Lipid Production in *Lipomyces starkeyi* LSR6

Siti Hajar Binti Abu Bakar

Bachelor of Science with Honours (Resource Biotechnology) 2015
Lipid Production in *Lipomyces starkeyi* LSR6

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(34605)

A thesis submitted in partial fulfilment of the requirement for the degree of Bachelor of Science with Honors (Resource Biotechnology)

Resource Biotechnology
Department of Molecular Biology
Faculty of Resource Science and Technology
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Lastly, I want to thank my fellow friends in the Microbiology Laboratory for their support and assistance throughout the progression of my final year project.
DECLARATION

I hereby declare that this Final Year Project entitled “Lipid Production in Lipomyces starkeyi LSR6” is based on my original work except for the quotations and citations which have been dully acknowledged also, declare that it has not been or concurrently submitted for any other degree at UNIMAS or other institution of higher learning.

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<tr>
<td>%</td>
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</tr>
<tr>
<td>°C</td>
<td>Celcius</td>
</tr>
<tr>
<td>C</td>
<td>Carbon</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>g/l</td>
<td>Gram per liter</td>
</tr>
<tr>
<td>hr</td>
<td>Hour</td>
</tr>
<tr>
<td><em>L. starkeyi</em></td>
<td><em>Lipomyces starkeyi</em></td>
</tr>
<tr>
<td>μ</td>
<td>Micrometer</td>
</tr>
<tr>
<td>ml</td>
<td>Milimeter</td>
</tr>
<tr>
<td>N</td>
<td>Nitrogen</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>SCO</td>
<td>Singe Cell Oil</td>
</tr>
<tr>
<td>TAGs</td>
<td>Triacylglycerols</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight per volume</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume per volume</td>
</tr>
<tr>
<td>YM Broth</td>
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Lipid Production in *Lipomyces starkeyi* LSR6

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**ABSTRACT**

The depletion of fossil-based resources is due to the high demand of petroleum-based fuels. Plant oil that is being used as biodiesel feedstocks are not suitable as it competes with human food production, causes deforestation and habitat destruction. Therefore, oleaginous yeast such as *Lipomyces starkeyi* is being chosen as it offers various advantages. However, wild type strain of *L. starkeyi* often face limited lipid production. The objective of this study was to observe the lipid production of *L. starkeyi* LSR6 cultured under 2.5% (w/v) of glucose medium. The LSR6 strain was cultured under optimal growth. In addition, the *L. starkeyi* ATCC 12659 was also co-cultured under the same condition on separate bottle as control. Phenol sulfuric acid test and lipid extraction was performed to determine sugar consumption and lipid production. The highest growth of *L. starkeyi* ATCC 12659 and *L. starkeyi* LSR6 were obtained at 144 hr at 9.80 g/l and 10.60 g/l while the highest lipid production were about 3.67 g/l for *L. starkeyi* ATCC 12659 and 3.93 g/l for *L. starkeyi* LSR6. From these results, the percentage of lipid content of *L. starkeyi* ATCC 12659 and *L. starkeyi* LSR6 were 37.4% and 37.1%, respectively.

**Keywords:** *L. starkeyi* LSR6, glucose, lipid production, sugar consumption.

**ABSTRAK**

Kekurangan sumber berasaskan fosil adalah disebabkan oleh permintaan bahan api petroleum yang tinggi. Minyak tumbuhan yang digunakan sebagai sumber biodiesel tidak sesuai kerana ia bersaing dengan pengeluaran makanan manusia, menyebabkan kemusnahan hutan dan habitat. Oleh itu, yis berminyak seperti *Lipomyces starkeyi* dipilih kerana ia memberi pelbagai kelebihan. Walau bagaimanapun, sumber asli yis ini sering berhadapan dengan masalah pengeluaran lipid yang terhad. Objektif kajian ini dijalankan untuk memerhatikan pengeluaran lipid hasil glukosa untuk *L. starkeyi* LSR6 menggunakan media glukosa 2.5% (w/v). Strain LSR6 telah dikultur di bawah pertumbuhan optimum. Di sameing itu, *L. starkeyi* ATCC 12659 juga dikultur pada keadaan yang sama dan digunakan sebagai kawalan. Kaedah ujian fenol sulfurik asid dan penegestrakan lipid telah dilakukan untuk menentukan pengambilan gula dan penghasilan lipid. Pertumbuhan tertinggi untuk *L. starkeyi* ATCC 12659 dan *L. starkeyi* LSR6 diperolehi pada jam 144 dengan 9.80 g/l dan 10.60 g/l manakala penghasilan lipid yang tinggi adalah 3.67 g/l untuk *L. starkeyi* ATCC 12659 dan 3.93 g/l untuk *L. starkeyi* LSR6. Daripada keputusan ini, peratusan kandungan lipid untuk *L. starkeyi* ATCC 12659 dan *L. starkeyi* LSR6 masing-masing adalah 37.4% dan 37.1%.

**Kata Kunci:** *L. starkeyi* LSR6, glukosa, pengeluaran lipid, pengambilan gula.
CHAPTER 1
INTRODUCTION

1.1 Introduction
Fossil-based resources are depleting because of the high demands of petroleum fuel to accomplish daily task. The increasing use of fossil fuel also increases atmospheric carbon dioxide and hastening the global warming (Khanal, 2008). Thus, scientists are trying to find other possible ways to overcome this matter and to reduce dependency on petroleum fuel.

One of the common alternative energy that being exploited is biodiesel which is originally produced by vegetable oils that then have been chemically modified (Cervero et al., 2008). The examples of vegetables oils that have been used to produce biodiesel are palm oil, soybean oil and cottonseed oil (Knothe, 2010). However the use of vegetable oils as biodiesel feedstock cannot satisfy the high demands for biodiesel. Furthermore, this practice is expensive due to high cost of raw materials while causing deforestation and habitat destruction (Pirozzi et al., 2013). Thus, oleaginous microorganism such as oleaginous microalgae, bacteria and yeast are currently being exploited.

Oleaginous microorganisms are potential sources of triacylglycerols, which have the ability to produce lipid more than 20% of their dry weight under nitrogen-limiting condition (Papanikolaou & Aggelis, 2011). However, the use of oleaginous microalgae and bacteria as biodiesel feedstocks are not suitable as it requires large bioreactors and display low lipid production in comparison with oleaginous yeast (Pinzi et al., 2013). Oleaginous yeasts are gaining more attentions because oleaginous yeasts can be cultured in fermentation tanks, thus, does not compete with human food production, free from endotoxin, display high lipid accumulation in their dry biomass and have ability to consume various carbon sources (Beopoulos et al., 2012; Ami et al., 2014).
*Lipomyces starkeyi* is an example of oleaginous yeast that can produce lipid up to 70% of its dry biomass. By using *L. starkeyi* as feedstocks of biodiesel, dependence on imported fossil oil in the transport sector can be reduced. However, wild type strains have limited lipid production (Tapia *et al.*, 2012). Thus, this study proposed the application of mutant strains of *L. starkeyi* that was produced by treating the wild type strain with ethidium bromide and it was obtained from the Microbiology Laboratory, Faculty of Resource Science and Technology in determining its lipid production rates using glucose as carbon source.

### 1.2 Objective

The present study was undertaken with the following objectives:

1. To optimize the growth of *L. starkeyi* ATCC 12659 and *L. starkeyi* LSR6.
2. To optimize lipid production of *L. starkeyi* ATCC 12659 and *L. starkeyi* LSR6.
3. To measure lipid and biomass production of *L. starkeyi* ATCC 12659 and *L. starkeyi* LSR6 cultured in 2.5% of glucose concentration.
2.1 Biodiesel

Biodiesel is a mono alkyl ester produced by transesterification process of vegetable oils with methanol in the presence of potassium hydroxide (Campbell, 2008; Cervero et al., 2008). Biodiesel is a clean burning fuel without sulfur emission and non-corrosive properties. It is a well known substitute for petroleum fuels and has the potential to reduce dependency on fuel based petroleum (Amaretti et al., 2012).

Figure 2.1 shows the equilibrium reaction where the TAGs is been processed into biodiesel with the presence of potassium hydroxide as catalyst.

\[
\begin{align*}
\text{CH}_2\text{-OOC-}R_1 & \quad \rightleftharpoons \quad \text{R}_1\text{-COO-}R' \quad \text{CH}_2\text{-OH} \\
\text{CH-}\text{OOC-}R_2 & \quad + \quad 3\text{R'}\text{OH} \\
\text{CH}_2\text{-OOC-}R_3 & \quad \rightleftharpoons \quad \text{R}_2\text{-COO-}R' \quad \text{CH-}\text{OH} \\
\text{Glyceride} & \quad \text{Alcohol} \quad \text{Esters} \quad \text{Glycerol}
\end{align*}
\]

Figure 2.1: Biodiesel productions via transesterification process (Ma et al., 1999).
Figure 2.2 shows the global biodiesel production from 2000 to 2012. The highest biodiesel production was in 2012 (22.5 billion liters) while the lowest biodiesel production was in 2000 with 0.8 billion liters being produced.

![Biodiesel Production Chart](image)

Figure 2.2: Ethanol and biodiesel global production from 2000 to 2012 (Retrieved from: www.ren21.net).

The United States is the world biggest biodiesel producer followed by Argentina, Germany, Brazil and France. Figure 2.3 shows the monthly biodiesel production in United State from 2011 to 2013. The highest biodiesel production was in December 2013, which is about 125 million gallons, while the lowest was in January 2011 with less than 50 million gallons production.
There are several advantages in using biodiesel to power engine such as to prevent damaging fires, because it has a lower flash point. It is also less offensive compared to petroleum. The engine life is also longer. More importantly, biodiesel burns cleaner than petroleum. Thus, it can lower nitrogen emission, unburnt hydrocarbons and carbon monoxide into atmosphere (Zhang et al., 2003).

2.2 Oleaginous yeast

Oleaginous microorganisms can be classified into oleaginous fungi, bacteria and microalgae (Subramaniam et al., 2010). Among these oleaginous microorganisms, yeast is of the highest interest as it has rapid growth rate and high lipid content compared to microalgae (Gohel et al., 2013). Oleaginous yeast is easy to handle and is suitable for large scale biodiesel production compared to oleaginous fungi (Papanikolaou, 2011). Examples of oleaginous yeast are *L. starkeyi*, *R. glutinis*, *C. curvatus* and *R. toruloides* (Pirozzi et al., 2013). These
oleaginous yeasts have high potential to produce biodiesel as they can produce lipid content up to 70% of their dry biomass.

2.3 Lipid

Lipids are organic compounds that are made up of carbon, hydrogen and oxygen. There are three main groups of lipids that are commonly found, which are triglycerides, phospholipids and steroids (Thye et al., 2012). Lipids from oleaginous yeast are stored in forms of TAGs in lipid bodies (Amaretti et al., 2012). The lipid of oleaginous yeast is also called as single cell oil (SCO) (Katre et al., 2012).

Production of lipid in oleaginous yeast requires a sugar rich medium, with nitrogen and phosphate in limited amount (Rattray et al., 1975). Under these conditions, oleaginous yeasts then convert excess carbon into lipid (Schulze et al., 2014). According to Botham and Ratledge (1979), there are two possible reasons that cause accumulation of lipid in oleaginous yeast which is oleaginous yeast will not cease to assimilate glucose during nitrogen exhaustion from the medium. Thus, acetate unit will be continuously generated. Another reason is, oleaginous yeast do not have lipid biosynthesis futile cycle with lipid oxidation that causes lipid accumulation. The factors that control lipid accumulation and composition depend on the environmental conditions, such as temperature and pH (Ratledge, 1989). The optimal temperature for the highest lipid production rate was reported at 28 °C (Suutari et al., 1993).
2.4 Lipomyces starkeyi

*Lipomyces starkeyi* belongs to the order *Saccharomycetales*. *L. starkeyi* can accumulate lipid up to 70% of their biomass. It has high flexibility in carbon source utilization and culture conditions. Its fatty acid compositions are similar to vegetable oils (Tapia *et al*., 2012).

Table 2.1 shows that lipids in bacteria such as *Arthrobacter* sp. and *Acinetobacter calcoaceticus* are lower compared to microalgae with average oil content of about 20% to 40% of dry biomass. However, oleaginous yeast and fungi shown large oil content which is about 60% to 80%.

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Oil content (% dry wt)</th>
<th>Microorganisms</th>
<th>Oil content (% dry wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Botryococcus braunii</em></td>
<td>25-75</td>
<td><em>Candida curvata</em></td>
<td>58</td>
</tr>
<tr>
<td><em>Cylindrotheca</em> sp.</td>
<td>16-37</td>
<td><em>Cryptococcus albidus</em></td>
<td>65</td>
</tr>
<tr>
<td><em>Nitzschia</em> sp.</td>
<td>45-47</td>
<td><em>Lipomyces starkeyi</em></td>
<td>64</td>
</tr>
<tr>
<td><em>Schizochytrium</em> sp.</td>
<td>50-77</td>
<td><em>Rhodotorula glutinis</em></td>
<td>72</td>
</tr>
<tr>
<td><em>Arthrobacter</em> sp.</td>
<td>&gt;40</td>
<td><em>Aspergillus oryzae</em></td>
<td>57</td>
</tr>
<tr>
<td><em>Acinetobacter calcoaceticus</em></td>
<td>27-38</td>
<td><em>Mortierella isabellina</em></td>
<td>86</td>
</tr>
<tr>
<td><em>Rhodococcus opacus</em></td>
<td>24-25</td>
<td><em>Humicola lanuginosa</em></td>
<td>75</td>
</tr>
<tr>
<td><em>Bacillus alcalophilus</em></td>
<td>18-24</td>
<td><em>Mortierella vinacea</em></td>
<td>66</td>
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</table>
The fatty acid composition of lipids in *L. starkeyi* is compared in Table 2.2 when cultured in different substrates. The lipids in *L. starkeyi* contained mainly of C16:0, C18:0, C18:1 and C18:2 (Wild *et al.*, 2010).

<table>
<thead>
<tr>
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<th>C16:0</th>
<th>C18:0</th>
<th>C16:1</th>
<th>C18:1</th>
<th>C18:2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starch</td>
<td>39.0</td>
<td>3.0</td>
<td>3.0</td>
<td>55.0</td>
<td></td>
</tr>
<tr>
<td>Sludge</td>
<td>55.9</td>
<td>13.8</td>
<td>1.8</td>
<td>25.8</td>
<td>0.1</td>
</tr>
<tr>
<td>Ethanol</td>
<td>31.8</td>
<td>6.7</td>
<td>2.3</td>
<td>53.2</td>
<td>5.0</td>
</tr>
</tbody>
</table>

### 2.5 Phenol sulfuric acid test

Phenol sulfuric acid test is often used as a qualitative test for the presence of carbohydrate. This method is also used to measure reducing sugar content that involve stoichiometric reactions. This assay can determine all classes of sugars including sugar derivatives, oligo- and polysaccharides. Oligo- and polysaccharides react to this method because they undergo hydrolysis in the presence of the hot and strong acid, releasing various monosaccharides. Phenol sulfuric acid test is accurate to ±2% under suitable conditions (Nielsen, 2010).

Dehydration of glucose to hydroxymethyl furfural in hot acidic medium that causes glucose to form reddish-black colour product with phenol is the principle of phenol sulfuric acid test. This reddish-black product has maximum absorption at 490 nm. The amount of total carbohydrate present in the sample solution is then determined by using a standard curve (Sadasivam & Manickam, 2005). The concentration used to make the standard curve must span the sample concentrations. The standard and the sample must be within the limits
reported for sensitivity of this method. Dilution is used when the concentrations are greater than the higher limit of the sensitivity range (Nielsen, 2010).

In this method, small amount of carbohydrate in aqueous samples are mixed with 5% (w/v) phenol solution, and, is then subjected to a direct stream of concentrated sulfuric acid. After a time period sufficient to permit color development, absorbances are measure at 490 nm (Stephen et al., 2006).

### 2.6 Glucose

Glucose is the most common of yeast nutrients. Glucose is preferred as carbon source because it can be used directly to produce energy for the cell by the glycolysis pathway (Russell et al., 2014). The use of yeast extract with glucose during culturing helps to maintain high growth rate during fermentation with low acetic acid secretion. The medium provides nutrient and precursors for the cell synthesis of building blocks while glucose is used mainly as an energy source (Ghose & Ghosh, 2003).

The glucose uptake is lowered during the cell growth and secretion of acetic acid is also lowered by the presence of yeast extract. The cell growth rate in a complex medium is affected by the low uptake of glucose (Ghose & Ghosh, 2003). Meanwhile, when the high-affinity system is repressed by high glucose concentration, a nonsaturable glucose uptake system appears. Regardless of the lack of saturability, the glucose transport is also catalyzed by a transport protein since it is substrate-specific and can be suppressed by inhibitors of protein synthesis (John & Dorothy, 1997).
3.1 MATERIALS

The materials that were used in this study are;

1. *Lipomycess starkeyi* ATCC 12659
2. *Lipomyces starkeyi* LSR6
3. 70% ethanol
4. Glycerol (R&M Marketing, U.K)
5. 5% Phenol
7. D-(+)-Glucose (SIGMA, U.S.A)
8. 250 ml Beaker (SCHOTT, Duran®, Germany)
9. 15 ml Falcon tube
10. Air pump
11. Test tube
12. Air filter
13. Microscope (BX51, Olympus, Japan)
14. Freezer (-20 °C)
15. Yeast Malt Broth (YM Broth, Sigma-Aldrich,Inc.,U.S.A)
16. Glass bottle 250 ml (SCHOTT, Duran®, Germany)
17. Glass bottle 500 ml (SCHOTT, Duran®, Germany)
18. Glass bottle 2 liter (SCHOTT, Duran®, Germany)
19. Analytical Balancer (Adventurer™ Pro Balancer, Ohaus Corporation, U.S.A)
20. Incubator shaker (Ecotron, Infors HT, Switzerland)
22. Vortex (VX-200, Labnet International Inc., U.S.A)
23. Hexane/Propanol (3:2, v/v)
24. Spectrophotometer UV (UV Mini 1240, Shimadzu, Japan)
25. Cuvette (Square cuvettes, Nerbe Plus, Deutschland)
26. Ammonium sulfate (Systerm, ChemAR®, Poland)
27. Potassium dihydrogen phosphate (Systerm, ChemAR®, Poland)
28. Magnesium sulfate (Bendosen Laboratory Chemical, Norway)
29. Calciumchlorid-2-hydrate (Hamburg chemical Ltd., Germany)
30. Sodium Monohydrogen Phosphate Heptahydrate (HmbG Chemicals, Germany)
31. Yeast extract (Conda Pronadisa, Spain)
32. Digital Stirring Hotplates (Cimarec™ Stirrers, Thermoscientific, Malaysia)
33. Homogenizer (Superfine Homogenizer, Fluko®, China)
34. Peristaltic controller (Masterflex Easyload L/S 7518-00, Cole Parmer, Malaysia)
3.2 METHODS

3.2.1 Stock preparation and media

*L. starkeyi* LSR6 was obtained from the Microbiology Laboratory in Faculty of Resource Science & Technology. A volume of 1 ml of *L. starkeyi* LSR6 was pipetted into the solution of 4.29 g in 200 ml of YM Broth (Broth, Himedia, India) in the 250 ml of glass bottle (SCHOTT, Duran®, Germany). YM Broth was used to cultivate cells of *L. starkeyi* LSR6. The culture was placed on the incubator shaker (Ecotron, Infors HT, Switzerland) at 150 rpm for 3 days in room temperature. After 3 days, the culture was checked for the possibility of contaminants by Crystal Violet staining and viewed under microscope (BX51, Olympus, Japan). The glycerol stock was prepared by adding 2.20 g of YM Broth in 100 ml of distilled water and mixed it with 50 ml of glycerol (R&M Marketing, U.K). Later, 7 ml of glycerol stock was added into 15 ml of falcon tube and then sterilized by autoclaving at 121 °C. A volume of 7 ml of *L. starkeyi* LSR6 was added into the falcon tube containing the 7 ml of glycerol stock. The culture stock was placed in the freezer at -20 °C until further used.

3.2.2 Optimization growth of *Lipomyces starkeyi* LSR6

The modified broth according to Wild *et al.* (2010) was prepared in glass bottle of 2 liter (SCHOTT, Duran®, Germany). The modified broth consist of glucose, (NH₄)₂SO₄, yeast extract, Na₂HPO₄·7H₂O, KH₂PO₄, MgSO₄·7H₂O and CaCl₂·2H₂O. Separate preparation of glucose in the modified broth was conduction to prevent caramelization. The glucose was prepared in 500 ml of glass bottle (SCHOTT, Duran®, Germany). The cap of the bottle glass of 2 liter was set with air outlet tube, sampling tube and air inlet tube. Next, the cap, the glucose and the remaining modified broth was autoclaved at 121 °C. After cooling down, the glucose was added and 14 ml of *L. starkeyi* LSR6 was cultured into 1 liter of the modified broth. Later, the bottle glass of 2 liter that filled with modified broth and culture was
attached to the air pump. The experiment was done in triplicates and all sets were cultured for 6 days. For each day, a volume of 150 ml from the modified broth was taken by using peristaltic controller (Masterflex Easyload L/S 7518-00, Cole Parmer, Malaysia) and then were centrifuged (CR21G, Hitachi, Japan) at 5000 rpm in 10 minutes at 4 °C. The highest dry mass weight of *L. starkeyi* LSR6 within 6 days was chosen.

### 3.2.3 Lipid production of *Lipomyces starkeyi* LSR6

After 5 days of incubation, 750 ml of 5% (w/v) glucose was added into 750 ml of the modified Wild broth respectively. The glucose concentration was lowered to 2.5% when mixed in the modified broth. This culture was incubated for 6 days. For each day (0, 24, 48, 72, 96 and 144 hr), 2 ml of the sample was taken for phenol sulfuric acid test to determine sugar consumption. A volume of 150 ml from modified broth were centrifuge (CR21G, Hitachi, Japan) at 5000 rpm in 10 minutes at 4 °C and the supernatant was discarded. The pellet was dried in the oven 80 °C for 1 day and then the dry mass was weighed by using the analytical balancer (Adventurer™ Pro Balancer, Ohaus Corporation, U.S.A). The dry mass was homogenized for lipid extraction.

### 3.2.3 Phenol sulfuric acid test

For the phenol sulfuric acid test, 1 ml of the sample was added into the test tube and diluted with distilled water in 1:5 ratios. The mixture was vortexed (VX-200, Labnet International Inc., U.S.A). In this test, every diluted solution was vortexed. After vortexed, 0.2 ml of the diluted sample was added with 0.2 ml of 5% phenol. Next, 1 ml of sulfuric acid was added to the sample and the mixture turns to reddish-black. Lastly, 5.6 ml of distilled water was pipetted into the mixture. A volume of 1 ml of sample was pipetted into the cuvette (Square cuvettes, Nerbe Plus, Deutschland) and the absorbance of glucose concentration was checked by using spectrophotometer (UV Mini 1240, Shimadzu, Japan) at 490 nm.