Microbial lipid production by the yeast Lipomyces starkeyi InaCC Y604 grown on various carbon sources

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Abstract. The use of non-renewable fuels in the transportation sector boosts the development of renewable biofuels, such as biodiesel. However, crops-based biodiesel production can cause the increment of deforestation and greenhouse gas emissions. This circumstance motivates the development of oleaginous microorganisms as alternative raw materials to overcome these problems. Lipomyces starkeyi is one of the potential oleaginous microorganisms due to its ability to produce higher lipid content. In addition, L. starkeyi is capable to grow in affordable carbon sources, such as lignocellulose and molasses, which can reduce the production costs. The aim of this study is to determine the ability of L. starkeyi InaCC Y604 in producing lipids from various carbon sources and its effects on the composition of fatty acid methyl esters (FAME) and lipid contents. L. starkeyi InaCC Y604 were cultivated in nitrogen-limited mineral medium (-NMM) with various carbon sources, namely glucose, xylose, fructose, galactose, mannose, cellobiose and a mixture of glucose and xylose. The results showed that mixed glucose and xylose gave the best result with the lipid content achieved at 64.19% (w/w). The results obtained indicated that L. starkeyi InaCC Y604 could be a potential candidate for further optimizing biodiesel production.

1. Introduction

The increasing demand for fuels, which is not followed by the increasing of fossil fuel reserves, makes exploring alternative energy sources more significant nowadays. In addition, the fossil fuels also have damaging impact to our environment. Those factors make more benign energy source highly demanded. One potential renewable energy is biodiesel, a non-crude oil fuel originated from vegetable or animal through transesterification [1]. Currently, biodiesel obtained from microbial lipid, especially one derived from oleaginous microorganisms is potential to be used as biodiesel feedstock [2]. Biodiesel originated from other sources like crops-based biodiesel can cause indirect land use change (ILUC), ultimately affect the increasing deforestation and greenhouse effect. On the other hand, using crops-based biodiesel will make the food cost increased [3].
Lipomyces starkeyi is one of potential oleaginous microorganism. This strain accumulate lipid more than 60% (wt/wt) and its main fatty acid content is oleic acid (C18:1) [4]. The high content of oleic acid is preferred to be used in biodiesel production [5]. In addition to its capability of accumulating high lipid content, this strain is able to assimilate various carbon sources such as glucose, xylose (as sole carbon sources or as a mixture) [6], glucose and mannose [7], xylose and cellobiose [8]. This strain can also be cultured in a medium containing inhibitor originated from hydrolysate pretreatment [9,10]. The ability to grow in low-cost carbon sources such as lignocellulosic hydrolysate is highly desirable. High production cost is the main hindrance for large-scale production of biodiesel originated from oleaginous microorganism. However, in the case of biodiesel from yeast, the efficiency of production cost can be achieved by using the more economical carbon sources as well as efficiency in downstream process. The production cost can be suppressed by employing such strategies, to make microorganism lipid competitive to biodiesel of other commodity [11].

In our previous study, Lipomyces starkeyi InaCC Y604 was found to be able to assimilate xylose and the mixture of glucose and xylose as carbon sources [12]. To further investigating the ability of L.starkeyi InaCC Y604 to grow in low-cost carbon source, a preliminary study using monosaccharides that constitute lignocellulosic hydrolysate is required. For that reason, we employed various carbon sources including glucose, xylose, mannose, galactose, fructose, cellobiose, and the mixture of glucose and xylose. Parameters including dry cell weight, carbon source consumption and lipid content of L.starkeyi InaCC Y604 were determined in this study.

2. Materials and Method

2.1. Yeast strain and inoculum preparation

The yeast Lipomyces starkeyi InaCC Y604 isolate is provided by Indonesian Culture Collection (InaCC), Indonesia. Recovery of this isolate from 20% (w/w) glycerol stock was performed using potato dextrose agar (PDA) plate while culture maintenance was conducted yeast extract-malt extract-peptone-glucose (YMPG) agar plate. Inoculum was prepared by inoculating single colony from an YMPG agar plate to pre-culture medium which contained 12 mL of YMPG broth in a 100 mL Erlenmeyer flask. This pre-culture was incubated for 24h at 30 °C in BioShaker BR-43FH MR (Taitec Corp., Tokyo, Japan) at 190 rpm. For microbial lipid production, nitrogen-limited mineral medium (-NMM) broth was used. The pH medium was adjusted at 5.5 with an appropriate amount of 1 M H₂SO₄ and sterilized by filtration. The composition of the media used in this study were described elsewhere [12]. All chemicals were of analytical grade.

2.2. Sugar analysis

The sugars concentrations during fermentation were analyzed using high-performance liquid chromatography (HPLC) (LC-20AB, refractive index detector RID-10A, Shimadzu, Kyoto, Japan) equipped with an Aminex® HPX-87H column (300 × 7.8 mm Bio-Rad). The instrument was operated at a column temperature of 60 °C, with 5 mM H₂SO₄ as the mobile phase at a rate of 0.6 mL/min. All samples were centrifuged (12,000 rpm, 4 min) to remove the cell mass and other water-insoluble substances and then filtered through a 0.22 mm filter before analysis.

2.3. Total lipid quantification

Total lipid accumulation was measured by gravimetric analysis following Folch method [13,14]. Briefly, triplicate 15-20 mg samples of freeze-dried cells were transferred to a 2 mL polypropylene microvial with an O-ring sealed cap containing 1.0 mm glass beads and 1.5 mL of Folch solvent (2:1 of CHCl₃: MeOH, v/v). The cells pulverization was conducted two times using a Mixer Mill MM 400 (Retsch, Germany) at 30 Hz for 10 min. Afterward, cells were centrifuged at 12,000 rpm for 5 min after pulverization and supernatant was removed. The cell pellets were washed with 1.5 mL of deionized water for second pulverization. Then cells were separated again by centrifugation, and the remaining
water was then removed. The cell pellets were dried at 60°C to a constant weight. The lipid content was determined by the weight difference and expressed as a percent of dry cell weight.

2.4. Total fatty acid methyl esters quantification

The transesterification was conducted by following the protocol from fatty acid methylation kit (Nacalai tesque, Inc., Japan). Then the fatty acid methyl esters were analyzed with a gas chromatography-mass spectroscopy (GC-MS QP 2010 Ultra, Shimadzu), equipped with a DB-23 capillary column 0.25 mm × 30 m (Agilent J&W Scientific, Santa Clara, CA, USA) and helium as the carrier gas. Operating conditions were as follows: the flow rate for helium gas at 0.8 mL/min and with a split ratio 1:5, the injection temperature was 250°C. The primary GC oven temperature was programmed at 50°C for 1 min, then increased at 25°C/min up to 190°C and finally increased at 5°C/min up to 235°C for 4 min. The volume of injection was one microliter. Fatty acids were identified and quantified by comparing of their retention times and peak areas with a standard mixture of fatty acids using Supelco 37 component FAME mixture (Sigma-Aldrich). The internal standard of C13:0 (Tridecanoic acid) was included in each sample. FAME (%) was calculated as the percentage of each fatty acid to the total number of fatty acids produced upon fermentation.

3. Results and Discussion

3.1. Fermentation profile

Microbial lipid production from *L. starkeyi* InaCC Y604 was conducted in two-stage. The first stage, the preparation of seed culture, and the second is the lipid production step. The seed was prepared in YMG broth medium until it reached OD₆₀₀nm 2.0–3.0 to initiate the lipid production step. Afterward, the seed inoculated to –NMM medium had been supplemented by 50 g/L monomer carbon sources or 100 g/L mixed carbon sources.

The fermentation profile, including the sugars consumption and cell growth from different carbon sources of *L. starkeyi* InaCC Y604 are shown in figure 1. The time courses varied from 2-4 days depend on the carbon source. Lipomyces starkeyi InaCC Y604 only required two days to fully consumed xylose, cellobiose, galactose and fructose, whereas glucose and mannose is completely depleted in the following day. Longer fermentation occurred with a medium containing glucose and xylose mixtures, which was exhausted in 4 days.

Based on figure 1, the utilization of xylose was faster than glucose. This might be because xylose can be metabolized through pentose phosphate pathway and phosphoketolase reaction [10], while glucose is assimilated through glycolysis pathway. On the other hand, mannose and glucose shared a similar profile consumption which was exhausted on three days. This is in accordance with the study of Yang, *et al*. using *L. starkeyi* As. 2.1560 where glucose and mannose have similar consumption rates [7].

The cellobiose consumption rate by *L. starkeyi* InaCC Y604 was higher compared to xylose and glucose. After 24 hours, cellobiose was consumed nearly half of its initial concentration. This result is consistent with Gong, *et al*. which showed that consumption rate of cellobiose is faster than glucose and xylose [15]. This might be due to cellobiose could be converted into glucose in the presence of β-glucosidase enzymes or through phosphorylation reaction [16,17].

On the other hand, galactose and fructose were also exhausted in two days. However, the consumption rate of these two sugars were different, whereas fructose has a higher consumption rate than galactose. In the first 24 hours, around 35 g/L fructose was assimilated by *L. starkeyi* InaCC Y604, while for galactose it was only around 17 g/L. The differences in term of consumption rate of fructose and galactose might be due to the form of intermediate compounds entering the glycolysis pathway.
Galactose enters the glycolysis pathway via glucose-6-phosphate [18], while fructose by fructose-6-phosphate, one step ahead in glycolysis pathway [19].

Figure 1. Time course of carbon source consumption and cell growth of *L. starkeyi* InaCC Y604. For single carbon source: the closed circle refers to carbon consumption in -NMM G, -NMM X, -NMM Gal, -NMM C, -NMM F, and -NMM M. For a mixture of carbon sources: the closed square refers to glucose consumption and the closed circle represents xylose consumption. The closed triangle charts represent the cell growth (dry cell weight (g/L)) in all type of mediums.

Figure 1 (-NMM GX) shows that *L. starkeyi* InaCC Y604 have the ability to assimilate a mixture of glucose-xylose almost simultaneously. At the first and second 24 hours, the xylose consumption rate seemed weak than glucose consumption. However, the xylose consumption was significantly increased while glucose in the medium dropped to a concentration that ranged 3-5 g/L. Indeed, at the first stage cultivation of a mixture of glucose and xylose, glucose consumption rates were higher than xylose, however at the late stage fermentation both sugars are assimilated at the same rate. Microorganisms are known tend to metabolize preferred sugars, usually glucose at the first stage when exposed to a mixture
of glucose and xylose due to glucose can repress the utilization of other sugar via either a catabolic repression mechanism or allosteric competition for sugar transporter [20,21].

3.2 Lipid and cell mass production

Table 1 shows the biomass and lipid production of L. starkeyi InaCC Y604 grown on various carbon sources. Generally, L. starkeyi InaCC Y604 accumulated lipid more than 50% (w/w) of dry cell weight. The highest lipid accumulation was achieved by cultivating L. starkeyi InaCC Y604 using cellobiose as carbon source at 65.05% (w/w), followed a mixture of glucose+xylose, xylose, glucose, and galactose at 64.19% (w/w), 63.24% (w/w), 62.26% (w/w), and 60.89% (w/w), respectively.

Table 1. Biomass and lipid production of L. starkeyi InaCC Y604 grown on various carbon sources

| Medium  | Final DCW (x, g/L)\(^b\) | Lipid Content (L, %, w/w)\(^c\) |
|---------|--------------------------|-------------------------------|
| -NMM G  | 13.49±0.10               | 62.26                         |
| -NMM X  | 19.08±0.69               | 63.24                         |
| -NMM F  | 18.05±0.27               | 54.24                         |
| -NMM Gal| 17.96±0.65               | 60.89                         |
| -NMM C  | 22.77±0.08               | 65.05                         |
| -NMM M  | 15.34±0.06               | 56.86                         |
| -NMM GX | 34.49±0.38               | 64.19                         |

\(^a\) The values are given as mean ± SD of duplicate determination.
\(^b\) Final cellular biomass at the end of cultivation time (x).
\(^c\) Lipid content = (mass before extraction (g) – mass after extraction(g))/ mass pellet (g) x 100%

A previous study by Huang, et al. also showed that lipid accumulation from cellobiose as carbon source was greater than mannose and galactose [22]. Furthermore, lipid content obtained by L. starkeyi InaCC Y604 higher compares to Cryptococcus curvatus which only accumulated lipid at 49% (w/w) using 70 g/L cellobiose [23].

As mentioned previously, L. starkeyi InaCC Y604 preferred to consume glucose over xylose at the initial stage of fermentation in the medium with a mixture of glucose and xylose (–NMM GX) as carbon sources. Nevertheless, xylose might be more efficient than glucose to produce lipid due to xylose can be metabolized through the pentose phosphate pathway and the phosphoketolase reaction [13,23]. The study of Juanssilfero, et al. showed that the lipid production from L. starkeyi NBRC10381 on glucose medium was lower than xylose medium [24]. However, if the two carbon sources are mixed, a higher lipid result will be obtained, even though the difference was not too significant.

However, when fructose (–NMM F) was used as sole carbon source, L. starkeyi InaCC Y604 produced the lowest lipid accumulation at 54.24% (w/w). The results obtained are in accordance with the research of Matsakas, et al. who showed that the lipid content of L. starkeyi CBS 1807 grown on media containing fructose was lower than glucose. This might be due to the distribution of carbon flux was dominantly for the cell growth [25]. In addition, Trichosporon fermentans grown on fructose also
showed the lowest accumulation of lipids compared to other carbon sources, such as glucose, xylose, sucrose, and lactose [26].

Other than that, mannose also produced lipid less than 60% (w/w). Although this sugar shared similar consumption rates with glucose, lipid accumulation in mannose was much lower than glucose in this study. This might be related to the decrement of dry cell weight on 3rd day (Figure 1). This circumstance indicates lipid turnover, a condition when the accumulated lipids are re-consumed by yeast as an energy source due to lack of main energy source [27].

The final dry cell weight (DCW) of L. starkeyi InaCC Y604 culturing in various carbon sources are shown in Table 1. The cell biomass (DCW) production using a mixture of glucose and xylose (-NMM GX) as carbon source was highest (34.49 g/L) compared to single carbon source, since the total carbons source concentration int the -NMM GX medium was twice that of either single carbon source. In comparison, other monomer carbon sources, for example cellobiose gave the highest DCW which was 22.77 g/L. Higher DCW is another crucial factor in addition of lipid content, because the productivity of lipids is comparable to cell biomass [28].

3.3. Fatty acid profile of intracellular lipids

The FAME profile of L. starkeyi InaCC Y604 grown on various carbon sources are shown in Table 2. Generally, there are no significant differences in fatty acid methyl ester (FAME) profile produced by L. starkeyi InaCC Y604 in various carbon sources. The major FAME produced either by all type of sugar monomer or a mixture sugars in this study were oleic acid (C18:1), palmitic acid (C16:0), stearic acid (C18:0), palmitoleic acid (C16:1) and linoleic acid (C18:2). This FAME profile is similar to the properties of vegetable oils. For all conditions, oleic acid was regarded for about 50% of the total fatty acids for L. starkeyi InaCC Y604 strain. A large amount of oleic acid may provide additional value for the microbial lipid produce from the L. starkeyi InaCC Y604.

| Carbon Source | FAME Compositions (%) |
|---------------|-----------------------|
|               | C16:0 | C16:1 | C18:0 | C18:1 | C18:2 | Trace fatty acids<sup>a</sup> |
| Glucose       | 27.7±0.9 | 3.8±0.4 | 8.8±1.0 | 48.8±1.2 | 2.2±0.0 | 3.1 |
| Xylose        | 32.4±1.0 | 3.1±0.6 | 8.7±0.8 | 51.4±0.9 | 1.8±0.3 | 2.5 |
| Fructose      | 31.8±0.7 | 4.0±0.2 | 8.2±0.2 | 50.7±0.3 | 1.7±0.2 | 3.3 |
| Galactose     | 28.9±0.2 | 3.4±0.3 | 7.7±0.1 | 50.9±1.1 | 1.6±0.2 | 4.5 |
| Cellobiose    | 33.5±0.8 | 2.8±0.5 | 9.4±0.5 | 48.2±0.4 | 2.9±0.6 | 2.5 |
| Mannose       | 28.4±0.9 | 3.3±1.0 | 6.9±0.5 | 49.5±0.8 | 2.3±0.7 | 2.9 |
| Glucose+Xylose| 32.5±1.0 | 3.1±1.0 | 7.9±0.8 | 53.2±1.5 | 1.6±0.4 | 2.8 |

<sup>a</sup>The values are given as mean ± SD of duplicate determination.

<sup>b</sup>The percentage of individual FAME below 1% were categorized as trace elements, which consists of C14:0, C15:0, C15:1, C17:1, C18:3, C20:0, C22:0 and C24:0.

Recently, an investigated study on several oleaginous yeast have been conducted by other researchers for practical biodiesel production and initiate that L. starkeyi strain could produce an enormous amount of oleic acid (over 75%) by using glucose as a sole carbon source [21,29]. Furthermore, high oleic oils has been considerable for biofuels due to their properties that can improve oxidative stability and cold-flow properties of biodiesel, and as an input for many renewable polymers, lubricants, elastomers, and...
other oleochemicals [30-32]. This study showed an initial understanding various sugars assimilation in *L. starkeyi* InaCC Y604, which is crucial for the further development of microbial lipid production by utilizing agro industrial waste such as lignocellulosic hydrolysates to reduce production cost.

4. Conclusion

This study has provided information of *Lipomyces starkeyi* InaCC Y604 grown on medium using various carbon sources. *L. starkeyi* InaCC Y604 showed the ability to grow and produce lipids in a wide variety of carbon sources, namely glucose, xylose, fructose, mannose, cellobiose, and mixture of glucose-xylose. The results indicate that this strain can assimilate the mixture of glucose and xylose at high rate and accumulate a high level of lipid content and cell biomass. However further research related to the ability of the yeast strain to detoxify the inhibitor compounds contained in the lignocellulosic biomass should be conducted.

Acknowledgement

This work was supported by a grant from the Central Government Ministry/Agency Budget Implementation-National Priority in Biorefinery (DIPA-PN Biorefinery 2019) under grant 3403.001 and Research Center for Biotechnology- Indonesian Institute of Sciences (LIPI).

Conflict of Interest

The authors declare that they have no conflict of interest.

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