contained in chitosan functions as an antimicrobial **[9]**.

4. CONCLUSION

Tempe fortified using rice bran and chitosan had a higher fiber content than tempe without fortification. The more bran added to tempe, the higher the crude fiber content. In addition, the number of probiotic bacteria cells will also increase as the fermentation time increases. Tempe with rice bran and chitosan added had a higher number of probiotic bacteria cells than tempe without fortification. So that tempeh that has been fortified with rice bran and chitosan has great potential as a prebiotic..

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