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# Linoleic and linolenic acids analysis of soybean tofu with *Rhizopus oryzae* and *Rhizopus oligosporus* as coagulant

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**Abstract.** *Sudaryatiningsih C, Supyani. 2009. Linoleic and linolenic acids analysis of soybean tofu with* Rhizopus oryzae *and* Rhizopus oligosporus *as coagulant. Nusantara Bioscience 1: 110-116.* The aims of this research are to know the potency of *Rhizopus oligosporus* and *Rhizopus oryzae* as a coagulant in tofu processing for increasing the amount of linoleic and linolenic acids, and to know the time that needed by *R. oligosporus* and *R. oryzae* for increasing the amount of linoleic and linolenic acids. It uses PDA for inoculating fungi, and it is done at Sub-Lab Chemistry, Central Laboratory for Mathematics and Natural Sciences, Sebelas Maret University, Surakarta. The tofu making was done in "Dele Emas" Tofu Factory, Surakarta. Analysis of linoleic and linolenic acids were done by Gas Chromatography, in LPPT-UGM Yogyakarta. The conclusion of this research are *R. oligosporus* dan *R. oryzae* having a potency as a coagulant in tofu processing for increasing the amount of linoleic and linolenic acids. *R. oryzae* needs 18 hours to coagulate the tofu, and *R. oligosporus* needs 12 hours for the same process. The highest amount of linoleic and linolenic acids were obtained by *R. oryzae* at 6 hours of fermentation (0.26% and 0.14%), and 24 hours of fermentation by *R. oligosporus* (0.06% and 0.04%).

**Key words:** linoleic acid, linolenic acid, tofu, coagulant, *Rhizopus oryzae*, *Rhizopus oligosporus*.

**Abstrak**. *Sudaryatiningsih C, Supiyani. 2009. Analisis kandungan asam linoleat dan linolenat tahu kedelai dengan* Rhizopus oryzae *dan* Rhizopus oligosporus *sebagai koagulan. Nusantara Bioscience 1: 110-116.* Penelitian ini bertujuan untuk mengetahui potensi jamur *R. oligosporus* dan *R. oryzae* sebagai koagulan pada proses pembuatan tahu dalam meningkatkan kadar asam linoleat dan linolenat tahu dan mengetahui waktu yang diperlukan oleh keduanya untuk memfermentasi tahu sehingga menghasilkan asam linoleat dan linolenat yang tinggi. Inokulasi jamur menggunakan PDA,dilakukan di Sub-Lab Kimia, Laboratorium Pusat MIPA, Universitas Sebelas Maret, Surakarta. Pembuatan tahu dilakukan di pabrik tahu Dele Emas Surakarta. Analisis kandungan asam linoleat dan linolenat dilakukan dengan metode kromatografi gas, dan dikerjakan di LPPT-UGM Yogyakarta. Dari hasil penelitian ini diperoleh kesimpulan *R. oryzae* dan *R. oligosporus* memiliki potensi sebagai koagulan dalam pembuatan tahu, dan waktu yang diperlukan untuk melakukan koagulasi *R. oryzae* adalah 18 jam *R.oligosporus* adalah 12 jam. Asam linoleat dan linoleat tertinggi diperoleh. Pada 6 jam fermentasi *R. oryzae* (0,26% dan 0,14%), dan 24 jam fermentasi oleh *R.oligosporus* (0,14% dan 0,08%).

**Kata kunci:** asam linoleat, asam linolenat, tahu, koagulan, *Rhizopus oryzae*, *Rhizopus oligosporus*.

## INTRODUCTION

Fat is a food substance that is always present in the daily diet. Various kinds of foods, ranging from fried bananas, fried potatoes, fried chicken, *rendang*, to pizza, are not apart from fat. The addition of fat in the form of oil is useful for the food to improve taste, texture, and improve the flavor (Muchtadi 2000). Fat is useful for the body as the source of energy, and also as a solvent to various vitamins (Campbell 1987). As a source of energy, fat has a high calorific value which is 9.3 kcal/g. This value is higher than carbohydrates and protein. In addition, during oxidation, fatty acid metabolic generates a lot of water, compared to carbohydrates and proteins (Harper et al. 1979). It is advantageous for organisms that live in dry areas.

Eating too much fat is dangerous for heart health (Forman and Bulwer, 2006; Muchtadi 2007), including the causes of obesity (Wilson et al. 2002; Panagiotakos et al. 2004) and hypertension (Houston et al. 2005). Foods that have high fat content, can lead to the formation of plaque in arteries. The formation of plaque will lead to the inside of the artery wall thickening, and narrowing the cross section. This disease is called atherosclerosis, and can cause strokes and heart attacks (Campbell 1987). Cooking oil used to fry more than once is also dangerous for our health, because it can cause cancer (Nadesul 2007).

Fats can be hydrolyzed by lipase into fatty acids and glycerol. In the process of metabolism, fatty acids can be harmful to health, but some are really needed for health. Fatty acids which are harmful are saturated fatty acids, i.e. fatty acids with no double bond on carbon chain constituent. Such saturated fatty acids can be found a lot in the animal fat, lard, milk, eggs, and poultry skin (Nadesul 2007). Fatty acids which are not harmful to health are the ones which are not saturated (Sipayung 2003). Unsaturated fatty acid is a fatty acid having double bond on carbon chain constituent. Examples of unsaturated fatty acids that are important for the health of the body are linoleic and

linolenic acids. Both types of these fatty acids are essential fatty acids, which are fatty acids that can not be produced by the body (Harper et al. 1979). Because they can not be produced by the body, it must exist in the consumed food. Linoleic acid plays an essential role in the prevention of coronary heart disease and healthy blood vessels (Nadesul 2007).

One source of linoleic and linolenic acids are soybean seeds (Harper et al. 1979). Indonesian people consume soy in various forms of processed foods such as soy sauce, tofu, *tempe*, *tauco*, soy milk, and others. In some processed foods, a change in the nutritional value of soybeans occurs, for example in the fermentation process of soybeans into *tempe*, there is a trend of increased fatty acid of linoleic and linolenic acids (Bisping et al. 1993; de Reu 1994; Naidoo and Hermana 1995). Tofu is one of the types of foods derived from soybeans widely consumed by the public. Tofu is made without the process of fermentation, so the content of linoleic and linolenic acids are not as high as *tempe*. That is why in this study the researchers tried to analyze the content of linoleic and linolenic acids on the tofu by using the fungus of *Rhizopus oligosporus* and *Rhizopus oryzae* as a coagulant.

This study aims to: (i) determine the potential for fungus of *R. oligosporus* and *R. oryzae* as a coagulant in the making process of tofu; (ii) determine the length of time needed by both to perform coagulation in the making process of tofu; (iii) determine the potential of both in increasing levels of linoleic and linolenic acids in the tofu; and (iv) determine the length of time needed by both to ferment the tofu so it produces high levels of linoleic and linolenic acids levels.

## MATERIALS AND METHODS

### Time and place

This research was conducted in June-July 2009. The inoculum of *Rhizopus oligosporus* and *Rhizopus oryzae* was created at the Laboratory of Biology, Sebelas Maret University, Surakarta. The tofu production was done at the tofu factory "Dele Emas" Surakarta. Unsaturated fatty acid test was conducted at the Integrated Research and Development Laboratory (LPPT), Gadjah Mada University, Yogyakarta.

### Material

Soybean imported from the United States (*Glycine max* (L.) Merr.), used as the material to make the tofu were obtained from soybean import wholesalers, in the market of Mojosongo, Surakarta. *R. oligosporus* and *R. oryzae* were obtained from the Laboratory of Microbiology, University of Setia Budi, Surakarta. The media of *Potato Dextrose Agar* (PDA) was obtained from the Laboratory of Biology, Sebelas Maret University, Surakarta, to breed *R. oryzae* and *R. oligosporus*

### Procedures

**The making of inoculum.** Media to make the pure culture of inoculum is the PDA. In the rules of use, it is said that to make 1 liter of gelatin requires 39 g PDA, so to make the culture of pure inoculum of 4 test tubes, 5 mL for each tube is needed {(4x5)/1000} x39 g = 0.78 g. How to the culture are as follows: as much as 20 mL of distilled water is inserted in a glass beaker, then added with 0.78 g of PDA (E. Merck) into it and stir it until well blended. PDA solution was poured into four test tubes 5 mL for each. The tubes were plugged with cotton, and inserted into the autoclave with a temperature of 121°C for 15 minutes. After it was placed on a board tilted up for the cold and dense, and then was left for 3 days. 1 ose pure culture of *R. oligosporus* was streaked on to tilt, and left for 48 hours to be ready for use. The same was done to make the pure culture of *R. oryzae*.

**Calculation of the spores of *Rhizopus* sp**. The surface and the lid of haemacytometer were cleaned by lens paper and distilled water. The cover glass of haemacytometer was placed on the surface of the counting machine. A total of 5 mL aquades was poured onto the culture of *Rhizopus* sp. By using a needle loop, *Rhizopus* sp. was scraped in order that all the spores are able to be lifted. Fungal suspension was poured into another tube, and shaken. Drawn 1 mL suspension of fungus with a pipette, and inserted into a V-shaped groove on the edge of the lid of haemacytometer. Haemacytometer was placed under the microscope, and the spores were counted. The result turned out that in 1 mL of suspense there were 8.3x106 *R. oryzae* spores; and to make tofu of 1 L of soymilk needed 107 spores (Purwoko 2001). Thus the number of suspension required is: 107/8.3 x106 = 1.2048 mL. While in 1 mL of suspense obtained 10.9 x106 *R. oligosporus* spores, so that for 1 L of material required 107/10.9x106 = 0.9174 mL.

**The tofu making.** One kilogram of soybean was washed, and then soaked for 6 hours. Next milled until smooth, strained and the juice was taken, the waste was removed. One liter of soymilk was cooked until boiling, then cooled, and when cool it was inoculated with 107 spores of *R. oligosporus*. The soybean which had been inoculated was made into 4 kinds of treatments, namely left for 6 hours, 12 hours, 18 hours, and 24 hours. The precipitate that had been formed was separated from the liquid and pumped by a fabricated pump layered with a coat. The same process was done to *R. oryzae.*

**Determination of linoleic and linoleic acids**. Determination of linoleic and linoleic acids was performed by the method of gas chromatography.

*Preparation of materials*. Each sample's weigh was 10 g, then crushed/grinded to be homogenized. The sample was put into the flask of 50 ml and then added with 4 mL of concentrated HCl, homogenized, then added with 7 mL of concentrated HCl.

*Hydrolysis*. The materials that had been homogenized were put into the water heater, then heated at 70°C, then continued to boil, and left for 90 minutes. While heating, the container was covered with a plastic so it did not evaporate.

*Extraction*. The materials were cooled, then added with 7 mL of ethanol, boiled, and then added with 25 mL diethylether and boiled again. 25 mL of Petroleum benzene fractions was added into the materials at 40-60 degrees

Celsius, then vortexted. The top layer that had been formed was separated from. The bottom layers were extracted again, using 15 mL of dietileter and 15 ml of petroleum benzene. The top layer was separated again, and then made into one with the top layer of the first. Next step is evaporation at a temperature of 50 degrees centigrade with the help of N2 gas, until it dried.

*Saponification*. 1 mL solution of 0.5 M NaOH-metanolic was added into the dry ingredients, heated to boil for 1 minute.

*Esterification*. Materials that had been saponificated were added with 2 mL of 20% BF3-methanol, and heated to boiling, for 1 minute. Then extracted with 1 mL of n-heptane and 1 mL of saturated NaCl, and until it formed 2 layers with the upper layer consisting of heptane and methyl esters. The top layer was taken and then injected into the GC. At the same conditions, the standard Methyl linoleic and linolenic methyl were also injected with a concentration of 0.125%.

**Research variables**

Variables examined in this study include: (i) Morphology of the tofu fermented by the fungus of *R. oligosporus* and *R. oryzae*. (ii) Changes in pH of the tofu during the fermentation process (iii) the time required for the fungus of *R. oligosporus* and *R. oryzae* to perform coagulation. (iv) The highest content of linoleic and linolenic acids formed during fermentation by the fungus *R. oligosporus* and *R. oryzae*. (v) the optimum time required by *R. oligosporus* and *R. oryzae* to produce the highest levels of linoleic and linolenic acids during the fermentation

## RESULTS AND DISCUSSION

**Morphology of tofu**

In the process of the tofu making, the fermentation was done for some long times, namely 6 hours, 12 hours, 18 hours and 24 hours. It was from the treatment above it can be seen different morphological forms of tofu , as in Table 4. In the table it can be seen the morphological changes of the tofu out of the shape that was initially soymilk. The changes can be seen at: discoloration, formation of cavities, the change becoming acidic odor, density, and the increase of water content.

**Changes of the tofu color**

In this event of tofu fermentation the change of color happened in soy milk from white color turned into yellowish, and then became brown. These changes occurred slowly over a long period of fermentation. These color changes in both Rhizopus oligosporus and Rhizopus oryzae fermentation began after the fermentation lasted for 6 hours. Furthermore, the color changed to yellow after 12 hours fermentation, then changed to brownish yellow after 24 hours fermentation. According to Cook (1994), the yellow color is caused by a pigment consisting of alpha carotene and beta carotene. This pigment is a natural dye ingredient contained in the material consisting of oil, or producing oil, for example the groups of fungi of the order of Mucorales. Wiesel et al. (1996), reported that the yellow color formed is the result of β-carotene biosynthesis by *Rhizopus* sp. and indicate the fermentation process going well. Denter et al. (1998) and Bisping et al. (1993) write that Rhizopus is a type of mold that is capable of forming β-carotene.

In this study, color changes that occur indicate the existence of fermentation activity by the fungus *Rhizopus* sp. During the fermentation, *Rhizopus* sp. synthesized β-carotene and released it to the media, making media changes color from white to yellow. This event at *R. oryzae* fermentation occurs continuously up to 24 hours. But not so in the fermentation by *R. oligosporus*. After the fermentation lasted for 24 hours, the fermented tofu with *R. oligosporus*, the color was no longer yellow, but changed to brown. This showed the change of the protein into peptide bond hydrolysis by the enzyme protease, resulting amine group, which can react with aldehyde or ketone group and produce the brown color. This is consistent with reports Subagio et al. (2002), which is in addition to the β-carotene, color changes in the process of fermentation by *Rhizopus* sp. is also due to the process of protein into peptide bond hydrolysis by protease enzymes. The result of hydrolysis is amine group, which then reacts with aldehyde or ketone group and produce brown color. Brown color appears in the fermentation by *R. oligosporus* after 24 hours, and has not appeared on the fermentation by *R. oryzae* after 24 hours. This proves that *R. oligosporus* has the ability to hydrolyze proteins faster than *R. oryzae*, or in other words *R. oligosporus* is more active in fermentation compared to *R. oryzae*.

**Table 4.** Morphology of fermented tofu.

| Time of fermention (hours) | *R. oryzae* | *R. oligosporus* |
|---|---|---|
| 6 | White color, the smell of soy milk | White color, the smell of soy milk |
| 12 | Soymilk was denser, yellowish-white color, smell like tempe. | Soymilk was denser (tofu was formed), yellowish color, smell like *tempe*, small cavities raised, some water was produced. |
| 18 | Soymilk was denser (tofu was formed), yellow color, smell of *tempe* mixed with sour, small cavities aroused, some water aroused. | Soymilk was denser, yellow color; smell like *tempe* mixed with a little sour, the cavities was larger, more water was produced. |
| 24 | Soymilk was solid, yellow, sour smell was stronger, the cavities grew more, water were produced more. | Soymilk was solid, brownish yellow color, acid smell was stronger, a lot of large cavities, water were produced more. |

**Tofu cavities**

In addition to color changes, in the fermentation process also arise out of the cavities. In the fermentation by *R. oryzae* cavities began to appear after 18 hours fermentation, and increased much after 24 hours fermentation. While in the fermentation by *R. oligosporus* small cavities already started to appear after 12 hours fermentation, and more and more in line with the length of fermentation. The cavities were formed because the proteins that were initially spread evenly on the liquid soymilk began to clot.

At first only a few proteins that were coagulated, so it only had a light molecular weight, and could still be spread on the fermentation container. As a result, cavities that were formed were only small. The incident occurred at the fermentation of *R. oligosporus* after 12 hours of fermentation. Further, after the fermentation by *R. oligosporus* lasted for 18 hours, the proteins that were formed got more and more, these proteins formed larger clumps, with large molecular weight, which tends to settle, and form greater cavities.

In the tofu production process carried out in the factory clotting proteins that occurred was because the soybeans were given an additional acid as coagulant. The given acid for example acetic acid, or the tofu liquid had been kept for one night. Furthermore, this acid reacts and binds to proteins contained in the material out, and together with lipids to form clots (Santoso, 1993). Coagulant material will determine the quality of the tofu. The better the coagulant, the more protein-bound, resulting more randemen (Suprapti 2005).

In this research, it was obtained that the coagulation by *R. oligosporus* were faster in forming the solid tofu than the fermentation by *R. oryzae*. *R. oryzae* took 18 hours to form thetofu, while *R. oligosporus* only took 12 hours, to agglomerate soybean into tofu. This is consistent with the reports of Sapuan and Sutrisno (1997), that *R. oligosporus* has a protein hydrolysis speed that is higher than that of *R. oryzae*. With the protein resulted from this hydrolysis, it will form clumps, and then it settles to form the tofu.

**The increase of the water content and the tofu density**

As a result of clots formed during the fermentation process there were areas that did not contain the protein mass. This area was not empty but filled with water. The existence of this water could not only be seen through the cavities that had appeared, but also through the condensation on the fermentation container lid. In the fermentation by *R. oligosporus*, the formation of water and water vapor on the container lid fermentation occurred after 12 hours fermentation, and the water content increased until 24 hours fermentation. Along with the presence of water and water vapor in the closed container, soya also turned into a solid, but not so with the fermentation by *R. oryzae*. Formation of water that was trapped in the cavities occurred after 18 hours fermentation, and continued to increase up to 24 hours of fermentation. The formation of solid soymilks was seen after 18 hours fermentation, showing fermentation by *R. oligosporus* turned out to be more quickly than that of *R. oryzae*. In the process of tofu making carried out in a factory, high water content may be removed by it being compressed using a mold out of wood covered with calico cloth (Suprapti 2005).

Fermentation of tofu by *R. oligosporus* produced a lot of clumps of protein, more solid tofu, compared to the fermentation by *R. oryzae*. This is in accordance with the opinion of Suhaidi (2003), that the more the clot is formed, the more protein that is trapped inside, so it will generate a lot of randemen.

**The smell of acid**

Tofu fermented by Rhizopus sp. has a sour smell. Sour odor occurs due to decrease in soymilk pH. The smell of acid can be observed by the senses in 18 hours fermentation, both by *R. oryzae*, and *R. oligosporus*. Observations soymilk pH using a pH meter shown in Table 5 below

**Table 5.** The degree of acidity (pH) of soymilk fermented by *Rhizopus* spp.

| Time of fermentation (hours) | pH | |
|---|---|---|
| | ***R. oryzae*** | ***R. oligosporus*** |
| 6 | 5.81 | 5.86 |
| 12 | 5.52 | 5.61 |
| 18 | 5.31 | 5.44 |
| 24 | 5.53 | 5.47 |

In Table 5 it can be seen that the fermentation by *Rhizopus* sp produced a product that has a pH of 5. According to the Sapuan and Sutrisno (1997), the decrease of pH in the fermentation media can occur until the pH of the media reached 4.5 to 5.3 and this pH change causes the mold to grow well, and there the process of fermentation occurred. Hidayat (2009), reported on the *tempe* fermentation process, the decrease of pH started at the immersion process of soybeans. The immersion process provided an opportunity for lactic acid bacteria to grow so that it decreased the pH of soybean seeds. This decrease in pH can inhibit the growth of bacterial contaminants that are decomposers. In addition, the decrease in pH caused the mold to be able to ferment properly.

Furthermore, when the fermentation is in progress, *Rhizopus* sp. will produce lactic acid, and cause the pH of soymilk to get decreased. This decrease of pH resulted in the soybean protein having clotting or coagulation (Gaman 1992). In the process of tofu fermentation, this clumping resulted in the protein which was originally spread out, becoming mutually bonded to one another, then forming the tofu. In this study, the sharpest decline in acidity occurred after 18 hours fermentation. The smell of acid can be observed even with the sense of smell. This shows the optimum coagulation activity in fermentation of *Rhizopus* sp. after 18 hours.

**Analysis of chromatography gas**

*The peaks of chromatogram*

The test of linoleic and linolenic acids content was conducted by the gas chromatography, using ethanol as

solvent, and Flame Ionization Detector (FID) as detector. The use of gas chromatography can be performed on samples that require high temperatures to evaporate, such as fatty acids. The gained results were in the form of chromatogram which had crests. From the observation of chromatogram analysis of the content of tofu fermented by *Rhizopus* sp., the gas chromatography could detect the three dominant peaks. Based on the comparison with the standard, it is certain the first peak was linoleic acid, and the second peak was linolenic acid. While the third peak was a compound formed from the oxidation of linolenic acid. Presumably that the third compound was arachidonic acid. This is in accordance with the opinion of fever (1997), that linoleic acid would be oxidized into the linolenic acid, and then the linolenic acid is oxidized into arachidonic acid. The oxidation process is strongly influenced by temperature and light intensity. The higher the temperatures and the greater the light intensity, the faster the oxidation will be.

High peaks that formed on the chromatogram of this experiment showed the concentration of a substance that was tested the samples, in this study it showed the levels of linoleic and linolenic acids. The levels of linoleic and linolenic acids can be seen in Table 6. The height of the peak from the bottom of the chromatogram showed retention time. According to Adnan (1997), the retention time is the time required to separate, and then evaporate, exit the column. A long retention time shows that those materials require high temperatures to separate. The high temperature is required to perform the separation of material that has a lot of double chains. So the longer retention time indicates that these substances have more double bonds.

Retention time can also indicate the speed of molecular motion. In this experiment the retention time was used to compare the standard molecular velocity with the velocity of the sample molecules. In this study, the standard retention time was 8.80 minutes for the linoleic acid, and 11.0 for the linolenic acid. While the retention time of the sample can be seen as Table 6.

**Table 6**. Retention time (Rf) fermented tofu by *Rhizopus* sp.

| Time of fermentation (hours) | Peak of | *R. oryzae* Rf (Minutes) | *R. oryzae* High peak | *R. oligoorus* Rf (Minutes) | *R. oligoorus* High peak |
|---|---|---|---|---|---|
| **6** | 1 | 8.998 | 1487980 | 8.983 | 8681484 |
| | 2 | 10.873 | 3561719 | 10.847 | 2593481 |
| | 3 | 11.029 | 604055 | 10.907 | 708088 |
| **12** | 1 | 8.996 | 1513297 | 8.987 | 1077314 |
| | 2 | 10.885 | 4157405 | 10.862 | 3169875 |
| | 3 | 11.031 | 646773 | 11.022 | 466356 |
| **18** | 1 | 8.990 | 1390400 | 9.009 | 2175045 |
| | 2 | 10.873 | 3711272 | 10.928 | 5733475 |
| | 3 | 11.027 | 619996 | 11.051 | 1090065 |
| **24** | 1 | 8.991 | 1283842 | 9.014 | 2484471 |
| | 2 | 10.873 | 3582183 | 10.941 | 6473468 |
| | 3 | 11.026 | 573739 | 11.060 | 1268570 |

From Table 6 above it is shown the analysis by gas chromatography was going well, as indicated by the retention times close to the standard, and the visible differences distance of time with each other.

**Linoleic and linolenic acids content**

The analysis results of linoleic and linolenic acids content using gas chromatography is shown in Table 7, which the tofu of the control group, made by using vinegar as a coagulant, contained linoleic and linolenic acids respectively 0.05% and 0.03%. Meanwhile, linoleic and linolenic acids content in the tofu with *Rhizopus* sp. as coagulant was higher. Allegedly linoleic and linolenic acids existing in the tofu were the linolenic and linoleic acids which already existed in soybean. This is consistent with the reports of Iskandar (2004), that soybean is a source of linoleic acid.

**Table 7**. Linoleic and linolenic acids content in the tofu.

| Name of sample | Content (%) | |
|---|---|---|
| | Linoleic acid | Linolenic acid |
| Tofu control | 0.05 | 0.03 |
| *R. oryzae* 6 hours | 0.26 | 0.14 |
| *R. oryzae* 12 hours | 0.10 | 0.05 |
| *R. oryzae* 18 hours | 0.09 | 0.06 |
| *R. oryzae* 24 hours | 0.07 | 0.04 |
| *R. oligosporus* 6 hours | 0.06 | 0.04 |
| *R. oligosporus* 12 hours | 0.07 | 0.05 |
| *R. oligosporus* 18 hours | 0.12 | 0.07 |
| *R. oligosporus* 24 hours | 0.14 | 0.08 |

The process of making the tofu distributed in the market usually uses vinegar or the remaining water of the tofu from the previous day that has been kept overnight, and without undergoing fermentation. Acetic acid will react with proteins, resulting in the occurrence of clotting proteins, and forming the tofu. The change of soymilk into a tofu lasts for about 90 minutes. While the tofu making using *Rhizopus* sp. changes soymilk into tofu slowly. These changes occur enzymatically, to form new substances. For example, changes with the enzyme lipase, resulting in the formation of linoleic and linolenic fatty acids (Pawiroharsono 1997).

**Tofu fermentation by *Rhizopus oryzae***

For the first 6 hours of fermentation by *R. oryzae*, tofu contained the highest linoleic and linolenic acids, and then there was the tendency to decrease. This happened because at the beginning of fermentation, *R. oryzae* would synthesize a high linoleic acid, after that this fatty acid was converted into the linolenic acid. Linoleic acid was derived from oleic acid which was the result of fat hydrolysis by lipase enzyme during the fermentation process. This is consistent with the theory of Teng et al. (2008), that *Rhizopus* produces lipase during the fermentation process. This lipase does hydrolysis activation. Initially the first acid that was formed was oleic acid, but then it was saturated acid into linoleic acid, and changed again into linolenic acid (Styme and Stobart 1986).

Mushrooms of the class of Zigomycetes have the desaturase enzyme, an enzyme which can alter the results of oleic acid hydrolysis of fat by lipase into linoleic and linolenic acids (Suharyanto et al. 2006). In the early stages of the hydrolysis of linoleic, the fatty acids were formed; further these acids had an addition of double bonds becoming the linolenic acid (Harper et al. 1979). Desaturation reaction of fatty acids is a chain reaction. Oleic acid is converted into linoleic acid with the aid of Δ12 desaturase enzyme, further into linoleic was changed acid linolenic acid with the help of the enzyme Δ6 desaturase (Suharyanto et al. 2006). Briefly, the changes of linoleic acid into linolenic acid can be written as follows:

| | |
|---|---|
| desaturase | desaturase |
| $\Delta^{12}$ enzyme | $\Delta^{6}$ enzyme |

Oleic acid → linoleic acid → linolenic acid

In the fermentation of *R. oryzae* for 12 hours and beyond, linoleic acid was not formed anymore but was changed into linolenic acid, so that the number of linoleic gradually decreased, followed by a decrease in linolenic acid levels. The declining trend in linoleic and linolenic acids continued, in 18 hours fermentation, up to 24 hours fermentation. This shows the optimum time of the formation of linoleic and linolenic acids in the tofu fermented by *R. oryzae* is 6 hours, and then the formation of linoleic acid stopped, and the linoleic experienced hydrolysis and become linolenic acid. The formation of linoleic acid presumably was caused by several factors, including: temperature, moisture content, and oxygen.

Purwoko et al. (2001) reported the increase in temperature on soybean tempe fermentation by Rhizopus sp. According Suharyanto et al. (2006), the increase in temperature in the fermentation process can inhibit the desaturation of the enzyme activity, so the formation of unsaturated fatty acids gets decreased. De Man (1997) reported that the oxidation process of linoleic and linolenic acids is strongly influenced by temperature and light intensity. The higher the temperature, the formation of fatty acids could decrease. In this study, the fermentation by *R. oryzae* was performed at the room temperature (25-26oC). At this temperature the formation of fatty acids occurred normally. Furthermore, along with the occurrence of fermentation, the temperature got increased. As a result, the formation process of linoleic acid got decreased and the formation of linolenic acid also got decreased.

The water content will affect the action of the enzyme lipase. With high water content, enzyme lipase will hydrolyze fats into glycerol and fatty acids. While at low water content, alcohol or other esters will be formed. In this research, the media used were soybeans in the form of liquid, so that the water content was high, and the resulting lipase enzyme activity that hydrolyzed fat ran well. Suharyanto et al. (2006) also reported that too high water content will affect the solubility of oxygen. At the beginning of the fermentation process, the available water was the water contained in the media. This water was to accelerate the formation of fatty acids of linoleic, and linolenic. Furthermore, the media got additional water from the fermentation process (Purwoko 2004). The increased water content in the tofu can be seen from the emergence of cavities in the tofu, and also from the existence of water vapor on the fermentation container lid. As a result the solubility of oxygen got reduced, and the formation of linoleic acid also got decreased.

Aerobic and facultative anaerobic microorganisms require oxygen in the process of fatty acid desaturation. Aeration can increase the solubility of oxygen thus increasing the degree of fatty acid's unsaturatability (Suharyanto et al. 2006). In this study, at the beginning of fermentation, the oxygen content in the media was high, but after several times of fermentation, the oxygen content decreased, so the formation of linoleic acid also decreased.

**Tofu fermentation by *Rhizopus oligosporus***

In the tofu Fermentation using *R. oligosporus*, there was a distinct tendency from the fermentation using *R. oryzae*. In the fermentation of 6 hours, the smallest amount of linoleic and linolenic acids were formed. This showed that the active fermentation has begun and *R. oligosporus* has been churning out a lipase enzyme in 6 hours of fermentation. Furthermore, in 12 hours fermentation, the content of linoleic acid was increased, and followed by linolenic acid. This proves, in 12 hours fermentation, the lipase enzyme was still actively working to produce linoleic acid. Besides the formation of linoleic acid, the hydrolysis change from linolenic into linoleic acid also occurred. This characteristic is very different from the fungal fermentation with *R. oryzae*, because the lipase activity in fermentation by *R. oryzae* after 12 hours they no longer produced linoleic acid.

If we have a look at Table 7, the fermentation by *R. oligosporus* for 18 hours, the fermentation activity looks stronger and more linoleic acid production. The speed of reaction of linoleic acid into linolenic was smaller than the speed of reaction formation of linoleic acid. As a result the increase in linoleic acid content was greater than linolenic acid. But this did not last long, because in the fermentation activity after 24 hours, although the formation of linoleic acid increased, but not as much as in 18 hours fermentation.

The increase of linoleic and linolenic acids occurred continuously throughout the time of fermentation. This is in accordance with the opinion of Sapuan and Sutrisno (1997) that *R. oligosporus* has higher lipase activity than *R. oryzae*, so as to produce more fatty acids. Also *R. oligosporus* can perform and produce the perfect fermented soy and *tempe* in less time. Steinkrauss et al. (1983) reported that *R. oryzae* required 48 hours to produce the perfect *tempe*, while *R. oligosporus* only took 24-36 hours to produce the perfect *tempe*. Meanwhile, Arias (2003) reported that *R. oligosporus* was actively doing fermentation until the 48$^{th}$ hour.

With the ability to perform activities of a long fermentation, then *R. oligosporus* was able to perform the metabolism of various substances, including linoleic acid, thus forming a high linoleic acid. Furthermore, while still active in fermentation, linoleic acid portion has been

changed into linolenic acid. Consequently, in the fermentation using *R. oligosporus* linoleic acid content tended to increase, while linolenic acid was also increased.

## CONCLUSION

*Rhizopus oryzae* and *R. oligosporus* have the potential to be used as a coagulant in tofu. The optimum time to perform coagulation for *R. oryzae* is 18 hours and for *R. oligosporus* is 12 hours. Tofu made using *R. oryzae* and *R. oligosporus* as a coagulant has higher content of linolenic and linoleic than in tofu using vinegar as a coagulant. The highest of linoleic and linolenic acids was obtained: (i) *R. oryzae* fermentation at 6 hours (0.26% and 0.14%), after that linoleic and linolenic acids tended to decrease, along with the length of fermentation. (ii) *R. oligosporus* fermentation of 24 hours (0.14% and 0.08%).

## REFERENCES


Adnan M. 1997. Chromatographic technique for the analysis of foodstuffs. Andi. Yogyakarta. [Indonesia]

Ariani SRD. 2003. Production of soycheese containing factor-2 compound bioconvertion product from isoflavone in tofu by Rhizopus oligosporus (L.41). Biosmart 5 (1): 8-12. [Indonesia]

Bisping B, Hering L, Baumann U, Denter J, Keuth S, Rehm HJ. 1993. Tempe fermentation: Some aspects of formation of γ-linolenic acid, proteases and vitamins. Biotechnol Adv 11 (3): 481-493.

Campbell NA. 1987. Biology. Benjamin/Cummings. California

Cook PE. 1994. Fermented foods as biotechnological resources Food Res Intl 27 (3): 309-316.

De Man JM. 1997. Kimia makanan. Penerbit ITB. Bandung. [Indonesia]

de Reu JC, Ramdaras D, Rombouts FM, Nout MJR. 1994. Changes in soya bean lipids during tempe fermentation. Food Chem 50 (2): 171-175.

Denter J, Rehm H, Bisping B. 1998. Changes in the contents of fat-soluble vitamins and provitamins during tempe fermentation. Intl J Food Microbiol 45: 129-134.

Forman D, Bulwer BE. 2006. Cardiovascular disease: optimal approaches to risk factor modification of diet and lifestyle. Curr Treat Options Cardiovasc Med 8 (1): 47-57.

Gaman PM, Sherrington KB. 1992. Introduction to food science, nutrition and microbiology. Gadjah Mada University Press. Yogyakarta. [Indonesia]

Harper HA, Rodwell VW, Mayes PA. 1979. Biochemistry. EGC. Jakarta. [Indonesia]

Hidayat N. 2009. Tahapan proses pembuatan tempe. Universitas Brawijaya. Malang. [Indonesia]

Houston MC, Basile J, Bestermann WH, Egan B, Lackland D, Hawkins RG, Moore MA, Reed J, Rogers P, Wise D, Ferrario CM. 2005. Addressing the global cardiovascular risk of hypertension, dyslipidemia, and insulin resistance in the southeastern United States. Am J Med Sci 329 (6): 276-291.

Iskandar Y. 2004. Determination of linoleic acid in Tempe by gas chromatography. FMNS, Padjajaran University. Jatinangor, Sumedang. [Indonesia]

Karyadi D. Hermana H. 1995. Tempe potential for nutrition and health. Proceedings of the National Symposium on Development of Tempe in Modern Food Industries. Indonesian Tempe Foundation. Jakarta. [Indonesia]

Muchtadi TR. 2000. Omega 9 fatty acids and health benefits. Media Indonesia, 29 November 2000. [Indonesia]

Nadesul H 2007. Healthy is cheap. Kompas. Jakarta. [Indonesia]

Panagiotakos DB, Pitsavos C, Chrysohoou C, Risvas G, Kontogianni MD, Zampelas A, Stefanadis C. 2004. Epidemiology of overweight and obesity in a Greek adult population: the ATTICA Study. Obes Res 12 (12): 1914-1920.

Pawiroharsono S 1997. Microbiological aspects of tempe. In: Sutrisno S (ed). Indonesian tempeh potpourri. Indonesian Tempe Foundation. Jakarta. [Indonesia]

Purwoko T, Gandjar I, Pawiroharsono S. 2001. Biotransformation of isoflavones by *Rhizopus oryzae* UICC 524. Biosmart 3 (2): 7-12. [Indonesia]

Purwoko T, Nurkhayati, Arumsari R. 2003. Antioxidative activity of fermented tofu against oxidation of soybean oil. Biosmart 5 (1): 12-16. [Indonesia]

Purwoko T. 2004. Content of isoflavone aglycone on tempeh fermented by *Rhizopus microsporus* var. *oligosporus*: the effect of immersion. Biosmart 6 (2): 85-87. [Indonesia]

Santoso HB. 1993. Making tempeh and tofu soy nutritious food. Kanisius. Yogyakarta. [Indonesia]

Sutrisno S. 1997. Indonesian tempeh potpourri. Indonesian Tempe Foundation. Jakarta. [Indonesia]

Sipayung R. 2003. Fatty acid biosynthesis in plants. Faculty of Agriculture, University of North Sumatra. Medan. [Indonesia]

Steinkraus KH, Cullen RE, Pederson CS, Nellis LF, Gavitt BK. 1983. Indonesian tempeh and related fermentations. In: Steinkraus KH (ed). Handbook of indigenous fermented foods. 1st ed. Marcel Dekker. New York.

Styme S, Stobart AK. 1986. Biosynthesis of gamma-linolenic acid in cotyledons and microsomal preparations of the developing seeds of common borage (*Borago officinalis*). Biochem J 240 (2): 385-393.

Subagio A, Hartanti S, Windrati WS, Unus, Fauzi M, Herry B. 2002. Review physicochemical and organoleptic properties of tempe hydrolyzate protease hydrolysis results. J Teknologi Industri Pangan 13 (3): 204-210. [Indonesia]

Suhaidi I. 2003. Effect of soy dipping time and type of coagulant substance to tofu quality. Faculty of Agriculture, University of North Sumatra. Medan. [Indonesia]

Suharyanto, Tripanji, Abdullah MI, Syamsu K. 2006. CPO bioconversion with immobilized desaturase semipilot continuous systems on the scale for the production of oil containing GLA. Biotechnology Research Institute for Estate Crops of Indonesia. Bogor. [Indonesia]

Suprapti L. 2005. Making out of tofu. Kanisius. Yogyakarta. [Indonesia]

Teng Y, Xu Y, Wang D. 2008. Production and regulation of different lipase activities from *Rhizopus chinensis* in submerged fermentation by lipids. State Key Laboratory of Food Science and Technology, Jiangnan University, PR China.

Wiesel I, Rehm HJ, Bisping B. 1996. Improvement of tempe fermentations by application of mixed cultures consisting of *Rhizopus* sp. and bacterial strains. Appl Microbiol Biotechnol 47 (3): 218-225.

Wilson PW, D'Agostino RB, Sullivan L, Parise H, Kannel WB. 2002. Overweight and obesity as determinants of cardiovascular risk: the Framingham experience. Arch Intl Med 162(16):1867-1872.