Lipid production from *Zygosaccharomyces siamensis* AP1 using sequencing batch method with acetic acid as carbon source

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Abstract. Microbial lipid made by microorganisms is a promising feedstock to produce biodiesel. One important parameter in the production of microbial lipids is carbon sources. Low-cost carbon sources are one of the considerations for large-scale microbial lipid production. Acetic acid is a low-cost carbon source and can increase the rate of lipid accumulation due to its shorter metabolic pathway. This research aims to evaluate sequencing batch method to increase the lipid production of *Zygosaccharomyces siamensis* AP1 using carbon sources of acetic acid. The optimum acetic acid for lipid production was first determined by growing the strain in Nitrogen Limited Medium (NLM) with varying concentrations of acetic acid (5 to 40 g/L). Effect of pre-culturing was determined next by growing the yeast in Nitrogen Rich Medium (NRM) with varied acetic acid concentration (5-40 g/L) before lipid production in optimised NLM. Lastly, sequencing batch cultivation was carried out using the optimised pre-culture medium (NRM) and the production medium (NLM). The result shows that *Z. siamensis* AP1 obtained its highest lipid production when the yeast was grown with 40 g/L of acetic acid. Total lipid increased by 64% when yeast was pre-culture in NRM containing 40 g/L acetic acid. Sequencing batch method resulted in biomass and total lipid of 0.61 g/L and 0.48 g/L, respectively. Half of the lipid was produced extracellularly. This study suggests that acetic acid can be used as a single carbon source in lipid production from *Z. siamensis* AP1. It also shows that sequencing batch method with pre-culturing can increase the lipid production. Besides, the method triggers excretion of the produced lipid out of the yeast cells. Further study to investigate the role of acetic acid in *Z. siamensis* AP1 extracellular lipid production is suggested.

Keywords: biodiesel, extracellular lipid, oleaginous yeast, pre-culturing, waste utilisation

1. Introduction

Energy is one of the most important resources for humanity and development in the future. Currently, the energy crisis is one of the global problems [1]. Biodiesel has become a common alternative to fossil fuels with the onset of an energy crisis globally [2]. Microbial lipid formed by microorganisms is a promising raw material to manufacture biodiesel [3]. Lipid is produced by oleaginous microorganisms such as yeasts, fungi, bacteria and algae in substrate containing higher carbon than other nutrients (nitrogen, phosphorus and sulphur) [4]. Oleaginous yeasts are reported can accumulate lipid more than 15% of their dry weight [5].
One important parameter in the commercial production of microbial lipid is the cost of carbon sources. Although glucose is an efficient carbon source for cell growth, its price is high and comprises around 60% of total production costs. Therefore, a low-cost carbon source is one of the considerations for commercial large-scale microbial lipid production [6]. One low-cost carbon source for microbial lipid production is acetic acid. Acetic acid can increase the rate of accumulation of lipids because the acid’s metabolic pathway is shorter than Acetyl-CoA, an important precursor in lipid biosynthesis [7]. Besides carbon sources, lipid extraction is also an important parameter because it requires energy which increases production cost [8]. Hence, strategies to increase extracellular lipid production is needed.

Biodiesel production from microbial lipid requires high cost so that it becomes an obstacle. A strategy is needed to increase the production of microbial lipid and reduce production cost. This study aims to increase the production lipid of *Zygosaccharomyces siamensis* AP1 using sequencing batch method with acetic acid as a carbon source.

2. Materials and Methods

2.1.Cultivation of microorganism

*Zygosaccharomyces siamensis* AP1 was obtained from the Faculty of Biology, UGM. The yeast was kept on YPG agar slants. The YPG medium was adjusted to include 20 g/L glucose, 10 g/L peptone and 10 g/L yeast extract with initial pH 6. Five ml of seed stock was inoculated into 45 ml of medium in 250 ml flasks and incubated for 48 h at 30°C with agitation 200 rpm.

2.2.Optimization of lipid production in the acetic acid medium

AP1 was washed twice with sterile distilled water after cultured in the YPG medium for 48 h. The initial cell density in the fermentation medium was 1 Abs (OD₆₀₀). Five ml of the seed culture was inoculated into 45 ml of Nitrogen Limited Medium (NLM) in 250 ml of flasks containing different amounts of acetic acid (5, 15, 30 and 40 g / L) and glucose (40 g / L) as a carbon source. *Z. siamensis* AP1 was cultured in the fermentation medium for 48 h at 30°C with agitation 200 rpm, and the initial pH was 7. Lipid from isolates in each variation of the concentration of acetic acid was extracted, and the concentration of acetic acid produced the highest lipid was used in the pre-culture stage.

The NLM contained 1.5 g/L of MgSO₄·7H₂O, 0.4 g/L of KH₂PO₄, a mixture of yeast extract and (NH₄)SO₄ as a source of Nitrogen with a ratio of 7.5:1, and acetic acid with concentrations of 5, 15, 30 and 40 g/L. The C/N ratio of the medium was maintained at a value of 225 by varying the source of N according to the concentration of acetic acid (Table 1).

| Acetic acid (g/L) | N source (g/L) |
|------------------|---------------|
| 5                | 0.027         |
| 15               | 0.083         |
| 30               | 0.166         |
| 40               | 0.222         |

2.3 Preculture cultivation

Five ml suspension cell of *Z. siamensis* AP1 was cultivated into 45 ml Nitrogen Rich Medium (NRM) (C/N = 20) in 250 ml flasks containing different concentration of acetic acid (5, 15, 30 and 40 g/L) and
glucose (40 g/L), incubated for 30 hours. Then 5 ml of the inoculum from each of the different variations of the concentration of acetic acid were put into 45 ml of NLM medium (C/N = 225) in 250 ml flasks containing acetic acid with optimal concentrations in optimization stage. *Zygosaccharomyces siamensis* AP1 was grown on NLM medium for 72 hours at 30 °C and pH 7 and with agitation 200 rpm.

NRM medium content was similar with NLM medium; however, the C/N ratio of the medium was maintained at a value of 20 by varying the source of N according to the concentration of acetic acid (Table 2).

| Acetic acid (g/L) | N source (g/L) |
|------------------|----------------|
| 5                | 0.312          |
| 15               | 0.937          |
| 30               | 1.875          |
| 40               | 2.5            |

### Table 2. Concentration of acetic acid and N sources in NRM used in preculture cultivation

2.4 Yeast cultivation with sequencing batch

*Zygosaccharomyces siamensis* AP1 was cultured in 45 ml of NRM medium (C/N = 20) containing the optimal concentration of acetic acid from the pre-culture stage for 30 hours. Then 5 ml of the inoculum was inoculated into 45 ml of NLM medium (C/N = 225) in 250 ml flasks with the optimum acetic acid concentration from the optimization stage. During cultivation, OD$_{600}$ of the culture was measured. When the yeast reached the stationary growth phase, the supernatant and cells were separated aseptically using centrifugation at a speed of 3600 rpm for 5 minutes. The obtained cells were inoculated into fresh NLM medium (C/N = 225). This stage was carried out up to four cycles. The supernatant collected from each cycle was measured for lipid content. After the last cycle, intracellular lipid and biomass were measured.

2.5 Measurement of biomass

Thirty-five ml of culture were harvested and centrifuged at 4000 rpm for 10 minutes. Harvested biomass was washed twice with 10 ml distilled water and then dried at 60 °C until the weight was constant [9].

2.6 Lipid extraction and measurement of lipid content

Lipid content in the yeast cell was extracted and measured based on the method of Bligh & Dyer [10]. Thirty-five ml of yeast cell suspension centrifuged at 4000 rpm for 10 minutes. After that, the yeast was washed twice with 10 ml of distilled water. Ten ml of 4 M HCl was added into the cells and incubated for 2 hours with agitation. After that, 10 ml of chloroform and methanol were added, and the mixture was incubated again at room temperature for 2 hours. The mixture was then centrifuged again with a speed of 4000 rpm for 10 minutes at room temperature to separate the liquid phase (top layer) and organic phase (bottom layer). The organic phase containing lipid was recovered with Pasteur pipette, and the solvents were evaporated. Lipid content was measured with the following formula [9]:

\[
\text{Lipid content} = \frac{\text{Lipid weight (g/L)}}{\text{Cell dry weight (g/L)}} \times 100\%
\]

### 2.7 Measurement of extracellular lipid

Thirty-five ml of supernatant was added with 17.5 ml of chloroform and then agitated using vortex to extract the lipid. The mixture centrifuged for 10 minutes at 4000 rpm. The organic phase was then taken
with Pasteur pipette, and the solvent was evaporated until the lipid weight can be determined using the following formulas:

\[
\text{Extracellular lipid content} = \frac{\text{Extracellular lipid weight (g/L)}}{\text{Total lipid weight (g/L)}} \times 100\% \quad (2)
\]

\[
\text{Total lipid} = \text{Extracellular lipid (g/L)} + \text{cell lipid (g/L)} \quad (3)
\]

3. Result and Discussion

3.1 Optimization of lipid production

Based on lipid calculations on yeast cells (Fig. 1), the highest lipid biomass was found in the concentration of acetic acid 40 g/L with lipid biomass of 0.21 g/L. While the lowest lipid biomass was found in yeast which is grown on glucose medium with lipid biomass of 0.10 g/L.

Total biomass is composed of lipid and non-lipid biomass. At low to moderate concentrations of acetic acid (5-30 g/L), non-lipid biomass reached the amount of lipid biomass. Whereas in fermentation medium with a high concentration of acetic acid (40 g/L), lipid biomass exceeds non-lipid biomass. These results suggest that more carbon tends to enter the lipid synthesis pathway when Z. siamensis AP1 is grown at a high concentration of acetic acid medium. On the other hand, yeast cultured on glucose medium tend to form non-lipid biomass rather than lipid biomass. Biomass growth of AP1 shows a big difference when cultured on glucose and acetic acid media. Glucose is a carbon source carried by various types of microbes. The abundance of glucose in nature encourages various types of microbes to adapt to use it in metabolism [11].

![Figure 1: Lipid biomass, non-lipid biomass, and total biomass of Zygosaccharomyces siamensis AP1 grown on medium with acetic acid and glucose as carbon source.](image)

Based on the results of lipid production optimization using acetic acid, the yeast grown on acetic acid medium have higher lipid biomass than yeast that which was grown on glucose medium. Lipid biomass increased (0.15 g/L to 0.21 g/L) along with the increase of acetic acid concentration. According to these results, acetic acid is an effective carbon source for lipid accumulation of Z. siamensis AP1 compared to glucose.

Acetyl-CoA is an essential precursor to triacylglycerol biosynthesis. If acetic acid is used as the only carbon source in lipid aggregation, acetic acid combines with CoA and ATP to produce Acetyl-CoA in
cells, resulting in a shorter metabolic pathway than glucose [12]. Some microorganisms that use acetic acid as the only carbon source will use the glyoxylate pathway for the biosynthesis of cellular material [13]. One of the enzymes that play a role in the glyoxylate pathway is isocitrate lyase. Under limited nitrogen conditions, the activity of the enzyme isocitrate lyase will be inhibited [14]. Lack of isocitrate lyase will increase fatty acids [15]. According to this study, AP1 can synthesize lipid until 0.21 g/L with a yield of 0.0053 g/g. In other words, 0.53% from 1 g acetic acid is synthesized into a lipid. Acetic acid is an effective, low-cost substrate for the accumulation of microbial lipid and is more suitable for growth and accumulation of oleaginous yeast lipid than other by-products such as furfural, 5-hydroxymethyl furfural, vanillin and vanillic acids [16].

3.2 Preculture cultivation

Preculture technique was suggested to alter the physiological properties of the yeast and to allow it to disperse extracellular lipid while grown on a medium containing acetic acid [12]. In the initial preculture, for 30 hours Z. siamensis AP1 was cultured on NRM medium with varying concentrations of acetic acid as stimulants. Then, the yeast was transferred to NLM medium with an acetic acid concentration of 40 g/L for lipid production. Fig. 2 indicates that extracellular lipid content was 0.059 g/L when yeast was precultured with 40 g/L acetic acid. Extracellular lipid increases from 26% to 32% when the concentration of acetic acid was increased from 5 g/L to 40 g/L. It can be concluded that acetic acid with high concentration can encourage the release of lipid from the cell. The results are consistent with the statement from Huang et al. [12] that high concentrations of acetic acid can trigger the release of extracellular lipid.

Total lipid increased by 64% when compared to yeast isolates cultured on glucose media. In glucose media, total lipid was 0.07 g/L whereas in yeast cultured on 40 g/L acetic acid media was 0.198 g / L. This shows that preculture cultivation is an effective method for increasing extracellular lipid production from Z. siamensis AP1 on acetic acid medium.

![Figure 2. Total lipid and extracellular lipid of Zygosaccharomyces siamensis AP1 in preculture cultivation using acetic acid and glucose as carbon sources.](image)

3.3 Sequencing batch cultivation

The profiles growth curve of Z. siamensis AP1 with sequencing batch (Fig. 3) shows that from day 0 to day 2 the growth of yeast is in a lag phase which is characterized by the slow growth of yeast. In this phase, the yeast is still adapting to the new environment. On day 2 to day 3, the yeast is in an exponential
phase which is characterized by the rapid growth of yeast. The slow growth of yeast marks the stationary phase achieved on day 5.

Figure 3. Growth curve of *Zygosaccharomyces siamensis* AP1 after sequencing batch with acetic acid for four cycles.

Transfers of the yeast from the old medium to the new medium and lipid analyses were carried out when the yeast entered the stationary phase. These were because the highest lipid accumulation of yeast is achieved during the stationary phase. In the stationary phase, the growth of yeast slows down due to nitrogen limitations in the medium. This condition causes the yeast to assimilate carbon sources for lipid synthesis [17]. Also, nutrient for yeast growth, such as carbohydrates, are becoming limited at stationary phase. Further growth in the phase will cause the accumulated lipid to be used by yeast as an energy source, thereby causing lipid production to decrease.

From Table 3, it is known that extracellular lipid production increases during the first to fourth cycle. From all cycles in sequencing batch, 50% of extracellular lipid (0.24 g/L) were produced from the total lipid. According to Huang et al. [12], repeated shocks with high concentrations of acetic acid can increase cell membrane damage, thereby raising the release of extracellular lipid. All four cycles produce extracellular lipid from 0.04 g/L to 0.072 g/L.

Table 3. Profile of Intracellular lipid, extracellular lipid and biomass in sequencing batch

| Cycle | Extramural Lipid (g/L) | Intracellular lipid (g/L) | Biomass (g/L) |
|-------|------------------------|---------------------------|---------------|
| 1     | 0.04                   |                           |               |
| 2     | 0.06                   | 0.24012                   | 0.61          |
| 3     | 0.068                  |                           |               |
| 4     | 0.072                  |                           |               |
| Total | 0.24                   | 0.24012                   | 0.61          |

For four cycles, cell density (OD<sub>600</sub>) increases continuously and then enters a stable phase at the end of the cycle. Four cycles in sequencing batch were carried out to achieve the highest biomass and lipid accumulation (Table 3). Intracellular lipid obtained from sequencing batch was 0.24 g/L so that the total lipid obtained was 0.48 g/L. On the other hand, the total biomass obtained from sequencing batch is 0.61.
g/L. Total biomass and total lipid content in sequencing batch are higher compared to the optimization stage. The increase shows that sequencing batch method is effective to increase the production of Z. siamensis AP1 yeast lipid.

Sequencing batch cultivation is a culture strategy used to increase biomass and lipid accumulation [17]. Repeated fed-batch processes are known to increase microbial culture productivity because the process will extend the production phase by replacing nutrient-poor medium in mature cultures with new medium [18]. According to the research of Huang et al. [19] note that sequencing batch cultivation can increase biomass and lipid production of Rhodosporidium toruloides when compared with batch culture. Liu et al. [20] reported that Cryptococcus curvatus cultured on volatile fatty acids (VFAs) by sequencing batch cultivation increased biomass and lipid production to 4.53 g/L and 0.614 g/L. Huang et al. [12] reported that the yeast Cryptococcus curvatus MUCL 29819 cultured on high concentrations of acetic acid media by sequencing batch cultivation, extracellular lipid production reached 50.5% in the last cycle with a total extracellular lipid concentration of 5.43 g/L.

In this study, the production of biomass and lipid of Z. siamensis AP1 increased 45.9% and 56.25% from the optimization stage. Besides, during sequencing batch cultivation extracellular lipid production can continue to be produced for four cycles. Sequencing batch cultivation can be an effective culture strategy to increase the lipid production of Z. siamensis AP1 using the acetic acid medium.

4. Conclusion
This study presents the results of research on increasing the lipid production of Z. siamensis AP1 with sequencing batch cultivation. 40 g/L acetic acid is the optimum concentration to increase the lipid production of Z. siamensis AP1 and sequencing batch cultivation effectively increase the lipid production of Z. siamensis AP1 to 56.25%.

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