Single Cell Oil Production by Wild Type Strain *Lipomyces starkeyi* Y604

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Abstract. As one of the promising oleaginous yeast, *Lipomyces starkeyi* has emerged as a great candidate of a single cell oil (SCO) producer. This is due to its ability to accumulate high lipid content and its ability to consume a diverse carbon sources. However, lipid production of the wild type strain is limited and efforts to obtain high producing wild-type strain are demanded. For this purpose, we conducted fermentation of the local wild-type strain of *L. starkeyi* InaCC Y604, derived from the collections of Indonesian Culture Collection (InaCC). In this study, we employed glucose, xylose, and a combination of glucose and xylose as carbon sources and conducted the fermentation in the batch flask scale. Following the fermentation, parameters such as dry cell weight (DCW), carbon source consumption, SCO production, lipid content and lipid yield were determined. In this study, we obtained that *L. starkeyi* InaCC Y604 grown in medium contained a mixture of glucose (50 g/L) and xylose (50 g/L) accumulate highest SCO production with 57.04±0.94 g/L and its lipid content reached out 0.17±0.00 w/w. Despite the result is not the best among previous reported literatures, this shows that the Indonesian local strain *L. starkeyi* InaCC Y604 can be a promising candidate to be further optimized.

1. Introduction

The need of energy source tends to increase from year to year. In 2018, the growth of global energy consumption increased 2.9%, the fastest growth since 2010, with the growth of global oil consumption increased 1.5% or equal to 1.4 million barrel per day. Despite the increasing need of oil, fossil fuel as one of energy source is getting limited. Another problem related to the use of fossil fuel is environmental problem. In 2018, carbon emission was reported to increase 2%, which was the fastest growth for the past seven years [1]. The above problems, scarcity and environment, urge the search for alternative and sustainable energy source.

The search for alternative energy has begun since long time ago. Among the available alternatives are oil from plants (such as rapeseed, sunflower, palm, and soy), oil from waste cooking oil, and oil from microorganism, which has attracted many researchers lately. Oil derived from microorganism (also known as single cell oil, SCO) is a potential candidate to be an alternative energy source. SCO is commonly defined as neutral storage lipids accumulated by oleaginous eukaryotic microorganisms (e.g. yeast, mold, microalgae) and it is often synonymous with lipid, oil, and triacylglycerol (TAG) [2]. The use of SCO will be free from the conflict between food supply and energy, no season problem, and it takes less time compared to the one derived from plants. Furthermore, the fatty acid composition of SCO is similar to plants oil [3].

Several microorganisms can produce oil or lipid beneficial as feedstock of energy source. Microorganisms considered as a potent producers are known as oleaginous microorganisms, those which can produce at least 20% (w/w) lipid of its biomass [2]. Oleaginous yeasts are viable SCO...
producers that can accumulate lipid up to 70% (dry mass basis) of their cellular biomass. They are unique compared to other oleaginous microorganisms in their ability to produce SCO heterotrophically from a variety of low-value input such as agricultural residues, food waste streams and industrial co-products [4]. Yeasts, as suitable candidates for biotechnological experiments, have many advantages over other microbial sources. Compared to filamentous fungi and microalgae, yeasts have a shorter duplication time and higher growth rates. In lipid production, yeasts have higher lipid content than microalgae and their cultivation is easier to scale up [5]. In the yeast fermentation process, bacterial contamination can be controlled by conditions of low pH growth. Yeasts have the ability to utilize various types of carbon sources for the production of biomass and lipids [6].

Species of oleaginous yeast include but are not limited to Lipomyces starkeyi, Yarrowiya lypoityca, Rhodoturula glutinis, Rhodosporidium toruloides, Crytococcus curvatus, and Trichosporon fermentans [4, 7, 8]. Lipomyces starkeyi is a well-known strain and promising candidate to produce SCO. This strain has the capability to accumulate over 70% of its cell biomass as lipid under defined culture conditions, and can produce lipid on various carbon sources as well as other wastes [9, 10]. L. starkeyi was also reported to employ various carbon sources, including lignocellulosic hydrolysate. This species can also survive in the presence of inhibitors, which are the by-products of the hydrolysate pretreatment [11, 12]. The lipid produced is mainly in the form of triacylglycerol (TAG). The remaining is in the form of phospholipid, sterol ester and ergosterol [13].

SCO from oleaginous yeast may serve as a renewable source of edible oil and as an intermediate “building block” for oleochemicals such as fuels, soaps, plastics, paints, detergents, textiles, rubber, surfactants, and lubricants, additives for the food and cosmetic industry and many other chemicals [14]. The production of SCO from oleaginous yeast for renewable oleochemicals is still in its early stage. Production of SCO has been successfully commercialized only for specialty oils containing polyunsaturated fatty acids (PUFAs) used in the food and supplement industries, including docosahexanoic acid (DHA), arachidonic acid (ARA), and eicosapentaenoic acid (EPA) [2]. SCO as a commodity-type oil has been hindered by competition from oilseed crops, which cost 10 to 20 times less [15]. Although the use of SCO as a feedstock for biodiesel has received interest in recent years, high manufacturing costs prevent the stand-alone production of biodiesel from SCO [15]. As more emphasis is placed on advancement of integrated biorefineries, the possibility of incorporating a yeast-based SCO biochemical platform for renewable fuels, chemicals, power, and products may become a reality [16].

In this research, we reported lipid production profile of L. starkeyi InaCC Y604. Despite the prominence and its potency to be applied, the study of wild type L. starkeyi with high lipid producing ability is still in need. For that reason, this research was performed to study the lipid production profile of Indonesian local L. starkeyi strain, which has not yet been reported. This will provide us with information of its potency to be further applied. In this study, glucose, xylose, and their combination are employed as carbon source with high C/N ratio. Several parameters including dry cell weight (DCW), carbon consumption, and SCO production of L. starkeyi InaCC Y604 were also reported in this study.

2. Materials and Method

2.1. Yeast strain

The yeast strain L. starkeyi InaCC Y604 used in this study was selected from the Indonesian Culture Collection (InaCC) Indonesia. The yeast strain was preserved in 20% (w/w) glycerol at -80 °C and was revived by streaking onto a potato dextrose agar (PDA) plate. Afterwards, the yeast strain was grown
on a yeast extract-malt extract-peptone-glucose (YMPG) agar plate (yeast extract 3 g/L, malt extract 3 g/L, peptone 5 g/L, glucose 10 g/L and agar 16 g/L).

2.2. Seed preparation
One colony from a YMPG agar plate was inoculated into pre-culture medium which contained 12 mL of YMPG broth (yeast extract 3 g/L, malt extract 3 g/L, peptone 5 g/L, glucose 10 g/L) in a 100 mL Erlenmeyer flask and incubated in an orbital shaker incubator (BioShaker BR-43FH MR, Taitec Corp., Tokyo, Japan) at 190 rpm for 24 h at 30 °C. Then, these seeds culture were transferred to 12 mL nitrogen limited mineral medium (-NMM) for lipid production. The medium for lipid production (-NMM) contained carbon sources (glucose and/or xylose) at 50 g/L form a single carbon source, and 100 g/L in a mix (glucose 50 g/L + xylose 50 g/L), nitrogen sources (yeast extract 1.5 g/L and (NH₄)₂SO₄ 0.25 g/L), MgSO₄ · 7H₂O 1.5 g/L, phosphate buffer (KH₂PO₄ 7 g/L and Na₂HPO₄ · 2H₂O 5 g/L), and a trace element solution (FeSO₄ · 7H₂O 0.08 g/L, ZnSO₄ · 7H₂O 0.01 g/L CaCl₂ · 2H₂O 0.1 g/L, MnSO₄ · 4H₂O 0.1 g/L, CuSO₄ · 5H₂O 0.002 g/L, CoCl₂ · 6H₂O 0.002 g/L). The -NMM medium were prepared into three categorize; -NMM G which contained glucose as sole carbon source, -NMM X contained xylose as sole carbon source and -NMM GX contained a mix of glucose and xylose as carbon source.

2.3. Cultivation condition
The seed volume of the pre-culture in YMPG broth was adjusted to an initial optical density (OD₆₀₀nm) of 2-4 for obtaining an appropriate active cell fraction. Afterward, the seed culture from the YMPG broth was transferred to -NMM with adjusted pH (at 5.5) media for cultivation with 12 mL working volume. These cultures were incubated in an orbital shaker incubator that was operated at 190 rpm and 30 °C until 5-d. Growth was analyzed gravimetrically by measuring the dry cell weight (DCW) of biomass. Following inoculation, samples for the measuring of DCW were taken on the initial day of cultivation and every 24 h thereafter until 5-d. The determinations of DCW and lipid quantity were conducted according to the method as previously described [11-17].

2.4. Sugar analysis
The concentrations of sugars 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) operated at a column temperature of 60 °C. The mobile phase was 5 mM H₂SO₄ at a rate of 0.6 mL/min. All samples were centrifuged to remove the cell mass and other water-insoluble substances and then filtered through a 0.22 mm filter before analysis.

2.5. Determination of total lipid quantity by gravimetric analysis
To measure total lipid content, a gravimetric analysis was used as previously described [17,18]. Briefly, triplicate 15 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). Cells were pulverized using a Mixer Mill MM 400 (Retsch, Germany) at 30 Hz for 10 min. Afterward, cells were centrifuged at 14,000 × g for 5 min, the supernatant was removed, and the pellets were washed with 1.5 mL of deionized water, then pulverized a second time, centrifuged, 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.
3. Results and Discussion

3.1. Fermentation profile

Single cell oil production by oleaginous yeast could be classified into two phase: the growth phase and the lipid production phase. After the seed was prepared in YMPG broth medium, the seed volume was adjusted to the initial $OD_{600nm}$ 2-4 to initiate the growth phase. Afterwards, the YMPG broth was transferred to the -NMM medium for the lipid production phase. The cultivation flowchart is presented in Figure 1.

![Lipomyces starkeyi InaCC Y604 cultivation flowchart.](image)

Figure 2 shows the time courses for the carbon source consumption and the cell growth of the InaCC Y604 strain during the fermentation in limited nitrogen mineral medium with different carbon sources (-NMM G, -NMM X and -NMM GX). For fermentation with -NMM G medium, it was clear that the strain only required 3 days to completely assimilate glucose as a sole carbon source. When xylose was used as a sole carbon source, the strain require 4 days to completely assimilate xylose in the medium -NMM X. Xylose consumption seemed weak in fermentation process with medium containing a mixture of glucose and xylose (-NMM GX), where some residual amounts of xylose remained that were not consumed ($\pm$ 20 g/L) during fermentation. This condition led to an half-completed consumption of xylose in the late stages of the fermentation (after 4 days). Many works have reported the inhibitory effect of high carbon source concentrations on the growth of oleaginous yeast due to high osmotic stress [19]. This study returned similar effect in the cultivation of L. starkeyi InaCC Y604 when the fermentation was performed in the medium containing a mixture of glucose and xylose as carbon source.

The fermentation of L. starkeyi InaCC Y604 in -NMM GX medium (Fig. 2) shows that the xylose consumption was significantly increased while glucose in the medium dropped to a concentration 15 to 30 g/L. Microorganisms are known to generally metabolize sugars sequentially when exposed to a mixture of glucose and xylose, because glucose can repress the utilization of other sugars via either a
catabolic repression mechanism or allosteric competition for sugar transporters [20]. However, recent studies by Juanssilfero et al., [21] found that by increasing the initial OD$_{600nm}$ to 16.0-18.0 when cultivating of *L. starkeyi* NBRC 10381 in the medium containing a mixture of glucose 50 g/L and xylose 50 g/L as carbon source, the inhibitory effect of the presence of highly concentrated carbon source in the medium was not observed. Higher level of inoculum concentration will leads to an increase in the number of active cells to utilize carbon sources. Consequently, a higher inoculum concentration leads to a higher cell/carbon source ratio and to an increase in the carbon-source consumption rate [17]. The cell biomass production by cultivation in -NMM GX medium was achieved higher (at 25.02 g/L) compared to cultivation in -NMM G and -NMM X (at 18.68 and 17.53 g/L, respectively) due to higher initial carbon source concentration.

Table 1 shows the carbon source consumption rate ($q$) and growth rate ($g$) of *L. starkeyi* InaCC Y604 in three different type medium. The carbon source consumption rate was determined during the first 24 h of the cultivation process, which provided insight into the reciprocation of consumption for each carbon source by the InaCC Y604 strain before one of them was exhausted. The glucose consumption rate in the medium -NMM G was achieved almost similar with the xylose consumption rate in the medium -NMM X at around 0.49 and 0.43 g/L h, respectively. When glucose and xylose was used as a mixed carbon source in the -NMM GX medium, the carbon source consumption rate data indicated that glucose was consumed at a higher rate than xylose (around 0.19 and 0.09 g/L h for glucose and xylose, respectively).

**Table 1.** Carbon source consumption rates, growth rates and SCO production of *L. starkeyi* InaCC Y604 during fermentation for lipid production phase. $^a$

| Media      | Consumption Rate ($q$) (g/L h) | Growth Rate ($g$) (g/L h) |
|------------|-------------------------------|--------------------------|
|            | Glucose                      | Xylose                   |                           |
| -NMM G     | 0.49 ± 0.01                  | -                        | 0.21 ± 0.01               |
| -NMM X     | -                            | 0.43 ± 0.1               | 0.15 ± 0.01               |
| -NMM GX    | 0.19 ± 0.00                  | 0.09 ± 0.0 $^b$          | 0.18 ± 0.02               |

$^a$ The values are given as the mean ± SD of triplicate determinations.

$^b$ Incomplete carbon source consumption in the late stage of cultivation process.

The SCO production of *L. starkeyi* InaCC Y604 growing on different carbon sources were shown in Table 2. Generally, the SCO production was reached levels more than 45% (w/w) of dry cell weight. The cultivation of *L. starkeyi* InaCC Y604 in the -NMM GX exhibited the highest SCO production at 57.04% (w/w), followed by -NMM X and -NMM G at 50.12 and 47.81% (w/w), respectively. The lipid yield obtained from the cultivation in the whole media was achieved similarly at around 0.18 for -NMM G and -NMM X respectively. However, the lipid yield obtained from the cultivation in the media with a single carbon source (-NMM G and -NMM X) was slightly higher (0.18 (w/w)) compared with that containing a mixed carbon source (-NMM GX, at 0.17 (w/w)), since the total carbon source concentration in the -NMM GX medium was twice that of either -NMM G or -NMM X.

Lipid yield and lipid content var depending on the oleaginous yeast species, feed stock type and cultivation condition. Lipid yield is an essential parameter that affects the cost of lignocellulosic lipid production. The theoretical yield of lipid production from oleaginous microbes is 0.32 w/w (mg/mg-
Figure 2. Time course of carbon source consumption and cell growth of *L. starkeyi* InaCC Y604. The closed triangle charts represent glucose consumption, the closed square refers to xylose consumption and the closed circle charts for the cell growth, -NMM G, medium with glucose as sole carbon source; -NMM X, medium with xylose as the sole carbon source; -NMM GX, medium with glucose and xylose as mix carbon source.
sugar) from glucose and 0.34 w/w (mg/mg-sugar) from xylose [3,22]. In this study the lipid yield of the theoretical maximum for *L. starkeyi* InaCC Y604 was achieved around 53-56%.

According to Jin et al., [23] lipid yields were often below 0.25 w/w (mg/mg-sugar) which equal to less than 74% of the theoretical maximum. Moreover, the data in Table 2 show that the SCO production from the conversion of xylose (-NMM X, 50.12% (w/w)) as a sole carbon source was higher than that of glucose (-NMM G, 47.81% (w/w)). Evans and Ratledge [24] reported that xylose was a better substrate for lipid production. Additionally, other studies [11,17,25] have suggested that xylose might be more efficient than glucose in terms of SCO production, theoretically. This could be due to xylose can be exclusively metabolized by the pentose phosphoketolase pathway beside the pentose phosphate pathway [26].

*L. starkeyi* InaCC Y604 proved to be a promising candidate for single oil production using different carbon sources. Generally, the dominant fatty acid produced by oleaginous yeasts are oleic acid (18:1) and palmitic acid (16:0) and overall, the fatty acid composition profiles produced by oleaginous yeasts were similar to those of vegetable oil, suggesting that microbial lipid could be explored for biodiesel production [27]. However, further analysis is required to analyze the fatty acid methyl ester (FAME) produced by *L. starkeyi* InaCC Y604 strain. Additionally, further study is necessitated to enable high SCO production from real biomass hydrolysates using oleaginous yeasts based on comprehensive understanding of the assimilation of sugars which is crucial for the further development of this industrial process and for the building of a suitable yeast platform for SCO production.

**Table 2.** Biomass and lipid production of *L. starkeyi* InaCC Y604 grown on different cultivation medium (carbon sources).^a^  

| Media                        | Final DCW ($x$, g/L)^b^ | DCW Yield (% of $x$, w/w)^c^ | SCO Production ($L_o$, %, w/w)^d^ | Lipid content ($L_t$, g/L)^e^ | Lipid Yield ($Y_L$, w/w)^f^ |
|-----------------------------|-------------------------|-----------------------------|----------------------------------|-------------------------------|-----------------------------|
| -NMM G (glucose Only)       | 18.68±1.16              | 36.9±0.67                   | 47.81±1.33                       | 8.93±1.10                     | 0.18±0.00                   |
| -NMM X (xylose only)        | 17.53±2.19              | 35±0.14                     | 50.12±1.61                       | 8.78±0.92                     | 0.18±0.01                   |
| -NMM GX (a mixture of glucose and xylose) | 25.02±1.37            | 30.45±0.88                  | 57.04±0.94                       | 14.27±0.81                    | 0.17±0.00                   |

^a^ The values are given as mean ± SD of triplicate determination.
^b^ Final cellular biomass at the end of cultivation time ($x$).
^c^ Gram of cell dry biomass per gram of sugar consumed × 100%.
^d^ Final lipid accumulation from the growth phase to the lipid production phase, determined by gravimetric analysis ($L_o$).
^e^ Lipid content ($L_t$) = ($x$) × (100 - $L_o$)/100.
^f^ Lipid yield ($Y_L$) = ($L_t$/total sugar consumed).

4. **Conclusion**

This study has provided the information for the achievement of cell mass and single cell oil production using glucose and/or xylose as the carbon sources. *Lipomyces starkeyi* InaCC Y604 exhibited promising strain in accumulated single cell oil under different carbon sources. The identification of oleaginous yeasts that can utilize both glucose and xylose appears to be a key aspect in the utilization of a lignocellulosic feed-stock and elucidation of the mechanism underlying the assimilation of these sugars is crucial for the further development of the industrial process and for the building of a suitable yeast platform for single cell oil production.
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