Effect of medium and incubation time on production of AA, DHA and EPA from *Aspergillus oryzae* by solid state fermentation

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Abstract. Lately, artificial surfactant is more often used than natural surfactant for petroleum industry. The main advantages of using natural surfactant is because the continuous availability. Single Cell Oil, which utilizes one-cell microorganism, can be a solution, such as Aspergillus oryzae, to produce AA, DHA & EPA. A. oryzae was cultivated on Potato Dextrose Agar (PDA), Czapek Dox Agar (CDA) and Malt Extract Agar (MEA), and then the incubation times are 2, 4, 5, 6 and 7 days in optimal medium. Lipids were extracted using ethanol and n-hexane. The characterization of lipid was done by gas chromatography (GC) method. The most optimal medium is CDA with a lipid concentration of 0.18 g. The best incubation time on CDA medium was 5 days with 0.216 g lipid content containing 0.123 g unsaturated fatty acid, 0.0613 g PUFA and 0.0615 g MUFA. The unsaturated fatty acid composition produced on the 5th day was 29.2% oleate; 29.1% linoleate and ± 0.046% EPA.

1. Introduction
Surfactants are widely used and have an enormous number of their ability to influence the properties of surface and interface. The ability of surfactant can be used in petroleum industry at all stages [1]. Lately, artificial surfactant is more often used than natural surfactant. The main advantages of using natural surfactant is because the continuous availability. Nowadays, there are few experiments using microorganism that considered as efficient producer of surface active agent [2].

Fungi have the ability to grow in low pH, easy to handle, able to degrade complex carbon source and can grow in waste rapidly [3]. A fungus that is used in this research is *Aspergillus oryzae*. *Aspergillus oryzae* has about 18-57% of lipid content [4].

Solid state fermentation (SSF) method that uses solid medium gives similar condition as fungi habitat, where fungi is the main group that is usually cultured by SSF method[5]. SSF method has lower bacterial contamination level because of the absent of free-water and low pH level in the medium [5]. Solid mediums that are used in this research are Potato Dextrose Agar (PDA), Czapek Dox Agar (CDA) and Malt Extract Agar (MEA) because those medium are the most common medium to grow fungi. But, within those three mediums, it has never been proved which mediums is the best to produce AA, DHA and EPA.

Single cell oil technology has been used long time ago. According to Nisha et al. (2011), fungi *Mortierella alpina* was used to produce AA until 40% (w/w) [6]. *Mucor circinelloides* was also used
for producing various type of fatty acid up to 50% (w/w) [7]. Fakas et al. (2009) used Cunninghamelata enchinulata and produced 40-60% of gamma-linolenic acid [8].

The other crucial factors in culturing Aspergillus oryzae is incubation time. Incubation time is time needed to control the growth of microorganism. Incubation time between fungi and other microorganism is different. The differences of incubation time between Aspergillus oryzae and other fungi raise a doubt, so this research will vary the incubation time to know the effect of incubation time on concentration profile of AA, DHA and EPA that produce by Aspergillus oryzae.

2. Method

2.1. Main Culture in Tilted Agar
Fungi Aspergillus oryzae that obtained from Indonesia Culture Collection (InaCC) grew on PDA and kept in ±4°C. Then, A. oryzae was regrown on PDA by tilted agar method in test tubes. A. oryzae was incubated for three days in ±25°C.

2.2. Modification of the Medium
Three mediums that were used in this research is PDA (4 g potatoes infusion; 20 g dextrose; 15 g agar), CDA (30 g sucrose; 2 g NaNO₃; 0.5 g MgSO₄; 0.5 g KCl; 0.01 g FeSO₄; 15 g agar) and MEA (30 g malt extract; 5 g mycological peptone; 15 g agar) manufactured by HiMedia Laboratories Pvt. Ltd. All mediums that were used during the experiment were modified. The ratio of C:N was modified into 30 : 1 [9]. Each medium was modified with certain carbon or nitrogen. The ratio of C:N of PDA was modified by adding (NH₄)₂SO₄, CDA was modified by adding sucrose and MEA was modified by adding glucose. All mediums was also supplemented with several compounds such as 7 g/L KH₂PO₄ and 2 g/L Na₂HPO₄ as buffer; 0.5 g/L CaCl₂·2H₂O; 0.1 g/L FeSO₄·7H₂O; 0.1 g/L ZnSO₄·7H₂O; 0.001 g/L MnSO₄·2H₂O; 0.005 g/L CuSO₄·5H₂O as micronutrients [10].

2.3. Growth Curve on PDA
Cultures for growth curve were made on 25 ml of modified PDA each dish. The C:N ratio of PDA was modified into 30 : 1 [9]. Each medium was modified with certain carbon or nitrogen. The ratio of C:N of PDA was modified by adding (NH₄)₂SO₄, CDA was modified by adding sucrose and MEA was modified by adding glucose. All mediums was also supplemented with several compounds such as 7 g/L KH₂PO₄ and 2 g/L Na₂HPO₄ as buffer; 0.5 g/L CaCl₂·2H₂O; 0.1 g/L FeSO₄·7H₂O; 0.1 g/L ZnSO₄·7H₂O; 0.001 g/L MnSO₄·2H₂O; 0.005 g/L CuSO₄·5H₂O as micronutrients [10].

2.4. Culture for Effect of Mediums
A. oryzae was cultured on three different mediums, which is PDA, CDA and MEA for five days according to the growth curve on PDA. Each petri dish was consists of 25 ml of each medium that had been modified. Cultures were incubated in ±25°C. These cultures were made in duplo. After five days of incubation, cultures were harvested and the lipid was extracted. The best medium was obtained based on the highest lipid productivity and would be used in the variation of incubation time.

2.5. Growth Curve on Highest-Lipid-Productivity-Medium
Based on the result of effect of medium, CDA was the medium with the highest lipid productivity. It needed a growth curve to determine the range of incubation time variation. Therefore ten cultures of 25 ml of modified CDA were made for a ten-days-growth-curve. Each petri dish was harvested every day for ten days. The cultures were incubated in ±25°C.

2.6. Culture for Effect of Incubation Time
Based on the growth curve on CDA, the range of incubation time that were going to use were 2nd (log phase), 4th (initial stationary phase), 5th & 6th (stationary phase) also 7th day (initial death phase). A. oryzae was cultured on 25 ml of CDA. These cultures were made in duplo. Two cultures were harvested and the lipid was extracted according to the range of incubation time.
2.7. **Harvesting**
Harvesting was done by autoclaving the culture in ±121°C for 15 minutes. After the medium melted down, the biomass could be taken and dried on filter paper for 24 hour in 60°C.

2.8. **Lipid Extraction**
Lipid extraction started by grinding dried biomass into powder. Then, the biomass powder was solved by ethanol 1:15 (w/v) and sonicated for three hour in 45°C according to Niawati et al. (2016). After it sonicated, the sampel was vortexed for five minutes then sentrifuged at 2000 rpm for 15 minutes [11,12]. Supernatant was dried over hot plate in <100°C until the odor of solvent was gone [12]. The fungus solid then solved by n-hexane with same ratio and sonicated for two hour in 60°C [13]. Next procedures were the same as the ethanol extraction.

2.9. **GC Analysis**
The composition of fatty acids, AA, DHA and EPA in lipids in incubation time variation were analyzed using Gas Chromatography (GC) SHIMADZU 2010 with the injector temperature was 250°C and the oven temperature was 400°C. The mobile phases for GC were UHP hydrogen gas, UHP helium and compressed air.

3. **Results & Discussion**
3.1. **Growth Curve on PDA and CDA**
The growth curves on PDA and CDA are shown on figure 1.

![Figure 1 Growth Curve on PDA and CDA](image1.png)

According to the growth curve on PDA, lag phase and acceleration phase was started on day 0 until day 1. Exponential phase happened on day 1 to day 2. On day 2 until day 4, fungi were in decelaration phase. On day 4 until day 6 were considered as stationary phase. Accelerated death phase happened on day 6 until day 10. The optimal harvesting time based on this curve was day 5.

On the experiment of effect of medium, the medium with the highest lipid productivity is modified CDA. To determine the range of incubation time, growth curve on CDA was made. The incubation times that were chosen were in exponential phase, initial stationary phase, two dots in stationary phases and decelaration phase. According to the growth curve on CDA, those phases were on day 2, day 4, day 5, day 6 and day 7.

3.2. **Effect of Medium on Dry Cell Weight and Dry Lipid Weight**
The comparison of DCW and dry lipid weight (DLW) of each medium are shown on figure 2. DCW that were obtained from all mediums were ranged between 0.15-0.43 g per 25 ml medium with error value <5%. The DCW of MEA was also similar to the DCW of CDA. Based on figure 2, CDA produced the highest DLW.
According to figure 2, the highest DCW obtained at CDA because Aspergillus sp. can grow on all types of carbon source, and growth as well as the maximum biological activity occurs at the time of sucrose as sole carbon source [14]. The DCW on MEA is also similar to the DCW on CDA because MEA has been modified to get the ratio of C:N 30:1 by adding glucose into it. Pepton is organic nitrogen source, which source of organic nitrogen provides vitamins, micronutrients and intermediate compounds needed by mould and can act as a stimulator and the essential precursor on the growth and ochratoxin production [15].

Medium CDA can produce highest DLW on figure 2 because the medium consists of simple sugar and pure synthetics. Mold will start forming lipids from the rest of the carbon on the medium after the nitrogen discharged. The remaining carbon in the medium that is available in CDA is simply sucrose. Meanwhile, in medium PDA and MEA, the amount of DLW produced by A. oryzae is much lower because the remaining carbon source in both medium is complex carbon compounds, causing fungus takes a longer time to break down the component that is used for the synthesis of cell and energy [16].

3.3. Effect of Incubation Time on Dry Cell Weight and Dry Lipid Weight

The comparison between DCW and DLW on several incubation times were shown below. According to figure 3, DCW on several incubation times ranged between 0.25-0.425 g/25 ml medium with error value <5%.

The highest DCW was obtained on day 6. The trend in DCW graph was inclining until day 6, and started to drop on day 7. The highest DLW was obtained on day 5. The trend in DLW graph was inclining until day 5. Dropping was found pretty drastic on day 6 and day 7.

Trends in figure 3 continue ascending until the day 6. The increase in DCW occurs because mold consuming carbon source and nitrogen source on the medium actively. On the 7th day, the DCW declined because of the lack of additional nutrients flow in the medium at that time period [17].

Trends in figure 3 from day 2 to 5 continue uphill because in the early phase of growth, there is sufficient nitrogen content in medium, therefore cells are more likely to reproduce it self rather than accumulate lipid. However, after certain cycles, microbes will start to confront the stress conditions that will trigger the accumulation of lipids [18]. The remaining carbon in the medium after a shortage of nitrogen will be assimilated by the cells and converted directly into lipids [19]. In the final phase of the growth cycle, the quantity of lipids will decrease because the mold cells will begin to use the stored lipids to meet energy needs for survival [18]. The results of this research are supported by statement of Dyal et al. (2005) that the amount of lipid is not always directly proportional to the DCW [20].
3.3.1. Analysis of Fatty Acid Composition

Analysis of lipid extracted from A. oryzae was done using GC. There are various fatty acid composition among several incubation times. The fatty acid compositions on each incubation time contain laurate (C12), myristate (C14), palmitate (C16-0), stearate (C18-0) as saturated fatty acid; 29,2-37,2% of oleic (C18-1) and 21,8-29,1% of linoleic (C18-2). Day 2 and day 4 also have 0,53% and 0,57% of linolenic (C18-3) respectively. AA, DHA and EPA appear to be not detected (n.d.).

Based on figure 4, unsaturated fatty acid that was produced ranged between 0,056-0,122 g. The highest unsaturated fatty acid weight was on day 5. The trend in unsaturated fatty acid graph is inclining until day 5 and starts to drop on day 6. The highest unsaturated fatty acid and PUFA, as our main target, were obtained on day 5. Unsaturated fatty acid concentrations, MUFA and PUFA in figure 4 continue ascending until day 5. Decreasing happens in the 6th day because of the lack of oxygen supply in medium. Fatty acid desaturation process is oxidative reaction that requires oxygen molecular [21].

4. CONCLUSION

Based on the results of the research, it can be concluded that the greatest concentration of lipids between PDA, CDA and MEA are 0.18 g obtained in medium CDA, therefore CDA is the best medium. The largest concentration of lipids between variations 2 days, 4 days, 5 days, 6 days and 7 days in medium CDA is 0.215 g on day 5. The best time variation is day 5 with unsaturated fatty acid content of 0.123 g, PUFA content of 0.0613 g and MUFA content of 0.0615 g. Unsaturated fatty acid composition are produced by Aspergillus oryzae on day 5: 29.2% oleic; 29.1% linoleic and ± 0.0467% EPA.

5. REFERENCES

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