LIPID ACCUMULATION IN *LIPOMYCES STARKEYI* CULTURED IN GLUCOSE MEDIA AND SAGO EFFLUENT

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Lipid Accumulation in *Lipomyces starkeyi* Cultured in Glucose Media and Sago Effluent

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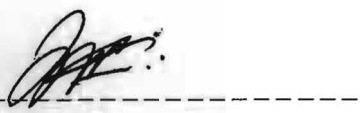
DECLARATION

I hereby declare that this Final Year Project entitled **"Lipid accumulation in *Lipomyces starkeyi* cultured in glucose media and sago effluent"** 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 to any other degree at UNIMAS or other institution of higher learning.



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LIST OF ABBREVIATIONS

TAG	Triacylglycerol
LS	*Lipomyces starkeyi*
DCW	Dry cell weight
WCW	Wet cell weight
HIP	Hexane:isopropanol
ml	milliliter
µl	microliter
°C	degree Celcius
nm	nanometer
g	grams
Mbp	Megabase Pair
kb	kilobase
t	tonne

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Lipid accumulation of *Lipomyces starkeyi* cultured in glucose and sago effluent

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ABSTRACT

Biodiesel production is a promising alternative for the rapid depletion of non-renewable resources. Utilization of waste residues, agricultural waste and feedstock for production of biodiesel are being implemented due to their potential as replacement for conventional diesel and burning fuels. Oleaginous fungi such as *Rhodosporidium* sp., *Lipomyces* sp., and *Rhodotorula* sp. can be used to hydrolyse these materials and turning them into usable biodiesel. In this project, *Lipomyces starkeyi* (*L. starkeyi*) was utilized to compare its performance between two different carbon sources, glucose and sago effluent. Natural sago effluent contain starch, another form of carbon source. Glucose was tested at 2.5% (w/v). Optimum growth of *L. starkeyi* was detected at 120 hour. The highest biomass production were recorded at 9.63 g/L at 144 h by glucose substrate while only 7.40 g/L was produced at 144 h when *L. starkeyi* utilizes sago effluent. The amount of carbon sources consumed per time were determined by using phenol-sulphuric test. From the lipid extraction stage, glucose substrate can yield about 1.83 g/L lipid at 144 h while sago effluent only manages to yield 1.20 g/L lipid at 144 h. From this data, *L. starkeyi* that consumes 2.5% glucose can produce 19.0% of its dry biomass into lipid and when sago effluent was used as the substrate, it can yield 16.2% of its dry biomass. In conclusion, 2.5% glucose is a better substrate in culturing *L. starkeyi* for lipid production rather than using sago effluent.

Keywords: Oleaginous fungi, *Lipomyces starkeyi*, sago effluent, phenol sulphuric carbohydrate test, lipid accumulation

ABSTRAK

Pengeluaran biodiesel boleh menjadi alternatif untuk menggantikan sumber yang tidak boleh diperbaharui yang kian berkurangan. Penggunaan sisa buangan, sisa pertanian dan bahan mentah bagi pengeluaran biodiesel boleh dilaksanakan kerana potensi mereka sebagai pengganti diesel konvensional dan bahan api. Yis berminyak seperti Rhodosporidium sp., Lipomyces sp., dan Rhodotorula sp. boleh digunakan untuk memakan bahan-bahan ini dan menukarkannya kepada biodiesel yang boleh digunakan. Dalam projek ini, Lipomyces starkeyi (L. starkeyi) telah digunakan untuk membandingkan prestasi antara dua sumber karbon yang berbeza, glukosa dan efluen sagu. Efluen sagu semulajadi mengandungi kanji, sejenis sumber karbon. Glukosa telah diuji pada 2.5% (w / v). Pertumbuhan optimum L. starkeyi dikesan pada 120 jam. Pengeluaran biomass tertinggi dicatatkan pada 9.63 g / L pada 144 jam dengan substrat glukosa manakala hanya 7.40 g / L telah dihasilkan pada 144 jam apabila L. starkeyi menggunakan efluen sagu. Jumlah sumber karbon yang digunakan pada setiap masa telah ditentukan dengan menggunakan ujian asid fenol-sulfurik. Dalam proses pengeluaran lipid, substrat glukosa boleh menghasilkan kira-kira 1.83 g / L lipid pada 144 jam manakala sagu efluen hanya berjaya menghasilkan 1.20 g / L lipid pada 144 jam. Oleh itu, ia telah dikenalpasti bahawa L. starkeyi yang menggunakan 2.5% glukosa boleh menghasilkan 19.0% daripada biojisim keringnya menjadi lipid dan apabila efluen sagu telah digunakan sebagai substrat, ia boleh menghasilkan 16.2% daripada biojisim keringnya menjadi lipid. Kesimpulannya, 2.5% glukosa adalah substrat yang lebih baik dalam pengkulturan L. starkeyi untuk pengeluaran lipid daripada menggunakan efluen sagu.

Kata kunci: *Yis berminyak, Lipomyces starkeyi, efluen sagu, ujian asid fenol-sulfurik, pengumpulan lipid*

1.0 INTRODUCTION

The potential of yeast to produce lipid can be the key in reducing human dependencies toward non-renewable sources. Currently, researchers are facing challenges with the fast depletion of non-renewable resources and at the same time, the increasingly intense emission of greenhouse gases that are released by the combustion of those resources (Tortura *et al.*, 2010). Most of these problems are from industrial and transportation sectors that severely damages the atmospheres. Mass utilization of geothermal, wind, solar, and hydroelectrical sources have been proposed for residential and industrial purposes. However, this is an extremely difficult task as replacement for burning fuels in vehicle is a gruesome challenge (Campbell *et al.*, 2012). Sheedlo (2008) stated that for an efficient combustion in an engine, the fuel injected must be of high density resources.

Through biodiesel production, researchers found out that it may give a golden opportunity in overcoming the non-renewable energy crisis. Sheedlo (2008) stated that biodiesel is more environmental-friendly because of the absence of corrosive polycyclic hydrocarbons as in petroleum. This can reduces the possibility of leakage when storing or transporting it from one place to another. This technology are mostly based on the fermentation of lignocellulosic materials such as wood residues as in those saw mill industries and agricultural waste and utilization of as a feedstock for biodiesel production (Ravikumar *et al.*, 2012). In United State alone, corn starch fermentation were chosen for their biodiesel production because corn plantation is abundant there. According to U.S. Energy Information Administration (2014), demand for biodiesel was proven high as there are 94.5 million gallons of biodiesel produced in March 2012, 97.9 million gallons in March 2013, and kept increasing to 98 million gallons in March 2014. In Malaysia, starch

industries are able to procure profit between US$3.4m to US$10.8m between 1988 to the 1990's. Bujang (2008) mentioned that with the decline of sago starch prices (US$9.15m at 61,000 t in 2000) and the rising price conventional petroleum, it shows how crucial the role of biodiesel in improving global economy.

Lipomyces starkeyi is an oleaginous yeast that are able to accumulate 70% of its dry weight in intracellular lipid production, more than other suitable yeast such as *Rhodosporidium toruloides*, *Rhodotorula glutinis*, *Yarrowia lipolytica*, or *Cryptococcus albidus* (Zhao *et al.*, 2008). The lipid in *L. starkeyi* is reported to be similar in composition when compared with vegetable oil (Ravikumar *et al.*, 2012). Thus, this project aims to utilize lipid-producing yeast, *L. starkeyi* which has shown to be able to digest carbohydrate present in sago effluent, waste product released by sago producing factories.

The objectives of this project are to:

1. Determine the biomass of *L. starkeyi* produced when grown in 2.5% (w/v) glucose medium and sago effluent as the carbon sources.
2. Quantify the total lipid produced by *L. starkeyi* when grown in 2.5% (w/v) glucose medium and sago effluent as the carbon sources.

2.0 LITERATURE REVIEW

2.1 Global crude oil and biodiesel production

Global population will increase exponentially by about 1.1 billion people and by 2025, the urban consumer class will increase by 1 billion people (LUKOIL, 2013). Most of the growth will emerge from the developing countries in Asia. In turn, the demand for infrastructures, vehicles, real estate, high-technology product will skyrocket, resulting in a demand for non-renewable energy resources (Campbell *et al.*, 2012). Nowadays, non-renewable energy resources are one of the main contributor for technological advancement of human civilization. This occur due to the high usage of mechanical equipment that assist the population in variety of ways. These equipment includes motorised vehicles, engine-based apparatus, military equipment, and so on (Tortura *et al.*, 2010). Some of the sectors that requires high dependencies with this energy resources are the marketing of goods, manufacturing, distribution, exploration, transportation, and urban industrialization (Bentley, 2002). LUKOIL (2013) stated that the energy resources that are currently in high demand are in the form of liquid hydrocarbons and the demand are foreseen to be growing at 1.2% per year and will be at 105 million barrels per day by the year 2025.

Crude oil are high demand liquid hydrogen because of various type of purposes. It is more preferred than natural gas because they are much cheaper. However, crude oil possess more threat toward the environment than natural gas (Yan, 2012). Yan (2012) stated that oil is a vital energy for most countries globally due to the huge fluctuation of its prices and the uncertainty of supply. Global oil price are controlled by several factors such as US dollar exchange rate, geopolitics, policies, financial markets, supply and demand,

macroeconomic situation, and depreciation of Dollar value (LUKOIL, 2013). The trend of international oil price alternates several times in the 21st century starting from 30 Dollars per barrel in 2003 and increased to 56.37 Dollar per barrel on 26th October 2004. The trend continues in 2005 and peaked at 70 Dollar per barrel during May, June, and July of 2006 (Energy Information Administration, 2007). It drops to 49.51 Dollars per barrel in January 2007 and skyrockets to 142.95 Dollars per barrel in July 2008. The oil price deteriorate under 40 Dollars per barrel in December 2008 (Yan, 2012). Several occasion leads to the stabilization of oil price until it drops again at about 80 Dollar per barrel in 2014. According to Yan (2012), these fluctuation does not only due to the supply and demand, but also the intense competition between countries that causes the oil price fluctuation factors to be far more complex.

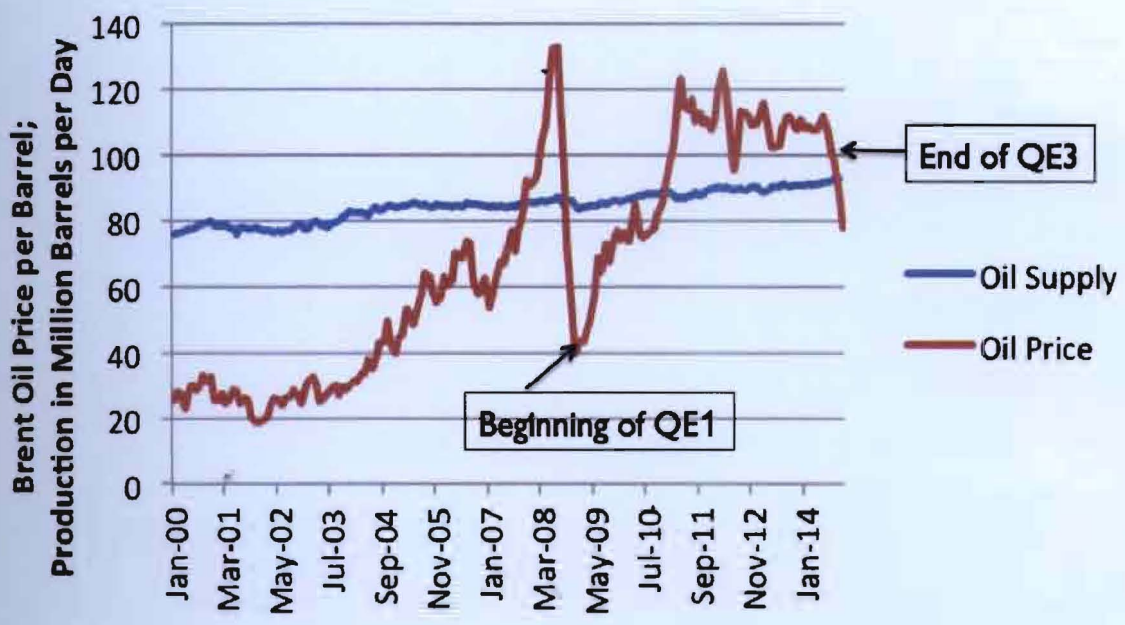

Figure 2.1 Comparison of oil supply and oil price from January 2000 to January 2014 (Retrieved from https://gailtheactuary.files.wordpress.com/2014/12/world-liquids-oil-production-and-price-with-qe-labels.png).

However, crude oil are not infinite and for the past 2 centuries, most of the deposits are being depleted quickly or depleted at all. Because of this reason, the global conventional

oil supply will be in a great risk as other solution for compensate the conventional oil are not approved for usage yet (Bentley, 2002). According to Bentley (2002), it is predicted that in the next 10 years, there will be a steep decline in the production of hydrocarbon resources. This will occur because within the next 20 years, oil deposit around the world will be depleted. Due to this reason, measures and researches had been conducted to avoid an event of global oil shortage. Thus, other forms of technologies and energy had been developed and several solutions had been proposed. The usage of high-technology production procedures as well as alternative fuels such as biofuel, biodiesel, Gas-to-Liquid (GTL) technology, and natural gas liquids (NGL) will occupy about 70% of the reservoir of liquid hydrocarbons from 2010 to 2025 (LUKOIL, 2013).

One of the promising solution in the global oil dependency are the utilization of biodiesel. Biodiesel is a mixture of mono-alkyl esters that are derived from TAG with long fatty acids chain, and, are usually produced from cheap raw materials such as fats and oil (Leesing *et al.*, 2011). Reece *et al.* (2011) mentioned that some crops such as soybean, cassava, and corn had been proposed as a possible feedstock for biodiesel production as the starch produced from them can be easily converted to glucose and fermented to become ethanol by microorganisms.

Biodiesel is more preferable than conventional diesel as it is more eco-friendly, more favourable in combustion emission profile, better lubricating mechanisms, and enormous energy density (Ravikumar *et al.*, 2012). Kirakosyan *et al.* (2009) agreed that due to the clean burning properties, it allows efficient combustion process, reducing the possibility of damage for the fuel injection system. Demand for biodiesel was high as 69 million gallons of biodiesel were produced in January 2013, 73 million gallons in January 2014, and 72

million gallons in January 2015 in United States alone. In total, about 545 million pounds of feedstock were used in January 2015 with soybean being the dominating group of feedstock at 306 million pounds. During January 2015, 39 million gallons of B100 biodiesel (100% biodiesel) were sold while about 22 milion gallons of it were sold in which it was combined with petroleum-based diesel fuel (U.S. Energy Information Administration, 2015).

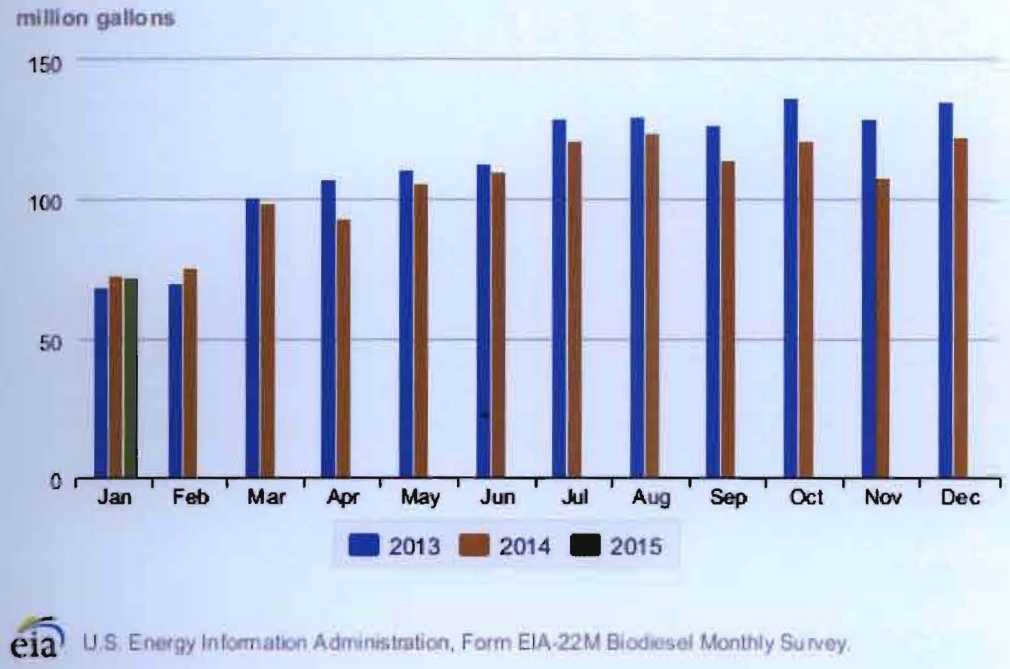

Figure 2.2 US monthly biodiesel production (Retrieved from http://www.eia.gov/biofuels/biodiesel/production/).

Currently, yeast and algae are being studied for biodiesel feedstock supplies, but, yeast are more preferable than microalgae, because according to Santamauro *et al.* (2014), yeast are able to yield its biomass at 10 to 100 g L^{-1} within a 3 to 7 days period, but microalgae can only achieve up to 0.15 to 0.25 g L^{-1} daily. Other than microorganisms, other potential biodiesel feedstock supplies are agricultural residues, industrial and sewage sludge, processing residues, and products that are grown mainly for bioenergy purposes (Taylor, 2014). As the depletion of fossil fuels are imminent in the near future, biodiesel production

might become one of the best solution in replacing human dependency with this non-renewable hydrocarbon source.

2.2 Kingdom fungi

Fungi are classified as a eukaryotes, as it has a true nucleus embedded within it (Brooker, 2012). They are of distinct classes with algae as they do not have chlorophyll in their cells. These diverse organisms can be found at almost all terrestrial and aquatic places (Reece *et al.*, 2011). The kingdom fungi includes moulds, yeasts, and mushrooms and each of them have their own classification as well (Campbell *et al.*, 2012). Tortura *et al.* (2010) mentioned that one of the crucial role of yeast other than lipid production are carrying plasmid that will allow foreign eukaryotic genes expression.

Fungi are capable of limitless growth if substrates are accessible at all the time. This is proven when there are evidence that the mycelium of *Armillaria bulbosa* are able to yield variety type of biomass from many species of single organisms (Brooker, 2012). Thus, when suitable substrates are available at a certain place, fungi have the ability to grow and colonise on it. Fungal hyphae constitutes their mycelium through the extension of each hyphae. Campbell *et al.* (2012) mentioned that this can allow limitless mycelium expansion if individual hyphae are able to extend themselves at the surface of the nutrient sources. With their hyphae, fungi can progress from one nutrient sources to another by using the energy obtained from the first source for their hyphae growth at the next one.

The hyphae can differentiate into specific fungal organs. Due to this, they are able to colonise nutrient sources at a relatively far from their sources either through root-like

organs (rhizomorphs) or spores formation. Fungi are known to be having high resistance toward antifungal agents, dryness, and temperature variant that may interrupt their life cycle (Tortura *et al.*, 2010).

Fungi are virtually everywhere due to their effective reproduction means and dispersal of spores. Fungi are, in nature, found growing without the presence of light and grown in the dark experimentally. However, researchers found out that there are the presence of sophisticated relation between fungi and the daily light-dark cycles, especially during the initiation of reproduction (Campbell *et al.*, 2012). The real factor that activates the response are not the light itself, but the starting of light after a darkness period. Spencer *et al.* (1997) stated that this dark-light changes are happening each day and fungal structure that are irradiated by light are grown repetitively. In turn, their extension rate are decreased, and have higher possibility of maturing into reproductive structures.

Besides that, Subhash *et al.* (2011) discovered that certain fungi can grow permanently on exposed habitat and thus, preventing any competition with those that are photosensitive and dark-light dependent. These fungi thrives on the surface of the leaves and such examples are *Alternaria alternate*, *Botrylis cinerea*, *Cladosporium cladosporioides*, *Phoma pomorum*, and many more. It is recorded that some fungi such as those of *Halosphaeriales* species strive on submerged timber while for *Loculoascomycetes* family, they grows on mangrove woods (Jones, 2000). Jones (2000) mentioned that temperature plays a crucial role in the distribution of fungi geographically.

2.3 Oleaginous fungi

Oleaginous fungi are fungi that are known to be having the capability of producing lipid. They were discovered in the 1970s (Reece *et al.*, 2011). Subhash *et al.* (2011) stated that some of the common oleaginous species are *Rhodosporidium toruloides*, *Rhodotorula glutinis*, *Yarrowia lipolytica*, and *Cryptococcus albidus*. All of these oleaginous fungi can usually accumulate lipid about 20% of their dry mass. Leesing *et al.* (2011) stated that some oleaginous fungi such as *Rhodosporidium* sp., *Lipomyces* sp., and *Rhodotorula* sp. can accumulate lipids exceeding 70% of their dry mass even with environment that have limited nutrients. From the total yeast population, it is reported that oleaginous yeast only made up only 5% of them (Ageitos, 2011).

Table 2.1 Oil content in several types of microorganisms (Meng *et al.*, 2009).

Microorganisms	**Oil content (% dry wt)**		**Oil content (% dry wt)**
Microalgae		Yeast	
Botryococcus braunii	25-75	*Candida curvata*	58
Cylindrotheca sp.	16-37	*Cryptococcus albidus*	65
Nitzschia sp.	45-47	*Lipomyces starkeyi*	64
Schizochytrium sp.	50-77	*Rhodotorula glutinis*	72
Bacterium		Fungi	
Arthrobacter sp.	>40	*Aspergillus oryzae*	57
Acinetobacter calcoaceticus	27-38	*Mortierella isabellina*	86
Rhodococcus opacus	24-25	*Humicola lanuginosa*	75
Bacillus alcalophilus	18-24	*Mortierella vinacea*	66

In oleaginous fungi, the lipid produced are usually in a discrete, globular deposits and known to be related with some of the cell's organelles. Oil produced from fungi are usually used in the manufacturing of triglycerides, polyunsaturated fatty acid, or surfactants (Ageitos *et al.*, 2011).

Transesterification is a process of chemical changes in alkoxy moiety, causing the

transformation of ester. In oil transesterification, a triglyceride undergoes reaction with methanol, producing the formation of glycerol and methyl esters. The methyl esters is the harvestable biodiesel. In the process, 1 mol triglycerides are reacted with 3 mol of methanol. This process is reversible with the formation of mono- and diglycerides as intermediates. The smoothness of the process were influenced by several factors. Some of those factors are the oil molar ratio, temperature, free fatty acid content, purity of reactants, and the type of catalyst used (Schuchardt *et al.*, 1998).

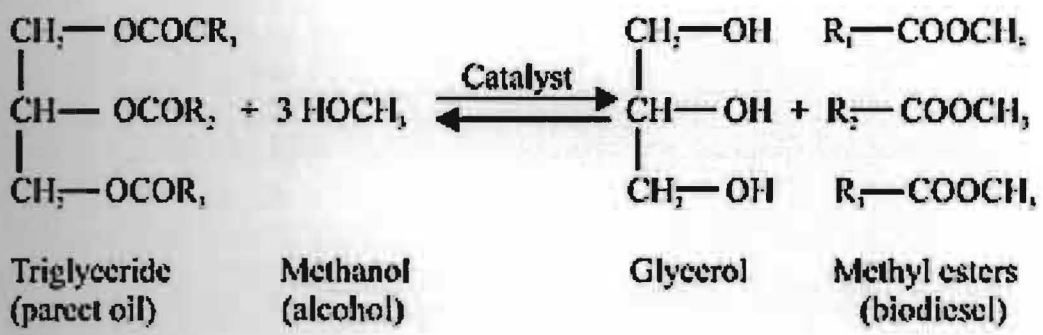

Figure 2.3 Transesterification of triglycerides (Retrieved from https://microbewiki.kenyon.edu/images/thumb/d/d7/Transesterification_eq.png/400px-Transesterification_eq.png).

2.4 Lipid and lipid production from oleaginous yeast

Lipid can be defined as a mixed clump of compound having several similar properties based on their morphology, mainly of non-polar group (Campbell *et al.*, 2012). They are partly water-soluble but readily soluble in organic solutions. "Lipid bodies", "lipid globules", "oil bodies", and "lipid particles" were some of the terms used in literatures as lipid droplet themselves and until 2011, about 1000 publications used the word "lipid droplet" within their study (Reue, 2011). There are three main classes of lipids that are recognised, which are triglycerides, steroids, and phospholipids (Brooker, 2012). Prokaryotes have plasma membrane that are made up of lipid molecules and protein. In

eukaryotes, they consist of double layer of lipids with various classes of protein embedded within it. Reue (2011) stated that the proteins involves are membrane-trafficking proteins and adipose tissue triacylglycerol lipase (ATGL). It have almost similar conformation with those of vegetable oil.

Nowadays, lipid production through oleaginous yeast can be done on many substrates such as sewage sludge, sugar cane molasses, and industrial glycerol. Oleaginous yeast accumulate lipids by hydrolysing glycerol, soluble starch, glycerol, and certain components from the growing medium (Xia *et al.*, 2011). An environment of high carbon sources are preferred with limited concentration of certain nutrients. Those nutrients are usually phosphate and nitrogen. Hence, Carbon:Nitrogen can affect the efficiency of lipid accumulation process of the yeast (Wild *et al.*, 2010). Subhash *et al.* (2011) mentioned that the amount of lipid accumulated can also be affected by the nature of microorganisms, substrate, and controllable parameters in cultures. They also stated that through genetic engineering, modification of oleaginous microorganisms for their lipid accumulation purposes is also possible.

2.5 Lipomyces starkeyi

Lipomyces starkeyi is an oleaginous fungi that is widely used for biodiesel production in an industrial scale. It belong to the phylum Ascomycota. According to Wild *et al.* (2010), this oleaginous yeast has the ability to digest soluble, unsaccharified potato starch. It is found that an increase in the Carbon:Nitrogen molar ratio will improves the overall lipid content at the cost of cell yield. According to Anderson *et al.* (1972), *L. starkeyi* can withstand herbicide at a great length. They can grow well at a wastewater environment

that do not significantly disrupt their growth and does not have high dependency toward foreign nutrients for favourable growth to occur. Angerbauer (2007) mentioned that because of this property, they had assessed the lipid accumulation potential of *L. starkeyi* when exposed with sewage sludge and from their study, lipid accumulation was the most at pH 5.0 and at pH 6.5, highest yield per litre was achieved. From their research, lipid accumulated from this fungi with sewage sludge as the main substrate can be dependable in biodiesel production sector.

L. starkeyi used for this project has the capability of producing lipid about 70% of its dry weight, provided that it is given an optimal environment (Zhao *et al.*, 2008). According to Bignell *et al.* (1996), *L. starkeyi* have a genome size of 15 Mbp, with eleven chromosomes varying from 0.7 kb to 2.8 kb. It is reported that the highest amount of lipid production can be achieved at 28 °C (Suutari *et al.*, 1996). *L. starkeyi* requires an aerobic atmosphere for it to grow and usually after 3 days of fermentation and at 25 °C, the morphological structure of the colony will be glistening, white to light cream-coloured, and smooth.

2.6 Sago and sago effluent

Sago, an edible starch, is one of cassava/tapioca plant and is one of the crucial agro-based industries in many Asian countries. Sago is an abundant resource in Sarawak. Sago palm (*Metroxylon sagu*) can grow normally with minimal care in swamp peat habitat and yield between 150 to 300 kg of starch in one harvest season (Bujang, 2008). It can grow optimally in a humid lowland up to 700 meters in altitude. The optimal condition for sago plantation are temperature of over 25 °C with air humidity at 70% (Rekha *et al.*, 2008). Even though it have a long maturation time (almost 10 years for it to became harvestable),

it can produce starch at a rate of 25 t per year. A study done by Rashid *et al.* (2010) suggested that 40,000 t of sago are exported per year. Solid residues that are found usually contain around 3% starch and 5% dry matter. A sago mill produce more than 500 t of effluent that consist of 25 t of fibres and 15 t starch (Bujang, 2008). These materials can be hydrolysed into fermentable sugars for ethanol production.

Sago effluent is an organic and untreated waste water that are discharged by the sago industries. In most countries, they are usually disposed into the surface water (Nizzy *et al.*, 2014). Before being discharged, these effluent have to be treated in order to safeguard natural resources and the health of the users as well.

2.7 Total carbohydrate test analysis determination

Carbohydrates is one of the organic substances that are abundant in most living organisms. Besides serving as one of the major component for energy production, it supports the rigid structure of plant cell in the form of cellulose (Reece *et al.*, 2011). It can be divided into three major groups according to the number of sugar unit embedded at them. Those three groups are monosaccharide, disaccharide, and polysaccharide (Berg *et al.*, 2012). In yeast, carbohydrate act as sugars that can provide carbon sources for the growth of yeast. Different type of carbon source will yield yeast colony with different growth curve and characteristics.

One of the commonly used test for total carbohydrate determination is the phenol-sulphuric test. Masuko *et al.* (2004) stated that this test have the highest reliability, sensitivity, and the most convenience one among other tests. This test can determine the

concentration of neutral sugar within glycoproteins, glycolipids, oligosaccharides, and proteoglycans. Many researchers prefer this test rather than other carbohydrate test because it does not require covering, shaking, and results can be obtained within 15 minutes (Masuko *et al.*, 2004). Due of this reason, large amount of samples can be tested in a short amount of time when this test is used. In this test, glucose will be dehydrated to form hydroxymethyl furfural, a yellow-brown phenolic compound with maximum absorbance of 490 nm. The value 490 nm is used because, according to Masuko *et al.* (2004), most sugars nears their own absorption maxima and can be measured clearly.

3.0 METHODOLOGY

3.1 Microorganisms conservation in glycerol media

Firstly, 250 ml of Wild's fermentation broth was prepared by mixing 2 proportion of media, the fermentation media and glucose media. Then, 200 ml of distilled water was mixed with 0.17 g of $(NH_4)_2SO_4$ (Systerm, ChemAR®, Poland), 0.24 g of yeast extract (Conda Pronadisa, Spain), 0.41 g of $Na_2HPO_4.7H_20$ (HmbG Chemicals, Germany), 1.16 g of KH_2PO_4 (Systerm, ChemAR®, Poland), 0.03 g of $MgSO_4.7H_20$ (Bendosen Laboratory Chemical, Norway), and 0.02 g of $CaCl_2.2H_20$ (Hamburg chemical Ltd., Germany) in a 500 mL Schott bottle. For the glucose media preparation, 2.25 g of glucose (Ee Syn Corporation, Malaysia) was mixed with 50 ml distilled water in a 250 mL Schott bottle. Then, both Schott bottles were sterilized using autoclave machine at 121°C for 15 minutes. After both Schott bottles have cooled down, the glucose media and one vial of 2 ml stock culture of *Lipomyces starkeyi* (*L. starkeyi*) (ATCC 12659) which was obtained from the Microbiology Laboratory UNIMAS collection were poured into the fermentation media aseptically. Then, it was left for 5 days on an incubator shaker (Ecotron, Infors HT, Switzerland) at 150 rpm at room temperature. At day 5, staining of the broth was conducted to observe the condition of *L. starkeyi*.

To prepare 250 ml of Rose-Bengal Dichloran Chloramphenicol (RBDC, HiMedia, India) media, 250 ml of distilled water was mixed with 4.83 g of RBDC agar powder in a 500 ml Schott bottle and sterilised using autoclave machine. When the media had cooled down, about 50 µl of 40 mg/ml chloramphenicol was added and poured into sterile petri dishes. Subsequently, streaking of *L. starkeyi* on RBDC agar plate was done and left for 2-3 days at 25 °C, followed by subculturing of the colony by spreading method on the same agar media plate to obtain a pure *L. starkeyi* isolate.