Growth and lipid production of I. starkeyi mutants

CHAPTER 1

INTRODUCTION

Diesel is one of the components in fossil fuel. However, the over-use of diesel is producing greenhouse gases such as carbon dioxide gases which are the major elements leading to global warming. Hence, due to increase in demand and source limitation, biodiesel is introduced as a substitute for diesel fuel (Wild *et al* ., 2010).

Biodiesel is a diesel fuel substitute that is extracted from renewable biomass. Biodiesel can be produced from plant oils, animal fats and microorganisms. Traditionally, biodiesel is produced from plant oils which were transesterify with methanol (Dai *et al.,* 2007). However, production of biodiesel from plant oils is not suitable due to the quality of tillable land (Li *et al.* , 2008) and competition with food production (Wahlen *et al* ., 2012). Furthermore, the increase in animal fats prices due to the increase in animal feed makes it not suitable as biodiesel feedstock (Li *et al.* , 2008). Hence, oleaginous microorganisms have been introduced as good candidates for biodiesel feedstock.

Oleaginous microorganisms can accumulate lipid up to 20% of its cell dry weight (Ageitos *et al.* , 2011). Oleaginous microorganisms have the ability to utilize different carbon source (Ageitos *et al.* , 2011). In this study, *Lipomyces starkeyi* will be used. This type of yeast has the ability to produce lipid up to 70 % of its cell dry weight (Wild *et al* ., 2010). *L. starkeyi* can utilize different types of carbon as its sole carbon and it is flexible in terms of culture conditions (Ageitos *et al* ., 2011). However, *L. starkeyi* is still not

economically practical because of the limitations in the wild-type strains (Ageitos *et al.* , 2011). Therefore, in our research, we will be using *L. starkeyi* mutants in an attempt to produce more lipid more lipid in the fungal cells.

The *L. starkeyi* mutants will be cultured in modified media consists of glucose, $(NH_4)SO_4$, yeast extract, $Na_2HPO_4 . 7H_20$, KH_2PO_4 , $MgSO_4 . 7H_20$, $CaCl_{2.} 2H_20$, $FeSO_4$, $ZnSO_4 . H_20$ and $CuSO_4$ supplied with 2. 5% (w/v) and 5. 0% (w/v) of glucose and sago effluents in separated schott bottles. pH 5 and pH 6 will also be used in order to optimize the production of lipid. The temperature that will be used is room temperature (± 27°C). In this experiment, sago effluent and glucose would serve as carbon source for *L. starkeyi.* The total carbohydrate that would be consumed by *L. starkeyi* will be tested using phenol-sulphuric test.

Our objectives in this research are:

1. To optimize growth and lipid production of *L. starkeyi* mutants
2. To measure the amount of lipid produced by *L. starkeyi* mutants cultured in 2. 5 % and 5 % of glucose medium
3. To measure the amount of lipid produced by *L. starkeyi* mutants cultured in sago effluent

CHAPTER 2: LITERATURE REVIEW

2. 1 Biodiesel

Biodiesel consists of alkly ester of fatty acids or triglycerides. Conventionally, triglyceride is produced from soybeans oil with the addition of alcohol and acid or base catalyst. This process is known as transesterifications which will produce Fatty Acid Methyl Ester (FAME) (Wahlen *et al* ., 2012). Basically,

biodiesel can be derived from 3 sources which are plants oil, animal fat and microorganisms (Meng *et al.* , 2008).

Plant oils that involve in the production of biodiesel are rapeseed, palm oil, soybeans, cottonseed, sunflower and many possible crops (Perritano, 2010). However, the practical used of plant oils raises critical issues on the decreasing in quality of land that is needed to plant the crops could affect the quality of the crops produced (Li *et al* ., 2008). In addition, it also competes with the food production (Wahlen *et al.* , 2012). Animal fat is also not a good biodiesel feedstock due to economical reasons (Meng *et al* ., 2008). Hence, oleaginous microorganisms stand out as a potential feedstock provider.

2. 2 Oleaginous microorganisms

Oleginous yeasts (OY) are known producers of single cell oil (SCO). SCO produced from this organism are triacylglycerides (TAG) that have long-chain of fatty acids and have similar properties with plant oils. TAG acts as source of energy and it assist in phospholipid membrane formation. OY also utilizes various its carbon sources from waste substrate thus the cost to culture this microorganism is low (El-Fadaly *et al* ., 2009).

There are four groups of oleaginous microorganisms that capable of producing biodiesel which are bacteria, algae, filamentous fungi and yeast (Kitcha and Cheirsilp, 2011). The genera of oleaginous yeast are *Yarrowia, Candida, Rhodotorula, Rhodosporium, Crytococcus, Trichosporon* and *Lipomyces* (Ageitos *et al* ., 2011) *.* The specific name for the most preferable candidates for production of lipid are *Cryptococcus albidus, Rhodosporidium*

toruloides, Rhodotorula glutinis, Lipomyces starkeyi and *Yarrowia lipolytica* . These microorganisms are capable of producing intracellular lipid more than 20% of its cell dry weight (Tapia *et al* ., 2012).

The duplication rate of yeast is lower than 1 hour and it is easy to culture compared to other microalgae. Other than that, certain oily yeast also has the ability to produce lipid up to 80% of their dry weight, while utilizing different carbon source including the lipid present in media (Ageitos *et al.* , 2011).

2. 3 Factors affecting lipid accumulations in Oleginous yeast

Lipid accumulations occur when yeast is cultured under high amount of carbon source but in limited source of nitrogen. This is due to the nutrient imbalance that helps in triggering the accumulation of lipid because the remaining substrate would be assimilated by the yeast's cells hence convert it into fat for storage (Ageitos *et al.* , 2011). The fat that accumulated could be extracted to produce biodiesel. In addition, the accumulations of lipid also affected by other factors such as the present of microelements and inorganic salts in media. These elements help in ATP (AdenosineTriPhosphate) citrate lyse which important in lipid production (Ageitos *et al.* , 2011).

2. 4 *Lipomyces starkeyi*

L. starkeyi is one of the members of *Saccharomycetales* and considered as true inhabitant of soil which have a worldwide distribution (Ansschau *et al.* , 2014). *L. starkeyi* have the ability to accumulate lipid up to 70% of its dry weight (Wild *et al* ., 2010). It also has a high flexibility in utilization of carbon source and culture environment. Other than that, fatty acid produced by *L.*

starkeyi is almost similar to the vegetable oil (Tapia *et al.* , 2012). According to Wild *et al.* (2010), *L. starkeyi* need a high ratio of carbon to nitrogen in order to optimize the production of lipid. The lipid bodies (LB) of *L. starkeyi* will receive the excess carbon source in the form of triglycerides (TAGs) (Ageitos *et al.* , 2011)

2. 5 Sago effluent

Sago effluent is a form of sago liquid waste. In normal processes, this effluent would be channeled into the river, thus polluting the river and environment (Awang-Adeni *et al.* , 2010). The releasing of sago effluent into the river can cause decreasing in water pH and increase in biochemical oxygen demand (BOD) and chemical oxygen demand (COD) (Ayyasamy *et al.* , 2008)

Sago effluent contains a high amount of organic materials and non-starch polysaccharide (NSP) (Awang-Adeni *et al.* , 2010). NSP are made of cellulose, hemicellulose and lignin. In cellulose, the sub-components are 89% glucose and small amount of xylose, rhamnose, arabinose, mannose, fructose and galactose. In contrast to cellulose, hemicellulose main components are glucose and xylose accompanied with arabinose, galactose, rhmnose, fucose and uranic acid. Lignin functions in rigidity and stability of the wood. To sum up, sago effluent contains up to 66% of starch, 14 % fiber and 25 % lignin (Awang-Adeni *et al.* , 2010).

Sago effluents which flow from the sago mill usually have the ratio of carbon to nitrogen high which is 105: 0. 12 (Awang-Adeni *et al.* , 2010). As stated by Ageitos *et al.* (2011), *L. starkeyi* have the ability to utilize starch as its sole

carbon. Hence, sago effluent is an excellent choice because it has a high amount of starch which can helps in optimizing the lipid production.

2. 6 Phenol-sulphuric test

Phenol-sulphuric test is the quantitative assays which often used in estimation of carbohydrate. This test could detect the presence of neutral sugar in oligosaccharides, proteoglycan, glycoproteins and glycolipids (Albalasmeh *et al* ., 2013). When phenol-sulphuric is added, the glucose that presence in samples would dehydrate thus forms hydroxymethyl furfurax. It would yield a yellow-brown product and the OD could be checked at 490 nm (Albalasmeh *et al* ., 2013).

CHAPTER 3: MATERIALS AND METHOD

3. 1 Materials

1. Modified media as suggested by Wild *et al* . (2010).
2. *Lipomyces Starkeyi* mutants (LS R1 and LS R2)
3. 2. 5 % (w/v) and 5. 0 % (w/v) of glucose (Ee Syn, Malaysia)
4. 2. 5 % (w/v) and 5. 0 % (w/v) of sago effluent (Pusa, Malaysia)
5. 80 % (w/v) of Glycerol stock (HmbG, Germany)
6. 5 % Phenol (Nacalai Tesque, Japan)
7. Hexane (Reagents, USA)
8. Isopropanol (Amresco, USA)
9. Microcentrifuge (Hettich EBA 21, England)
10. Schott’s bottles (Duran, Germany)

3. 2 Glycerol stock

A single colony of *L. starkeyi* mutants R3 will be inoculated into 100 ml of modified media. 800 µl of *L. starkeyi* mutants R3 that have grown will be transferred into vial that contained 1200 µl of glycerol stock. The glycerol stock steps of *L. starkeyi* will be repeated for *L. starkeyi* mutants R4. The solution will be stored in freezer at -20 °C.

3. 3 Propagation of cell

1. 5 L of modified media with pH 5 will be prepared into two Liter schott bottles and *L. starkeyi* mutants R3 and R4 will be inoculated in respective bottles (Wild *et al* ., 2010). This step will be repeated for pH 6.

For day 1 until day 6, three (3) falcon tubes will be autoclave and weight. After that, 50 ml of the cultured from first bottle will be transferred into each three (3) falcon tubes and it will be weighted again. The sample will be sent for centrifuge for 5 minutes at 5000 rpm. The supernatant will be discarded and the pellet with falcon tube will be weight again for its wet weight. The sample will be dry in the oven for 1 or 2 days. After that, the sample will be weight again for its dry weight. All experiments will be performed in duplications.

3. 4 Standard curve for *L. starkeyi*

1 ml of culture which will be incubated for 3 days earlier will be added into 9 ml of modified media in test tube. Serial dilution will take place with the factors of 10^{-1} until 10^{-7} . For factors of 10^{-1} until 10^{-7} , their OD will be checked for 600 nm. For factors 10^{-5} until 10^{-7} , 300 µl from each sample

will be taken and poured onto plate count agar. The plate will be incubated overnight before colony counting will be performed.

3. 5 Lipid accumulation stage for *L. starkeyi* mutants

The *L. starkeyi* mutants culture will be incubated for 3 days (optimum growth) at room temperature. After 3 days, 750 ml of 10. 0% (w/v) of glucose will be added into 750 ml modified media to achieve final concentration of 5% (w/v) in the schott bottle and it will be incubated further for 6 days. From day 1 to day 6, 150 ml of cultured will be harvested into each three (3) falcon tubes. This step will be repeated for pH 5 with 5. 0% (w/v) of glucose and pH 6 with 10. 0% (w/v) and 5. 0% (w/v) of sago effluent.

3. 6 Sampling biomass

The samples will be weighted in wet condition before dry in the oven. After that, the samples will be dried in the oven for 3 days. The dried mass will be taken and weighted again for dry weight.

3. 7 Lipid extraction

Hexane: propanol in the ratio of 3: 2 will be added into the falcon tubes consists of the dry mass. The mixture will be homogenized for 2 minutes. The homogenized sample will be incubated for 1 hour before centrifuge for 5 minutes. The supernatant will be taken and placed in an empty beaker and weight. The supernatant will be heated until the hexane and propanol solution have evaporated completely. The remaining oil will be weighted again. This step will be repeated for 5. 0% (w/v) of glucose, 2. 5% (w/v) of sago effluent and 5. 0% (w/v) of sago effluent.

3. 8 Phenol-sulphuric carbohydrate test

Phenol test is used to detect the amount of carbohydrate that is not consumed by *L. starkeyi* . For each sample, phenol-sulphuric carbohydrate test will be performed by adding 0. 2 ml of 5% (w/v) of phenol and 1 ml of 96% (w/v) of sulphuric acid. After that, 1 ml from each mixture will be placed into a clean cuvette and read at 490 nm in a spectrophotometer.

EXPECTED OUTCOME

By the end of this experiment, we expect to measure the amount of lipid produced by *Lipomyces starkeyi* mutants in 2. 5% (w/v) and 5. 0% (w/v) concentration of glucose and sago effluent at different pH.

WORK SCHEDULE

Project Activities	2014					2015			
	Aug	Sept	Oct	Nov	Dec	Jan	Feb	March	Apr	May
Data collection	â-									
Proposal writing and presentation	â-º	â-º	â-							
Bench work and sample processing			â-º	â-º	â-º	â-				

Progress report	â-º	â-º	â-º	â-▨						
Data analysis			â-º	â-º	â-▨					
Data validation: Statistical analysis			â-º	â-º	â-º	â-▨				
Report writing and presentation				â-º	â-º	â-º		â-º	â-▨	

Legends

â-º: In progress

â-▨ : End of progress

REFERENCES

Ageitos, J. M., Vallejo, J. A., Veiga-Crespo, P., & Villa, T. G. (2011). Oily yeast as oleaginouscell factories. *Applied Microbiology and Biotechnoogy, 90* (12), 1219-1227.

Albalasmeh, A. A., Berhe, A. A., & Ghezzehei, T. A. (2013). Method for rapid determination of carbohydrate and total carbon concentrations using UV spectrophotometetry *. Carbohydrate Polymers, 97* (2), 253-261.

Anschau, A., Xavier, M. C. A., Hernalsteens, S., & Franco, T. T. (2014). Effect of feeding strategies on lipid production by *Lipomyces starkeyi* . *Bioresource Technology* , 157, 214-222.

Ayyasamy, P. M., Banuregha, R., Vivekanandhan, G., Rajakumar, S., Yasodha, R., Lee, S., & Lakshmanaperumalsamy, P. (2008). Bioremediation of sago

industry effluent and its impact on seed germination (green gram and maize). *Journal of Microbiology and Biotechnology, 24* (11). 2677-2684

Awang-Adeni, D. S., Abd-Aziz, S., & Hassan, M. A. (2010). Bioconversion of sago residue into value added. *African Journal of Biotechnology, 9* (14), 2016-2021.

El-Fadalay, H. A., El-Naggar, N. E., & Marwan, E. M. (2009). Single Cell Oil Production by an Oleginous Yeast Strain in a Low Cost Cultivation Medium. *Research Journal of Microbiology, 4* (8), 301-313.

Kitcha, S., & Cheirsilp, B. (2011). Screening of Oleaginous Yeasts and Optimization for Lipid. *Energy Procedia, 9* , 274-282.

Li, Q., Du, W., & Liu, D. (2008). Perspectives of microbial oils for biodiesel production *. Applied Microbiology and Biotechnology, 80* (5), 749-756.

Meng, X., Yang, J., Xu, X., Zhang, L., Nie, Q., & Xian, Mo. (2008). Biodiesel production from oleaginous microorganisms. *Renewable Energy, 34* (2009), 1-5.

Perritano, J. (13, December 2010). *10 top biofuel crops* . Retrieved from HowStuffWorks: http://auto. howstuffworks. com/fuel-efficiency/biofuels/10biofuelcrops. htm#page= 2

Tapia, E. V., Anschou, A., Coradini, A. L., Franco, T. T., & Deckmann, C. (2012). Optimization of lipid production by the oleaginous yeast Lipomyces starkeyi by random mutagenesis coupled to cerulenin screening. *AMB express, 2* (64), 1-8.