CO-CULTIVATION OF LOCALLY ISOLATED YEAST *Rhodotorula* sp. AND MICROALGAE *Chaetoceros muelleri* FOR ENHANCEMENT OF LIPID PRODUCTION

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ABSTRACT

CO-CULTIVATION OF LOCALLY ISOLATED YEAST *Rhodotorula* sp. AND MICROALGAE *Chaetoceros muelleri* FOR ENHANCEMENT OF LIPID PRODUCTION

Microbial-producing oils such as yeast and microalgae have attracted researchers' interests as an alternative to the conventional biodiesel feedstocks. The simplicity of operation due to the oleaginous microorganisms' rapid growth and similar fatty acid profiles with the vegetable oils making them versatile microbes with valuable applications. The co-cultivation of lipid-producing yeast and microalgae has been recognized having a synergistic activity that is beneficial to increase biomass and lipid production compared to monoculture. In this study, the cultivation conditions of yeast *Rhodotorula* sp. and microalgae *Chaetoceros muelleri* as well as co-culture were optimized to enhance lipid production. The yeast and microalgae were obtained from Mamut Copper Mine and Kota Kinabalu Wetland, respectively. The studied growth conditions include the initial pH of media, light intensity, co-culture inoculum ratio and harvesting time. The growth of the culture was determined using UV spectrophotometer while the lipid production was analyzed using methanol: chloroform: water (1:1:1) lipid extraction method. The detection of gene responsible in lipid production known as malic enzyme was also employed. The findings of the study revealed that the optimal pH for *Rhodotorula* sp., *C. muelleri* and co-culture were 7.0, 7.5, and 6.0 respectively. The light intensity at 15,000 lux and inoculum ratio of 1: 3 (yeast: microalgae) were found providing the best conditions for the co-cultures. The optimum harvesting time for microalgae and co-culture was 10 days, while the yeast could sustain up to 30 days for greater lipid production. In comparison with monocultures, the co-culture had successfully increased the biomass productivity from 0.01 to 0.16 g/l/day and lipid productivity from 1.1 to 2.4 g/l/day. These findings, which supported by the detection of malic enzyme gene in yeast and co-culture cultivations, indicated that both are potential lipid producers. Co-culturing of yeast and microalgae enhanced the lipid production that is promising for a sustainable and industrially viable microbial-oil biofuel production.

ABSTRAK

Mikrob-penghasil minyak seperti yis dan mikroalga telah menarik minat penyelidik untuk dijadikan alternatif kepada bahan mentah biodiesel konvensional. Kemodahan dari segi pengendalian mikrooganisme oleaginous disebabkan oleh kadar pertumbuhannya yang cepat dan profil asid lemak yang serupa dengan minyak sayuran menjadikannya mereka mikrob serba-boleh dengan aplikasi yang berharga. Pengkulturan yis dan mikroalga mempunyai tindak-balas sinergistik yang bermanfaat untuk meningkatkan peningkatan biomas dan lipid berbanding dengan monokultur. Dalam kajian ini, parameter pengkulturan *Rhodotorula* sp. dan mikroalgae *Chaetoceros muelleri* serta ko-kultur dioptimumkan untuk meningkatkan pengeluaran lipid. Yis dan mikroalgae masing-masing diperoleh dari Mamut Copper Mine dan Wetland Kota Kinabalu. Parameter pertumbuhan yang dikaji termasuk pH media, intensiti cahaya, nisbah inokulum bagi ko-kultur dan masa penuaian. Kadar pertumbuhan ditentukan menggunakan UV spektrofotometer manakala pengeluaran lipid dianalisis menggunakan kaedah pengekstrakan lipid, metanol: kloroform: air (1: 1: 1). Pengesanan gen yang bertanggungjawab dalam pengeluaran lipid yang dikenali sebagai enzim malik juga dilaksanakan. Hasil kajian menunjukkan bahawa pH optimum untuk *Rhodotorula* sp., *C. muelleri* dan ko-kultur masing-masing adalah 7.0, 7.5 dan 6.0. Keamatan cahaya pada 15,000 lux dan nisbah inokulum 1: 3 (yeast: microalgae) didapati memberikan keadaan terbaik untuk ko-kultur. Masa penuaian optimum mikroalga dan ko-kultur adalah 10 hari, manakala yis boleh mengekalkan pengeluaran lipid yang lebih tinggi sehingga 30 hari untuk. Dibandingkan dengan monokultur, ko-kultur telah berjaya meningkatkan produktiviti biomas dari 0.01 hingga 0.16 g/l/hari dan produktiviti lipid dari 0.06 ke 2.4 g/l/hari. Penemuan ini, yang disokong dengan pengesanan gen enzim malik dalam pengkulturan yis dan ko-kultur, menunjukkan bahawa kedua-duanya adalah pengeluar lipid yang berpotensi. Pembiakan yis dan mikroalga meningkatkan pengeluaran lipid yang menjanjikan pengeluaran biofuel yang mampan daripada mikrob penghasil minyak.

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LIST OF ABBREVIATIONS

ATP	-	adenosine triphosphate
BLASTN	-	basic local alignment tool for nucleotide
bp	-	base pair
EM	-	enriched media
CDW	-	cell dry weight
CTAB	-	cetyltrimethylammonium bromide
dH_2O	-	distilled water
DNA	-	deoxyribonucleic acid
dNTP	-	deoxynucleoside triphosphate
EDTA	-	ethylenediamine tetraacetic acid
EtBr	-	ethidium bromide
FAs	-	fatty acids
L	-	liter
m	-	meter
$MgCl_2$	-	magnesium chloride
NADPH	-	nicotinamide adenine dinucleotide phosphate
OD	-	optical density

LIST OF SYMBOLS

°C	-	degree Celsius
µg	-	microgram
µm	-	microliter
µM	-	micromolar
%	-	percent
g	-	gram
nm	-	nanometer
s	-	second
U	-	unit
w/v	-	weight over volume

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

To date, with the increasing demand for energy requirement globally, the search for an efficient substitute for the source of energy is critical. With the rapid increase of population and wealth globally, additional energy is required. Furthermore, the over-exploitation of coal, natural gas and petroleum has triggered vast ecological distresses such as the climate change which is resultant of the global warming effects (Yen *et al.*, 2015). Thus, the development of renewable energy has to meet the increasing energy demand, in addition to reducing the environmental impacts caused by the over-exploitation of the traditional energy source, hydrocarbon oils (Vlysidis *et al.*, 2011).

With an increasing awareness to the shortcomings of using fossil fuels which led to global warming and greenhouse effect as a result of carbon dioxide emission (Abdullah *et al.*, 2009), biodiesel has received increasing approval as an alternative and renewable source to substitute fossil fuels. Palm oil produced by the oil palm trees were the main source of income for farmers in South East Asia, in fact, Malaysia is the second largest palm oil producer in the world (Young, 2011). It is generally used as cooking oils, in fact, exported for commercialization in food industries, personal care merchandises as well as converted into biofuel. Palm oil produces 10 fold more oil per unit area than the vegetable oils for instance soya beans, rapeseed or sunflowers (Sumathi *et al.*, 2008). Despite the potential of palm oil to replace the fossil fuels as biodiesel feedstock, the palm oil plantations are under elevating pressure due to their side effects to the environment. The risks of loss of carbon-sequestering forest land such as seen in Sarawak, where a large areas of peat swamp forest was destroyed for palm oil plantations (Young, 2011). This is one of the many issues in palm oil plantations which make it less ideal substitute for the traditional fossil fuels.

Recently, the improvement of microbial oils is emphasized in order to use them as the substitute for energy source. Vast microorganisms had been identified of having the ability to store lipids, this include bacteria, yeast, fungi, and algae (Li *et al.*, 2008). Compared to other plant oils, microbial oils have many advantages, such as rapid life cycle, less regard for venue, season and climate, and ease of scale up. With the increasing demand of biodiesel in recent years, microbial oils have become as one of the promising feedstocks for biodiesel production. The production of key fatty acids (FAs) in plant cells that was highly recommended for the production of biodiesel showed similarity with the key FAs produced by an oleaginous yeast (Steen *et al.*, 2010). Rapeseed and soybean oil are among the most commonly used feedstocks for biodiesel production. Biodiesel that has determined properties such as viscosity, density and melting point can be mixed with the common diesel fuel. Furthermore, biodiesel can be applied as a substitute to low carbon heating oil.

High amount of lipid content can be produced by yeasts using different sources of carbon including glycerol and molasses (Yen *et al.*, 2015). Beopoulos and Nicaud (2012) in their study reported that lipid content and lipid profile varied amongst species. The yeast species including ***Cryptococcus albidus, Lipomyces lipofera, Lipomyces starkeyi, Rhodosporidium toruloides, Rhodotorula glutinis, Trichosporon pullulan*** and ***Yarrowia lipolytica*** were found able to accumulate oils. Different yeast species accumulate different lipid compositions. *Rhodotorula* genus especially *Rhodotorula glutinis* was reported yielding high lipid content (Zhang *et al.*, 2014). Microorganisms derived from extreme environment may exhibit unique properties and high tolerance towards diverse conditions. The *Rhodotorula* sp. used in this study is a new locally isolated strain from Mamut Copper Mine. The soil sample from which the yeast strain was isolated contained high iron, aluminium, and copper. In addition to that, the seepages water was extremely acidic, around pH 2-4. Hence, investigation further about the potential of the strain will be a biotechnological interest.

Microalgae also have been recognized as a potential source for biodiesel due to their high lipid content, photosynthesis capacity and carbon dioxide reduction efficiency. Microalgae can convert carbon dioxide into lipids through the process of photosynthesis which makes it known as cell factories of sunlight-driven. With the availability of organic and inorganic substrates, some microalgal cells are able to grow in a heterotrophic or mixotrophic system instead of strictly photosynthesis system (Eugenia, 2012). Under mixotrophic and heterotroph systems, the algal cells ingest or absorb organic carbon rather than fixing carbon from inorganic sources such as carbon dioxide as in photosynthesis process. The fixation of carbon by algae may end up in three different paths. The carbon may either be used in respiration or converted into an energy source, in fact, it can end up in the formation of more cells by using the carbon as raw materials (Berman-Frank and Dubinsky, 1999). Inorganic carbon source is required for algae to execute photosynthesis. Different carbon form such as carbon dioxide, bicarbonate or carbonate can be used for algal growth (autotrophic) while for heterotrophic growth, carbon in the form of glucose or acetate is used. In water, depending on temperature, nutrient content and pH, carbon dioxide may exist as one of these forms. Thus, the algae cultivation is known by its ability to fix carbon (Ho *et al.*, 2011; Kumar *et al.*, 2011; Yen *et al.*, 2015). Carbon fixed by microalgae is incorporated into carbohydrates and lipids. Therefore, chemicals, energy or food can be produced from algal biomass (Olaizola, 2003).

In lipid biosynthesis pathway of oleaginous microorganisms, three enzymes have been identified as crucial for lipid accumulation including isocitrate dehydrogenase, ATP-citrate lyase (ACL) and malic enzyme (Christophe *et al.*, 2012). As indicated by Tang *et al.* (2010), malic enzyme is regarded as one of the main NADPH supplier that is vital for fatty acid intracellular storage. Previous studies revealed that malic enzyme plays an important role in lipid accumulation. Malic enzyme proportionally altered with lipid accumulation in *Mortierella alpine* (Wynn *et al.*, 1999). In fact, the lipid content of *Mucor circinelloides* was increased by 1.5-fold when the gene encoded malic enzyme was overexpressed (Zhang *et al.,* 2007). This further supports the vital role of malic enzyme for lipid biosynthesis in oleaginous organisms which makes it the highlighted gene to be evaluated in the present study.

Microalgal feedstock has been considered as an effective substitute to the conventional oil-bearing crops due to the advantage in regards to demand for natural resources and arable land are eliminated (Singh and Dhar, 2011). The *Chaetoceros muelleri* microalgae strain used was provided by the Borneo Marine Research Institute, Universiti Malaysia Sabah from the culture collection of the Unit for Harmful Algal Blooms Studies (UHABs). This study was a continuation from previous research by Andrew *et al.* (2014) which indicated that *C. muelleri* showed top potential for biofuel production. This was proved in an indoor mass cultivation, under optimized conditions, whereby *C. muelleri* recorded the highest increment in lipid productivity with 178.4%. *C. muelleri* also synthesized the highest amount of C14 - C18 FAs with 30.95%. In fact, the combinations of high SFAs (saturated fatty acids) and MUFAs (monounsaturated fatty acids) as well as low PUFAs (polyunsaturated fatty acids) are considered as the fittest FAs profile as a biofuel feedstock candidate.

In nature, mixture cultivations of microorganisms usually with non-specific microbial strains are found under non-sterile conditions. Many microorganisms under mixed cultures may strive for substrates, at the same time they may act symbiotically. Contrary in pure culture, different microorganisms play a critical role by interacting with each other under a co-cultivation condition (Bader *et al.*, 2010). The growth of one strain may be affected by another, whether the growth can be improved or repressed by the growth activities of the other strain existing under the same medium.

Yen *et al.* (2015) hypothesized that in co-culture, as both yeast and microalgae produce lipids, the yeast will provide the microalgae carbon dioxide while microalgae mutually provides oxygen to the yeast. On the other hand, Kleerebezem and van Loosdrecht (2007) study showed that co-cultivation possibly will result in an increased yields, process costs reduction due to cheaper substrates, finally quality control of product. Fermentation of co-culture and mixed culture have the potential on the products advancement of bioenergy, biofuels and bio-based (Bader *et al.*, 2010).

The cultivation for *Rhodotorula* sp. has been comprehensively studied. However, isolates from different sources may exhibit different growth requirements attributed to their respective adaptation and acclimatization at their origin. While the cultivation of other microalgal strains such as *Chlorella vulgaris*, *Scenedesmus obliquus*, and many others have been studied extensively, the *C. muelleri* strain however has not been widely published. Hence, this study will provide an understanding of the potential of co-cultured microorganisms as a biofuels feedstock. In this study, the effect of co-cultivating yeast and microalgae *Rhodotorula* sp. and *C. muelleri* respectively was investigated as potential biodiesel feedstock. The study aimed to confirm that the co-culture of yeast and microalgae could significantly enhance biomass and lipid production. The growth characteristics of the pure cultures were initially established prior to the optimization of culture conditions such as the initial pH of media, light intensity, ratio of yeast to microalga and harvesting time. Finally, the key gene (malic enzyme) involve in lipid production was identified using the molecular characterization, polymerase chain reaction, PCR.

1.2 Problem Statement

The co-culture cultivation of yeast *Rhodotorula* sp. and microalga C. *muelleri* can mutually benefit in enhancement of lipid production as biodiesel feedstock. However, both yeast and microalgae have been recognized growing optimally under different conditions. In previous studies, yeast was reported to grow optimally in an acidic condition while microalga showed preference towards basic condition. In addition, the cultivation of microalgae is also very sensitive to light intensity. While yeast requires agitations for greater biomass productivity, microalga is recognized as a shear-sensitive culture where agitations cause destruction of the cells. Therefore, the operating conditions of the co-cultivation need to be investigated. It includes fine-tuning the ratio of yeast/microalgae inoculum for an optimum symbiotic effect of the co-culture for lipid and biomass production as well as the right harvesting time for the cultures.

1.3 Research Hypothesis

Co-cultivation of two lipid producers, *Rhodotorula* sp. and *C. muelleri* will induce synergistic activity which is beneficial to enhance the biomass growth of the co-culture and consequently may increase lipid productivity.

1.4 Research Objectives

The objectives of the study were as follows:

i. To optimize the cultivation conditions of yeast *Rhodotorula* sp. and microalgae *Chaetoceros muelleri* co-culture for the enhancement of lipid production.
ii. To detect the key gene responsible for lipid biosynthesis in pure and co-culture cultivations of yeast *Rhodotorula* sp. and microalgae *C. muelleri*.

1.5 Significance of the Study

To date, the studies on co-culture for lipid production as a biofuel feedstock are still lacking. This research will lead to the discovery of the potential co-cultures of *Rhodotorula* sp. and *C. muelleri* as biodiesel feedstock. The study is expected to unravel the significance of co-culture in enhancing the production of lipid in comparison to monoculture. On the other hand, this study will provide important findings related to the potential of natural resources from Sabah in biofuels production. Both strains were derived from Sabah environment that are promising for a locally sustainable and industrially practical feedstocks for biodiesel.

CHAPTER 2

LITERATURE REVIEW

2.1 Production of Biodiesel

Biodiesel can be applied in vehicle up to 5-20% blends including for railway and aircraft usage. In addition to the transportation benefit, biodiesel has been employed as a heating oil fuel in domestic and commercial boilers and as an effective solvent to clean oil spill due to its methyl ester component that can reduce the viscosity of crude oil. Various lipids sources have been used as a feedstock for the production of biodiesel including plant oils, animals fats and waste cooking oils (Poontawee *et al.*, 2017). Microorganisms present many advantages that make them an interest of biotechnological research. There are great varieties among microbes and they grow very rapid (multiply once every 20 to 90 minutes). In addition to that, they have the ability to consume cheap substrates and ease of cultivation at large scale (Beopoulos and Nicaud, 2012). Oleaginous microorganisms including yeasts and moulds, bacterium and algae have been studied comprehensively due to the similarity of soybean lipid components, a promising feedstock for the production of biodiesel.

In oil production industry, each microorganism's class and species displays benefits as well as drawbacks. There are several benchmarks that are used as guidance to identify the microorganism's potential commercially. According to Ahmad *et al.* (2015), microorganisms' selection and ranking for production of lipid and biodiesel, the key measures likely to be vital are shown in Figure 2.1.

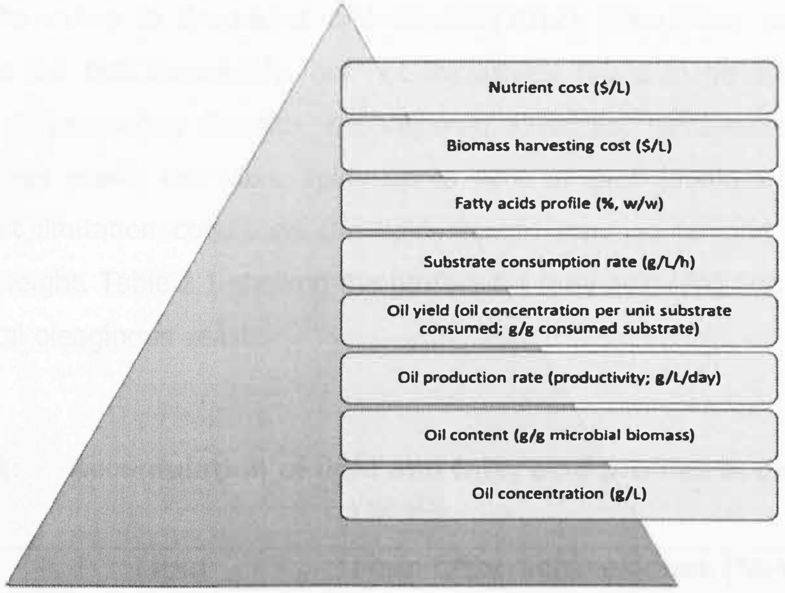

Figure 2.1: Microorganisms' selection and ranking for biodiesel production

Source: Ahmad *et al.* (2015)

Among oleaginous microorganisms, the yeast species of *Rhodotorula* genus especially *Rhodotorula glutinis* was reported to have great compliant of lipid strain that can consume wastewater to produce lipid (Zhang *et al.*, 2014). Alternatively, due to the high lipid content in microalgae as well as photosynthesis and carbon dioxide fixation efficiency, they are regarded as one of the outstanding source for production of biodiesel. In fact, when the organic and inorganic substrates are present, some microalgal cells are able to grow in a heterotrophic or mixotrophic system instead of strictly photosynthetic system (Eugenia, 2012).

2.1.1 Biodiesel from Yeast

Yeast is perceived as highly manipulated microbes for the applications of biotechnological due to the drawbacks represent by bacteria. Bacteria keep their carbon in the form of polysaccharides and lipids in polyhydroxyalkanoates (wax ester), on the other hand, yeasts store carbon as lipids (triacylglycerides (TAG)) and glycogens. Out of the 600 recognized yeasts, not more than 30 were identified as