Tapioca and tofu waste utilization to produce AA, DHA, and EPA

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Abstract. Human body has limited ability to synthesize unsaturated fatty acids such as AA, DHA, EPA. These fatty acids are essential for the body. This study initiated the efforts to produce unsaturated fatty acids of microorganisms, with low cost and high percentage. This study used tapioca and tofu waste as a medium for Aspergillus. In addition, this study analyzed the varying of carbon concentration to the maximum lipid production from Aspergillus oryzae and determined the optimum rate of agitation against lipid production from Aspergillus oryzae. The results show that the optimum composition of AA, DHA, EPA are 0.18% (w/w), 0.33% (w/w), and 2.96% (w/w) respectively in concentration carbon of 9% (w/w) and agitation rate of 120 RPM.

1. Introduction
Sustainable Development Goals (SDGs) is to reduce the increasing malnutrition. According to the Basic Health Research Data (Riskesdas), malnutrition is characterized by an increase in malnutrition prevalence rate which increased to 19.6 percent in 2013. Malnutrition in children leads to growth and development disruption and impacts on the quality of future generations. One of the nutrient deficiency factors is caused by the lack of fatty acid intake in children under five years who are not optimally fulfilled, whereas the composition of human brain cells about 60% consists of fatty acids [1].

Alternative sources of fatty acid producer is microorganism. Fatty acid producing microorganisms consist of yeast, yeast, bacteria and microalgae. One of the best microorganism producing fatty acids is yeast where yeast has advantages such as the time required to produce relatively short and fatty acids that can produce is high [2]. Safety yeast for food so far is Aspergillus oryzae. Aspergillus oryzae used in the manufacture of soy sauce, tauco, and miso, so it is rated as a safe food grade [3]. Aspergillus oryzae is an easy-to-cultivate oleaginous microorganism that can survive in conditions with high levels of sugar or salt, energy saving, easy handling and easy use of technology that makes the Aspergillus oryzae potentially and highly prospective to be developed as an alternative source of AA, DHA, EPA producers [4]. Aspergillus oryzae does not require light in its growth, making it more energy efficient [5]. In addition, Aspergillus oryzae forms a filament in its infancy so it does not require filtration with a special membrane or special technology in its harvesting [6].

Factors which are affecting the growth of Aspergillus oryzae, among others, are the concentration of carbon source and the rate of agitation. According to Lopez et al. (2013), carbon concentrations can naturally regulate lipid production through catabolic repression processes. The presence of variations in
carbon concentrations determines the flexibility and ability of *Aspergillus oryzae* to consume food [7]. During this time, there has been no research in the variation of carbon concentration on the growth of *Aspergillus oryzae*. Therefore, further research is needed related to the relation of carbon concentration given to *Aspergillus oryzae* growth so it can be concluded whether the higher variation of carbon concentration determines the amount of lipid produced.

The rate of agitation plays an important role in the homogenization of nutrients in the fermentation medium. In general, the optimum agitation rate determines the transfusion of hyphae between the yeast affecting *Aspergillus oryzae* growth [8]. Low agitation rates result in slower growth and lower lipid rendement. Overly high agitation rates cause shear stress in the *Aspergillus oryzae* [9]. This is reinforced by Fadaly et al. (2009) that each type of *Aspergillus oryzae* has a large optimum agitation rate that varies depending on the type and magnitude of the reactor. Therefore, an optimal condition of carbon concentration and an appropriate agitation rate are required to produce an optimum unsaturated fatty acid.

In this study, the type of culture method used is submerged fermentation method using tapioca and tofu waste as a medium of fermentation. It aims to facilitate the manipulation of culture conditions so that more lipid rendement can be obtained. The researchers used industrial waste, i.e. tapioca starch and tofu waste as a fermentation medium for *Aspergillus oryzae*. In this research, two extraction methods were used to optimize the fatty acid yield, namely sonification extraction using ethanol and then followed by extraction using n-hexane. The ethanol and n-hexane extraction methods have several advantages such as safe and easy to apply in the food industry [10]. Lipids analyzed using GC (Gas Chromatography) to determine the composition of AA, DHA, EPA.

2. Experimental Methods

2.1. Tools and Materials
This study used gas chromatography, centrifugator, spectrophotometer, autoclave, water bath shaker, laminar flow, and oven. The ingredients used for cultivation medium are tapioca waste, tofu waste, KH₂PO₄, Na₂HPO₄, CaCl₂·2H₂O, FeSO₄·7 H₂O, and CuSO₄·5 H₂O. The composition of carbon content using concentrated H₂SO₄ 98%, K₂Cr₂O₇ 2N, and Glucose. The solvent used for the extraction is ethanol and n-hexane.

2.2. Research Variable
The variables that will be varied in this research are carbon concentration and agitation rate in *A. oryzae* fermentation medium. The carbon concentrations used in this study were 6% (w/w), 7% (w/w), 8% (w/w), 9% (w/w), and 10% (w/w), and the agitation rate used in this study were 70 rpm, 120 rpm, 170 rpm, 220 rpm. The variables that will be measured in this research are percentage of AA, DHA, and EPA content. The fixed variables in this study were temperatures in the range 25-30 °C, pH in the range of 4.5-6.5, and the incubation time to enter the stationary phase.

2.3. Preparation
Preparations include the manufacture of tapioca waste and tofu waste, testing of carbon content (Walkey and Black) and nitrogen (Kjeldahl) in tapioca waste and tofu waste, and sterilization of the tool.

2.4. Preparation of Stock Culture
Proliferation of yeast is done by culturing on PDA (Potato Dextrose Agar). Besides aiming to multiply the cells, culture on PDA medium also aims to increase the *Aspergillus oryzae* spores that will be used in liquid culture. Cultures on PDA medium were incubated for 3-4 days at 25-30 °C.

2.5. Liquid Medium Culture
The culture of the liquid medium was carried out 3 times, the first stage of the culture of liquid medium was carried out using variations of carbon concentration at concentrations of 6% (w/w),
7% (w/w), 8% (w/w), 9% (w/w), and 10% (w/w). The second stage of liquid medium culture is carried out using variation of agitation rate of 70 rpm, 120 rpm, 170 rpm, 220 rpm with carbon concentration is the optimal carbon concentration obtained in the previous stage. Liquid medium culture was carried out on a batch reactor using a medium of tapioca and tofu waste as a source of carbon and nitrogen, and also micronutrients added to the medium. Liquid culture is carried out at 25-30 °C, pH 4.5-6.5, with 6 days incubation time in water shaker bath.

2.6. Harvesting
Harvesting starts from filtration stage using filter paper, then weighing wet biomass. The biomass was then dried oven at 65 °C for 24 hours until a constant weight.

2.7. Extraction
The extraction process uses two solvents, ethanol and n-hexane. Fractionation method used is liquid-liquid partition. Partition separation techniques are based on differences in the degree of polarity of the solvent used. The separation technique involves two non-mixed solvents in a separating funnel, and then separates according to the partition coefficient [10]. Fractionation was carried out using a polar solvent using extraction with ethanol and continued for a non-polar solution using n-hexane.

According Ambarsari (2013), ethanol is a semipolar solvent that can dissolve polar and non-polar solutes. Extraction using ethanol was introduced as a simple extraction method because of its relatively easy processing and is often used in the process of sifting for microorganisms because it is food grade [11]. The definition of food grade is safe for use in producing foodstuffs. In this extraction process is also done simultaneously with the sonication process to optimize lipid production. Sonication is done for the process of breaking the cell wall. When the sonication process takes place, cavitation occurs due to the treatment of ultrasonic waves [12].

Furthermore, the nonpolar part was again extracted using n-hexane extract. Selection of n-hexane as solvent, since n-hexane is stable and volatile, selective in dissolving substances, extracting small amounts. Then the mixture was centrifuged to separate between the biomass and the ethanol-lipid mixture. Furthermore, the lipid and solvent mixtures are evaporated to separate the solvent from the lipids and regenerate the solvent. Lipid obtained in the form of dry lipid or concentrate which will then be tested. Then measure lipid mass to calculating the lipid yield.

2.8. Composition Analysis of AA, DHA, EPA
The composition analysis of AA, DHA, and EPA conducted by using GC in the Agro Industry Center (BBIA), Bogor. The test method using GC is done by GC-IUPAC 2.301 7th Edition method.

3. Result and Discussion

3.1. Medium Waste Quality Analysis
The carbon analysis was performed using a UV VIS spectrometer with a wavelength of 651 nm. The following table is an analysis results of carbon and nitrogen content in the effluents carried out.

| Component | Tapioca Waste (% w/w) | Tofu Waste (% w/w) |
|-----------|------------------------|-------------------|
| Carbon    | 34.43                  | 29.04             |
| Nitrogen  | 0.12                   | 5.2               |

The analysis of carbon and nitrogen content in tapioca waste and tofu waste shows that tapioca waste has a function as the highest carbon source compared to tofu wastes. The tofu waste basically has a fairly high carbon content, but the carbon in the tofu waste comes in part from the crude fiber and starch.
that are more difficult to degrade by microorganisms [13]. The tofu waste has the highest nitrogen content compared to the tapioca waste. Therefore, the tofu waste is known to act as a source of nitrogen.

3.2. The Growth of A. oryzae

The growth and proliferation of *Aspergillus oryzae* is characterized by increasing weight and number of cells [14]. The addition of dry weight of the cell during the fermentation process shows the growth of *Aspergillus oryzae* so that it can be made a *Aspergillus oryzae* growth profile. The addition of the dry weight of the cell is plotted on the Y axis, while the incubation time is plotted on the X axis (Fig. 1). The calculation of the dry weight of the cell is carried out to determine the number of cells present in the medium over a given period.

![Growth curve of Aspergillus oryzae](image)

**Figure 1.** Growth curve of *Aspergillus oryzae*

Observations were made every 24 hour period. It aims to identify the growth curve of *Aspergillus oryzae*. According to Subrahamanyam et al. (2001) states that *Aspergillus oryzae* growth is not like a bacterium whose cell division is relatively rapid within a few hours. Early *Aspergillus oryzae* growth is transparent white, thin cotton-shaped, and floating-floating. This form is defined as a single spore or conidia that has grown into mycelium [14]. The mycelium continues to grow until it enters the stationary phase.

Figure 1 shows that *Aspergillus oryzae* passes through lag phase, log, and stationer. From the growth curve it can be seen that the first day there is a lag phase (or adaptation phase). Yeast began to grow hovering like fine white cotton. In this phase, the increase in the dry weight of the cells has not been significant because of the *Aspergillus oryzae* in the adaptation process so that the nutrients present in the medium have not been used for the growth process [14]. The exponential phase occurs on day one to day 5 is marked by a significant increase in cell dry weight.

The stationary phase begins on the 6th day. On the 6th and 7th day there is no significant biomass increase. The largest dry weight was obtained on day 6 so it can be proven that day 6 is the best time to harvest biomass. The same is also stated by research conducted by Sukma et al. (2010), rice bran oil fermented with *A. terreus* for 6 days resulted in an abundance of unsaturated fatty acids higher than the variation of incubation time for 5 days and 7 days.

On day 7 to day 10, *Aspergillus oryzae* begins to decrease dry weight. According Firdiana (2007), this is due to reduced nutrients and oxygen so that biochemical activity decreases. This causes many mycelia to tend to coagulate with each other, so at the time of sampling, many are not carried away.

According to Abu-Elreesh (2013), *Aspergillus oryzae* is harvested in the stationary phase yielding optimum fats. Abu-Elreesh (2013) using the DGB1 strain proves there is a relationship of lipid accumulation with *Aspergillus oryzae* growth. In the stationary phase there is maximum lipid accumulation and further decreases as the nutrients in the medium decrease, the accumulated lipids are used by the microorganisms for the metabolism [15].

According to Ratledge and Wynn (2003), lipid accumulation can occur when the nitrogen contained in the substrate has been used up for *Aspergillus oryzae* growth, then excess carbon is converted to
After its optimum conditions, a decrease in lipid accumulation caused by the use of lipids by microorganisms to produce biomass. Therefore, the harvesting process is carried out at the stationary phase on the 6th day of the fermentation process.

3.3. **Effect of Carbon Concentration on Production of AA, DHA, EPA**

The process of catabolic repression to regulate the production of lipid microorganisms is influenced by the concentration of carbon sources [16]. To optimize the process of catabolic repression is still needed sources of nutrients such as mineral elements. *Aspergillus oryzae* growth depends on the concentration of carbon present in the medium. This is reinforced by Prihastuti and Yuliatun (2002) that higher carbon content provides a longer logarithmic phase than lower carbon content. The enzyme activity produced by oleaginous microorganisms is greater when the carbon and nitrogen sources in the substrate are sufficient to support the growth of stem cells and their metabolism, and can increase the ability of *Aspergillus oryzae* to produce lipids [17].

The yield of lipid with variation of carbon concentration in this study is presented in the following graph.

![Figure 2. The yield of lipid biomass on the variation of carbon concentration](image)

Based on Figure 2, the yield of lipid from variation of carbon concentration of 6% (w/w) was 14.01% (w/w). The lipid yield is relatively increased with an increase in carbon concentration of 7% (w/w), 8% (w/w), 9% (w w). The lipid yields were 14.29% (w/w), 19.6%, respectively, where the greatest lipid yield was obtained at a concentration of 9% (w w) of 23.59% (w/w). This increase occurs because *Aspergillus oryzae* are able to synthesize lipids by breaking carbon chains and releasing hydrogen atoms to produce oxidized compounds (pyruvic acid) (Sumanti et al., 2005). At 9% concentration (w/w), *Aspergillus oryzae* is able to optimize growth for the process of glycolysis where the resulting pyruvic acid enters the Krebs cycle in the mitochondria. This triggers the optimum lipid synthesis [18].

The lowest lipid yield was obtained at a concentration of 10% (w/w) of 7.96%. This is because the concentration of 10% is too concentrated so that the osmotic potential increase at substrate concentration becomes too high [18]. The high osmotic potential causes *Aspergillus oryzae* growth to be less optimum. According to Fadaly et al. (2009), less optimum *Aspergillus oryzae* growth is characterized by low biomass which has an effect on cell conversion to synthesize lipid to low. This is reinforced by Sumanti et al. (2005), the low yield lipid obtained because most of the carbon consumed is used for cell poliferation, whereas the carbon used in the lipid accumulation process becomes low.

Carbon concentrations of 6% (w/w) and 7% (w/w) had a significantly lower lipid yield of 14.01% and 14.29%, respectively. At a concentration of 8% (w/w) yielded a 19.6% lipid yield. Different carbon concentrations result in differences in cell growth in *Aspergillus oryzae*. As carbon concentration increases, cell growth and metabolism increase with increasing enzyme activity, thus affecting the ability of yeast to produce lipids [19]. In addition, the power of contact between nutrients with *Aspergillus oryzae* becomes intense thus increasing the resulting lipid.
The lipid yields obtained in this study had greater results than Putri, et al. (2016). The highest lipid yields produced by Putri, et al. (2016) is 8.2% (w/w). While in this research yield highest yield of lipid equal to 23.59% (w/w). This is due to the extraction factor. In a study conducted by Putri et al. (2016), the extraction carried out using only extraction using ethanol. In this study using fractionation extraction of ethanol and n-hexane which can optimize the amount of yield of lipid obtained.

The use of food grade solvents aims to produce safe lipids for consumption because the resulting lipids are designated as man materials [20]. The choice of n-hexane as a solvent because n-hexane is stable and volatile, selective in dissolving substances, extracting small amounts of solution and able to extract non-polar substances in sufficient quantities [21]. Based on the consideration of dry weight and yield of lipids produced for cultivation stage with variation of agitation used carbon concentration of 9% (w/w).

Lipid samples obtained from the extraction process. Furthermore, it was analyzed using GC-IUPAC 2.301 7th Edition method. The chromatogram of the lipid sample analysis results detected the fatty acid composition present in the lipid sample.

![Figure 3. Percentage of unsaturated fatty acid of A. Oryzae with variation of carbon concentration](image)

The largest unsaturated fatty acid composition was obtained at a concentration of 9% (w/w) in which the dry weight and high lipid yield affected the resulting fatty acid composition. In previous studies, the highest unsaturated fatty acids were obtained at a concentration of 6% [22]. This is due to an increase in concentration triggering nutrient transfer between medium and Aspergillus oryzae [23].

The maximum unsaturated fatty acid yields obtained in A. oryzae with RSM (RSM hydrolysate, molasses, and salt) mediums performed by Yangmin et al. (2015) is 8.55 mg/L or equivalent to 0.855 mg/100 ml of medium. While in Muniraj et al. (2013) yields a 3.5 gram/L lipid yield of 57.9% unsaturated fatty acid (w/w) or an unsaturated fatty acid of 202.65 mg/100 mL of medium. The study obtained 61.2% (w/w) unsaturated fatty acids under lipid conditions 122.44 mg/100mL medium. The unsaturated fatty acid yields produced by Muniraj et al. (2013) higher. This is because the medium used in the form of liquid waste potato containing starch is 31.2% (w/w).

The results of this study are higher than Yangmin et al. (2015) other than because the carbon source is also caused by the gradual extraction method using ethanol and n-hexane. The extraction method used can optimize the yield of lipid yield and the conversion of unsaturated fatty acid produced to be higher. The difference in the results is also due to differences in the medium used, Yangmin et al., (2015) using RSM medium whose main composition is cellulose, hemicellulose, and lignin with a tendency more difficult to be degraded by microorganisms.

AA, DHA, EPA are read only at a concentration of 9% (w/w) with a percentage of 0.18% (w/w), 0.33% (w/w), 2.96% (w/w) respectively. In a study conducted by Muslimah, et al. (2015), the
composition of DHA is higher by 2.54% (w/w). Meanwhile, this study has a higher EPA than Muslimah, et al. (2015). This is due to the difference in concentration used by Muslimah by 2% (w/w).

Based on the results of this study, the concentration of carbon that can produce lipids by producing AA, DHA, and EPA is the optimal concentration of 9% (w/w). At this concentration, the yield of dry lipids and biomass has the highest percentage. Unsaturated fatty acids, other than PUFA and MUFA, the presence of AA, DHA, and EPA are considered in the selection of optimum concentrations. Therefore, Submerged Fermentation of the Aspergillus oryzae by using tapioca and tofu waste has the potential to be the source of production of AA, DHA, and EPA.

3.4. Effect of Agitation Rate on Production of AA, DHA, EPA
The agitation rate is able to influence the contact power between medium and nutrient [24]. In the medium of tapioca waste and tofu waste know the optimum agitation rate is required to produce high lipid yield on biomass. The optimum agitation rate can facilitate carbon storage in the form of a lipid reserve. According to Firdiana (2007), the agitation rate plays an important role in the homogenization of nutrients in the fermentation medium and the increased surface area of oxygen displacement in the fermentation medium. Agitation rates are too low resulting in slower growth and lower lipid yield. However, too high agitation rates cause shear stress in the Aspergillus oryzae [25].

The results obtained were linear with the yield of lipid yields obtained:

![Figure 4](image_url)

**Figure 4.** The percentage of lipid yields in variations in agitation rate

In Figure 4, agitation rate of 120 RPM and 170 RPM, the yield of lipid are 23.59% (w/w) and 26.1% (w/w), respectively. The resulting lipid yield has a correlation to dry weight. The amount of dry weight produced determines the amount of lipid obtained. The lipid yields obtained are quite high compared to other studies obtained by Hui et al. (2010) at 180 rpm agitation yields an 18% lipid yield (w / w). In the test of AA, DHA, EPA, GC-IUPAC 2,301 7th Edition was performed on the variation of agitation rate obtained in Figure 5.
Unsaturated Fatty Acid (% w/w)

Figure 5. The percentage of unsaturated fatty acids of lipids \textit{A. oryzae} with agitation rate variation

The results obtained at agitation rates of 120 RPM and 170 RPM have differences in unsaturated fatty acids that are not significantly different. The unsaturated fatty acids each obtained were 61.2% (w/w), while for the agitation rate of 170 RPM was 59.9% (w/w). These results indicate that the fungus is able to perform metabolism well with the agitation of 120 RPM and 170 RPM resulting in high unsaturated fatty acids. According [26], \textit{Aspergillus oryzae} is able to transfer the hyphae well in the range of agitation of 120 RPM. Sari et al. (2015) obtained an unsaturated fatty acid of 59.42% (w/w). The percentage of unsaturated fatty acids obtained in this study was higher than that of Sari et al. (2015). This is because the medium of tapioca waste and tofu waste used in this study still contains nutrients that can help \textit{Aspergillus oryzae} growth.

The agitation rate of 220 RPM has an unsaturated fatty acid content composition of 40.73% (w/w). According Purwanto et al. (2009), this is due to high contact between the mycelia causing \textit{Aspergillus oryzae} can not do optimum growth. According Purwanto et al. (2009), at too high agitation rates the production of acetyl-KOA becomes unoptimum and results in low fatty acids being produced. The agitation rate of 70 rpm yields an unsaturated fatty acid of 34.84% (w/w). The low agitation rate makes the \textit{Aspergillus oryzae} unable to perform the optimal metabolism because the nutrients in the medium are not dissolved properly.

AA, DHA, and EPA can be generated on agitation of 120 RPM and 170 RPM. At the agitation rate of 120 RPM AA, DHA, the resulting EPA was 0.18% (w/w), 0.33% (w/w), 2.96% (w/w). At the agitation rate of 170 RPM AA, DHA, and the resulting EPA was lower by 0.07% (w/w), 0.25% (w/w), and 1.51% (w/w). Meanwhile, the agitation rate of 220 RPM was detected only DHA and EPA of 0.1% (w/w) and 0.03% (w/w). In the study of Muslimah et al. (2015), AA, the resulting DHA is greater. Meanwhile, in this study, EPA contained higher.

Based on the results of this study, the agitation rate values with the highest unsaturated fatty acids as well as the composition of AA, DHA, and EPA optimum is 120 RPM. In this condition, \textit{Aspergillus oryzae} undergo optimal hyphae transfer and can carry out the homogenization of nutrients in the maximum fermentation medium. This is due to the large yield of lipid yield and biomass yield, as well as the magnitude of the composition of AA, DHA, and EPA.

4. Conclusion

Carbon concentration and agitation rate with optimum biomass yield and lipid yield, i.e. carbon concentration of 9% (w/w) and 120 RPM of 23.59% (w/w). The content of the largest unsaturated fatty acids was obtained in the variation of 9% carbon concentration and the agitation of 120 RPM of 61.2% (w/w) with the unsaturated fatty acid composition comprising 35.68% omega-9 fatty acid, 22.58% Omega-6 fats, and 4.02% omega-3 fatty acids.
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