Effect of pH, temperature and medium agitation rate in production of AA, DHA, EPA from *Aspergillus oryzae* with submerged fermentation

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Abstract. There are several substances that needs to be fulfill to keep the brain cell growth such as AA, DHA and EPA. Fungi is one of the alternative source of omega 3, omega 6, omega 9 especially AA, DHA and EPA. This research variates operating condition that is suitable for the growth of *Aspergillus oryzae* in AA, DHA, and EPA fatty acid production with *Submerged Fermentation* using synthetic medium. *Aspergillus oryzae* cultivated in medium using glucose as carbon source and Ammonium sulfate and yeast extract as nitrogen source. The extraction method using ethanol and n-hexane as solvent. The result shows that optimum agitation rate for unsaturated fatty acid production of *Aspergillus oryzae* is 120 RPM, lipid yield 28.28% and unsaturated fatty acid content 50.36 % and EPA content 2.42 %. Optimum medium pH for PUFA production of *Aspergillus oryzae* is 6, lipid yield 22.35 % and unsaturated fatty acid content 45.5 %. optimum incubation temperature for unsaturated fatty acid production of *Aspergillus oryzae* is 25 °C, lipid yield 13.19 % and unsaturated fatty acid content 62.15 %. Unsaturated fatty acids produced from *Aspergillus oryzae* are oleic, linoleic, linolenic and EPA.

1. Introduction

Food problems became one of the biggest problems related to population growth. To guarantee the availability of foods for humans in large numbers, require large natural resources, both in terms of availability of land as well as the variety of food sources. According to economic experts who are members of the forum of Economic Intelligence Unit (EIU) in 2014, the food security index of Indonesia ranks 64 in the world, far below other ASEAN countries.

Indonesia food security index numbers are worrisome to be one of the causes of the poor condition of Indonesian public health. According to the World Health Organization (WHO) in 2013, the level of Indonesian health is one of the lowest in ASEAN. To meet nutrient intake on toddlers, there are some substances that have to be fulfilled for the process of development of brain cells mainly include carbohydrates, proteins, iron, vitamins and fatty acids such as DHA and EPA, which is divided into saturated fatty acids and unsaturated fatty acids.

Currently, omega 3 and omega 6 are derived from fish oil. However, the availability of fish in terms of food security are not proportional to the need that continue to increase. So the alternative source of fatty acids, especially the omega 3 and omega 6 besides fish oil is needed. Alternative sources of omega 3 and omega 6 besides fish oil should have several advantages both in terms of land use, the time it takes to produce fatty acids as well as cheaper cost [1]. A lot of research
have been done on fatty acids fish oil replacement. Such research produces conclusion for using microorganisms as an alternative source of fatty acids [1]. The uses of microorganisms as an alternative source of fatty acids also have an advantage as mentioned.

There are different types of microorganisms that can be used as an alternative source of fatty acids. These microorganisms have a high fat content, such as yeast, fungi, bacteria and mikroalga. In this study used microorganisms fungi (mold). Mold used in this study because the oleaginous microorganisms including mold, has various advantages such as easier handling, able to grow in low pH and can degrade a complex carbon source [2]. In this research the type of mold, Aspergillus oryzae is used which has a lipid content of approximately 18-57% [3].

In this research, molds were cultured in the synthetic medium with the ratio of C:N of 30:1 and submerged fermentation. Submerged fermentation method was chosen as a method of culture because nutrients spread more evenly so that yield production of fatty acids expected to be greater. So, this research will find profile concentrations of unsaturated fatty acids from Aspergillus oryzae against variations in temperature incubation medium pH, as well as the rate of agitation of the medium in the production of unsaturated fatty acids, DHA and EPA

2. Methods

2.1. Culture stock in agar medium
Aspergillus oryzae cultured using PDA (Potato Dextrose Agar) medium containing 0.39 gram medium agar dissolved with 10 mL aquades.

2.2. Culture to make growth curve
Aspergillus oryzae cultured in medium for 10 days then harvested every day. Dry cell weight counted to make growth curve.

2.3. Culture in submerged fermentation
Previous cells cultured at medium agar transferred into liquid medium with glucose as the carbon source composition 4.5 g, ammonium sulfate and yeast extract 0.15 grams of micro nutrient sources, along with (NH₄)₂SO₄ as 0.25 g KH₂PO₄, 0.15 g K₂HPO₄, 0.05 grams, MgSO₄.7H₂O 0.2 grams, FeCl₃ 0.015 grams into 150 ml aquades. Culture is carried out for 6 days with each variation rate of agitation, pH and temperature. Culture agitation rate variation of 70, 120 and 170 rpm. The ratio of C and N 30:1, pH 7 medium, incubation temperature 35 °C made during 144 hours (6 days). After obtained the rate of agitation with the optimum yield, a culture with pH variations variations medium conditions 5, 6 and 7, the ratio of C and N 30:1, incubation temperature 35 °C. Incubation time for 144 hours (6 days). After the pH and agitation rate obtained with optimum yield, a culture with temperature variations 25, 30 and 35 °C. Incubation time for 144 hours (6 days).

2.4. Cell harvesting
Cells harvested and dried with oven for 24 hours in temperature 60 °C. The cells then extracted with sonicator using ethanol as solvent for 3 hours. After that centrifuge 4000 rpm for 15 minutes. Then extracted again with n-hexane as solvent for 2 hours. After that centrifuge with 4000 rpm speed for 15 minutes to separate the lipid and biomass content.

2.5. Fatty acid composition analysis
Lipid extracted then analyzed using GC-MS in Balai Besar Industri Agro (BBIA) Bogor, Jawa Barat.

3. Result and discussion

3.1. Effect of agitation rate
The rate of agitation would affect the state of the mold as the impact both positive and negative. Positive impact caused by agitation is increasing the solubility of oxygen and nutrients distributed evenly or mold growth will increase the production of lipids [4]. But, like a double-edged blade, negatively impact the rate of agitation that is too high will damage the mycelium cells and tissues.
Therefore, this study tried to find out the relationship between the rate of agitation with the growth of mold.

In the figure it can be seen that on a medium with a higher rate of agitation, biomass growth mold tends to be faster, as seen on the medium with agitation 120 and 170 RPM compared to 70 RPM. From the graph we can say that optimum growth of the mold Aspergillus oryzae occurred at a range of 120 RPM agitation. On the agitation with 120 RPM mold growth is the highest with a biomass of 0.8391 g. This is because the medium mixed with good (perfectly mixed), so that the solubility of oxygen in high nutrient medium are mixed evenly. Another case with agitation 70 RPM which showed the lowest results, i.e. 0.3071 grams. It is thought to be caused by nutrients not distributed evenly in the medium.

3.2. Production of Lipids on the variation rate of Agitation
The influence of rate of agitation against the weight of lipid obtained there is in figure below. On the picture, seen that the resulting lipid ranged from 0.096 – 0.237 grams. The maximum amount of lipids formed at 120 RPM agitation. The abundance lipids produced at 120 RPM agitation is directly proportional to biomass mold that grows at a rate of agitation. It shows that the medium at a rate of 120 RPM agitation is the optimum medium for incubation mold either in terms of the growth of the biomass as well as the formation of lipids.

![Figure 1](image1.png)  ![Figure 2](image2.png)

**Figure 1.** Effect of Agitation Rate to Cell Dry Weight.

**Figure 2.** Effect of Agitation Rate to Lipid Weight.

The condition is the best agitation conditions where oxygen can be dissolved and the medium can be mixed well. While contact between Hypha can be maintained properly. Hypha quite contacted with each other and form a mycelium, later the mycelium does not suffer fragmentation and damage cells.

3.3. Saturated and unsaturated fatty acid analysis

| Fatty Acid Composition | Agitation rate (RPM) |
|------------------------|----------------------|
|                        | 70  | 120  | 170  |
| Saturated Fatty Acid (% w/w) |
| Lauric (C12:0)        | 13.9| 1.27 | 6.81 |
| Miristic (C 14:0)     | 6.92| 1.3  | 3.62 |
| Palmitic (C 16:0)     | 34.3| 19.7 | 21.9 |
| Stearic (C 18:0)      | 6.93| 22.9 | 22.8 |
| Unsaturated Fatty Acid (% w/w) |
| Oleic                 | 31.6| 24   | 17.7 |
| Linoleic              | 6.31| 25.9 | 20.5 |
| Linolenic             | n.d | 0.46 | n.d  |
| Linolenic             | n.d | n.d  | n.d  |
| AA                    | n.d | n.d  | n.d  |
Saturated fatty acids resulted in variations of agitation 70, 120 and 170 RPM-including lauric, Palmitic, and stearic myristate. These fatty acids are produced with different compositions on any variation of the rate of agitation. The rate of 70 RPM agitation dominated Palmitic acid with percentage of 34.3%. The rate of 120 RPM agitation dominated stearic acid with percentage 22.9%. While the rate of 170 RPM agitation is also dominated by stearic acid with 22.8% percentage. The variation rate of agitation 70 RPM produces the largest saturated fatty acids with the largest percentage is a type of Palmitic (C16) and 34.3%. While saturated fatty acids produced in the smallest variations in the rate of 120 RPM agitation with the largest percentage of types of stearic (C20) namely 22.9%. Type of saturated fatty acids are produced depending on the success of elongase enzymes in adding chain lengths of fatty acids[5]. It showed variations 70 RPM elongase enzymes do not work in optimum. Elongase enzymes working the most optimum at 120 RPM agitation.

3.4. Effect of the pH of the incubation medium
The result of the growth of mold on the medium pH variations 5, 6 and 7 show the profile as shown in the figure below. At pH 5 obtained biomass mold of 0.98 g, in a medium with a pH obtained biomass mold of 1.2 grams. Whereas in the medium with a pH of 7, obtained biomass mold 0.3 grams. In General, on a medium with a slightly acidic pH of biomass produces a larger mold than a neutral pH. This indicates that the pH of the medium affects the growth of mold and mold can grow optimally at pH slightly acidic to neutral.

![Figure 3. Effect of pH to Cell Dry Weight.](image)

![Figure 4. Effect of pH to Lipid Weight.](image)

3.5. Production of lipids on the medium pH variation
The success of the accumulation of acetyl Coa to be transformed into a secondary metabolic products namely lipid depends on enzymes that synthesize lipids. The enzyme has a optimum pH to work in particular. Study on production of lipids on the mold known by using the weight of the lipids that formed during incubation.

On the picture, we can see that the resulting lipid range between 0.06-0.27 grams. The maximum amount of lipids are formed at pH 6. The abundance lipids produced at pH 6 is directly proportional to biomass mold, which grows at pH 6. It shows that the medium with a pH of 6 is the optimum medium for incubation and mold, both in terms of the growth of the biomass as well as the formation of lipids. Different results shown on a medium with a pH of 5. Mold growth well with the amount of biomass that is growing, though not as big a pH of 6. But the amount of the biomass is not followed by the amount of lipid produced.

3.6. Saturated and unsaturated fatty acid analysis

| Table 2. Fatty Acid Composition on pH Variation. |
|-----------------------------------------------|
| Fatty Acid Composition | pH 5 | pH 6 | pH 7 |
| Saturated Fatty Acid (% w/w) | Lauric (C12:0) | n. d\(^a\) | n. d\(^a\) | 13.9 |

\(^{a}\) n: not detected
The highest concentration of saturated fatty acids found in the variations of the medium with a pH 7. This condition is an appropriate pH for enzyme-substrate complex formation. The corresponding medium pH will cause enzymatic activity can work well. On the formation of lipids by microorganisms especially mold, strongly influenced by the activity of the enzyme FAS (Fatty Acid Synthases). FAS Enzyme (Fatty Acid Synthases) can create a bond with NADPH well at pH 6-8.5 [6].

3.7. Effect of incubation temperature

![Figure 5. Effect of Temperature to Cell Dry Weight](image)

![Figure 6. Effect of Temperature to Lipid Weight](image)

The result of the growth of mould on temperature variation of 25 and 30 °C shows the amount of biomass that is higher than the temperature of 35 °C. This is in accordance with the theory that summed up by Fardiaz (1992) whereby the mould Aspergillus can grow well in the temperature range 25-30 °C [7]. This caused mold Aspergillus mold types with type mesofilik which is a species that fits on a medium temperature. The type of the mould Aspergillus will hampered its growth at temperatures below 18 °C [8].

3.8. Production of Lipid on Temperature Variation

In terms of the production of lipids, temperature variations show a slightly different profile with the acquisition of dry biomass. The lowest lipid production occurs at a temperature of 25 °C, while the highest at temperature 35 °C. The range of lipid production in incubation temperature variation ranged from 0.08 – 0.16 grams. The resulting lipid yield per dry weight biomass has a range between 13%-52%. At a temperature of 25 °C, the production of lipids are not too big, as well as biomass mold. This indicates that the temperature of 25 °C is the temperature which is more suitable to mold growth than temperature 35 °C. A similar case is found at a temperature of 30 °C, the temperature at which the production of biomass is the highest. However, the magnitude of the biomass is not comparable to the lipids produced. Can be drawn the conclusion that the temperature 30 °C is the best temperature for growing of biomass mold, but not for the production of lipids on the mold.

3.9. Saturated and unsaturated fatty acid analysis

| Unsaturated fatty acid (%) w/w |
|-----------------------------|
| Saturated fatty acid (%)     |

| Fatty Acid     | 1,2 n.d. | 6.92 |
|----------------|----------|------|
| Miristic (C 14:0) | 1.2      | n.d. |
| Palmitic (C 16:0) | 21.4     | 54.5 |
| Stearic (C 18:0) | 22.1     | n.d. |

| Unsaturated fatty acid (%) w/w |
|-----------------------------|
| Saturated fatty acid (%)     |

| Fatty Acid     | 1,2 n.d. | 6.92 |
|----------------|----------|------|
| Oleic          | 27.7     | 45.5 |
| Linoleic       | 21.8     | n.d. |
| Linolenic      | 0.39     | n.d. |
| AA             | n.d.     | n.d. |
| DHA            | n.d.     | n.d. |
| EPA            | n.d.     | n.d. |

The result of the growth of mould on temperature variation of 25 and 30 °C shows the amount of biomass that is higher than the temperature of 35 °C. This is in accordance with the theory that summed up by Fardiaz (1992) whereby the mould Aspergillus can grow well in the temperature range 25-30 °C [7]. This caused mold Aspergillus mold types with type mesofilik which is a species that fits on a medium temperature. The type of the mould Aspergillus will hampered its growth at temperatures below 18 °C [8].

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3.9. Saturated and unsaturated fatty acid analysis
Table 3. Fatty Acid Composition on Temperature Variation

| Fatty Acid Composition | Temperature (°C) |
|------------------------|------------------|
|                        | 25               | 30               | 35               |
| Saturated Fatty Acid (% w/w) |       |       |       |
| Lauric (C12:0)         | n.d\textsuperscript{a} | 1.64 | 13.90 |
| Miristic (C 14:0)      | n.d\textsuperscript{a} | 1.84 | 6.92  |
| Palmitic (C 16:0)      | 18.03            | 34.36           | 34.30            |
| Stearic (C 18:0)       | 19.81            | 16.48           | 6.93             |
| Unsaturated Fatty Acid (% w/w) |       |       |       |
| Oleic                  | 21.13            | 19.87           | 31.60            |
| Linoleic               | 41.03            | 23.57           | 6.31             |
| Linolenic              | n.d\textsuperscript{a} | 1.58 | n.d\textsuperscript{a} |
| AA                     | n.d\textsuperscript{a} | n.d\textsuperscript{a} | n.d\textsuperscript{a} |
| DHA                    | n.d\textsuperscript{a} | n.d\textsuperscript{a} | n.d\textsuperscript{a} |
| EPA                    | n.d\textsuperscript{a} | n.d\textsuperscript{a} | n.d\textsuperscript{a} |

On the table, fatty acids produced from lipid is broken down into saturated fatty acids and unsaturated fatty acids. The percentage of saturated fatty acids are produced varies between 37.84% - 62.05%. Meanwhile, the percentage of unsaturated fatty acids that are generated vary between 37.91-62.16%. The highest saturated fatty acids obtained on incubation temperature 35 °C. Whereas, the highest unsaturated fatty acids are obtained at a temperature of 25 °C incubation.

The concentration of saturated fatty acids increase at the temperature variation range 25 – 35 °C. A different matter is shown by the acquisition of unsaturated fatty acids that decrease in temperature variation range 25 – 35 °C. The highest concentration of saturated fatty acids found in 35 °C temperature variations i.e. of 62.05%. It shows that saturated fatty acids dominated at a temperature of 35 °C. Concentrations of unsaturated fatty acids found on the highest variation in temperature 25 °C, namely in the amount of 62.16%. Thus, the unsaturated fatty acids are produced optimally at a temperature of 25 °C.

4. Conclusion

Optimum Agitation Rate for production of unsaturated fatty acids from Aspergillus oryzae is 120 RPM with yield lipid by 28.28% and yield unsaturated fatty acids of 50.36%. Optimum agitation Rate for production of EPA is 120 RPM with the composition of EPA obtained is 2.42%. Optimum pH medium for the production of unsaturated fatty acids from Aspergillus oryzae is pH 6 with yield lipid 22.35% and yield unsaturated fatty acids 45.5%. Optimum incubation Temperature for the production of unsaturated fatty acids from Aspergillus oryzae is 25 °C with yield lipid 13.19% and yield of unsaturated fatty acids 62.15%

5. Reference

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