STUDY ON MICROALGAE GROWTH AND LIPIDS CONTENT USING WASTE WATER AS MEDIUM

BY

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ABSTRACT

Nowadays there is great and continuous increase in industrialization, infrastructure and urban- expansions, which has contributed to a significant wastes demand and water shortage due to water pollution. Industry including agro-based industry is one of the major sectors discharging great amount of waste water annually affecting the other water sources from one hand and the human life from another hand. Palm oil industry in Malaysia is producing the largest organic pollutant loads into rivers. Palm oil mill effluent (POME) is highly polluted waste, having unpleasant smell and containing high amount of pollutants. There is great need to find alternative way on how to utilize those organic pollutants for the good benefit of both human being and environment. Microalgae are unicellular, photosynthetic microorganisms having the ability of both reducing the amount of pollutants from these waste waters and giving rapid biomass growth which can be great source for many biofuels and renewable green energy. Major drawback growing these microorganisms in waste water is low amount of biomass due to deplete of the essential nutrients. This can be adjusted by supplementing the waste water with addition of the essential nutrients in order to enhance the growth. This study aims at growing microalgae source of biofuel in the biologically treated POME and identification of bioremediation role of these microorganisms. The first step of this study consisted of screening two microalgae strains namely: *Botryoccocus sudeticus* and *Chlorella vulgaris* are two microalgae species knowing by their high lipids content and biomass growth. When comparing the results *B sudeticus* seemed to be a better source for biofuel production due to higher lipids content reaching 26.6%. The same microalgae was used for evaluation of the media components for biomass production using full factorial design and optimisation of the biomass growth by using central composite design. The results showed that the presence of the organic carbon, nitrogen and phosphorus can increase the growth significantly and all of these three nutrients were having positive effects on the growth of the biomass. This biomass was optimized to reach 2.35g/l with R^2= 0.9414. The waste water used to grow the algae was characterized before and after the biomass collection and it was found that these microorganisms reduced the BOD, COD, TOC, TN and TP to 75%, 51.75%, 54.54%, 44.20%, and 57.6% respectively. The growth curve was also determined by measuring the daily optical density and the total suspended solids in the medium of the growth with and without addition of nutrients. Finally, the fatty acids in the final extracted lipids were also identified and it was found that the algae oil contains saturated fatty acids represented by caproic acid, palmitic acid and stearic acid. However the unsaturated fatty acids found were: palmitoleic acid and oleic acid. *Botryoccocus sudeticus* can be grown on biologically treated POME and its oil can be a good candidate for biodiesel production due to higher level of unsaturated fatty acids as compared to saturated fatty acids.

ملخص البحث

في الوقت الحاضر هناك زيادة كبيرة ومستمرة في التوسع إلى المناطق الحضرية,التصنيع، والبنية التحتية، والتي ساهمت في الطلب المفرط ونقص كبير في المياه بسبب التلوث. الصناعة بما في ذلك الصناعة القائمة على الزراعة هي واحدة من القطاعات الرئيسية التي تساهم في تفريغ كمية كبيرة من المياه العادمة سنويا التي تؤثر على مصادر المياه الأخرى من جهة، وحياة الإنسان من جهة أخرى. صناعة زيت النخيل في ماليزيا تنتج أكبر كميات الملوثات العضوية في الأنهار. النخيل مطحنة النفايات السائلة (التفاح) هو النفايات الملوثة للغاية، غير سامة يتميز بوجود رائحة كريهة ويحتوي على كمية عالية من الملوثات. هناك حاجة ماسة لإيجاد طريقة بديلة للعثور على كيفية استكشاف تلك الملوثات العضوية لفائدة كلا الإنسان والبيئة. الطحالب هي عبارة عن كائنات دقيقة, وحيدة الخلية, ضوئية لديها القدرة على تقليل كمية الملوثات من مياه الصرف وإعطاء النمو السريع للكتلة الحيوية التي يمكن أن تكون مصدرا كبيرا لإنتاج العديد من الوقود الحيوي والطاقة الخضراء المتجددة. العيب الرئيسي في نمو هذه الكائنات الدقيقة في مياه الصرف هو كمية قليلة من الكتلة الحيوية نظرا لنقص العناصر الغذائية الأساسية. ويمكن تعديل هذا عن طريق المكمل لمياه الصرف مع اضافة العناصر الغذائية الأساسية من أجل تعزيز النمو. تهدف هذه الدراسة إلى تنمية الطحالب التي تعد كمصد رللوقود الحيوي في مياه الصرف المعالجة بيولوجكياً وتحديد دور المعالجة البيولوجية لهذه الكائنات الحية الدقيقة. تتألف الخطوة الأولى من هذه الدراسة على فحص سلالتين من الطحالب الدقيقة وهي: Chlorella vulgaris, Botryoccocus sudeticusمعروفين بمحتوى عالي من الدهون و القدرة على نمو الكتلة الحيوي عند مقارنة النتائج بدا ان: B sudeticus هو أفضل مصدر لإنتاج الوقود الحيوي بسبب المحتوى العالي من الدهون تصل إلى 26.6٪. نفس الطحلب تم استخدامه لتقييم مكونات الغذاء لإنتاج الكتلة الحيوية وتعظيم الاستفادة من نمو الكتلة الحيوية باستخدام تصميم مركب المركزية. أظهرت النتائج أن وجود

النيتروجين والكربون العضوي والفوسفور لها آثار إيجابية على نمو الكتلة الحيوية. و يمكن أن تزيد بشكل كبير في النمو. و قد تم تحسين هذه الكتلة الحيوية لتصل إلى 2.35 g/l مع $R^2=0.9481$. حددت ميزات المياه العادمة المستخدمة لزراعة الطحالب قبل وبعد جمع الكتلة الحيوية ووجد أن هذه الكائنات الدقيقة هي قادرة على الحد منBOD، COD، TOC، TN، TP إلى75٪، 51،75٪، 54،54٪، 44،20٪، 57.6٪ على التوالي .كما تم تحديد منحنى النمو عن طريق قياس الكثافة الضوئية اليومية وجملة المواد الصلبة العالقة في متوسط النمو مع وبدون إضافة مواد مغذية. وأخيرا، تم تحديد الأحماض الدهنية في الدهون المستخرجة النهائية.تبين ان زيت الطحالب يحتوي على الأحماض الدهنية المشبعة التي يمثلها حمض الكابرويك، حمض البالمتيك وحمض الستياريك. اما بالنسبة للأحماض الدهنية غير المشبعة تمثلت في: حمض الأوليك وحمض البلميتولي. Botryoccocus sudeticus يمكن زراعتها في المياه معاملة بيولوجيا و يمكن أن يكون مرشحا جيدا لإنتاج وقود الديزل الحيوي بسبب ارتفاع مستوى الأحماض الدهنية غير المشبعة بالمقارنة مع الأحماض الدهنية المشبعة.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Master of Science in Biotechnology Engineering.

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DECLARATION

I hereby declare that this dissertation/thesis is the result of my own investigation, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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LIST OF ABBREVIATIONS

BOD	Biological oxygen demand
COD	Chemical oxygen demand
TOC	Total organic carbon
TP	Total phosphorus
TN	Total nitrogen
rpm	Rotation per minute
DOE	Department of environment
POME	Palm oil mill effluent
CG/MS	Gas chromatography mass spectroscopy
CO_2	Carbon dioxide
FAME	Fatty acids methyl ester
R^2	Regressions fit
TG	Triacylglycerols
WW	Waste water
EFB	Empty fruit bunches
g/l	Gram per liter
ml	Milliliter
MPOB	Malaysian palm oil board
ANOVA	Analysis of variance

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Nowadays, there is heavy dependence on the petroleum which poses a great threat on its sources and on the environment due to the release of carbon dioxide in the air. For this reason, there is great need to find another source which relief these environmental dangers and provide an alternative renewable energy source (Chisti, 2007). The only solution to protect our economy from the incredible rises of the petroleum price is to reduce our reliance on the petroleum in our economy.

As an alternative solution, biomass can be proposed as better and natural source of energy (Kulkarni et al., 2006). Biodiesels which are known as monoalkyl esters are famous to be substitute fuel obtained through the process of tranesterification of triglyceride oil in combination with monohydric alcohols. These substitute fuel have two major sources namely vegetable oils and animal fatty acids. The main sources of the vegetable oils can be from sunflower (Antolin et al., 2002) olive (Dorado et al., 2003), soybean (Noureddini et al., 2005), cotton (Keskin et al., 2008), hazelnut (Usta, 2005), rubber seeds (Ramadhas et al., 2005), mahua, jojoba (Canoira et al., 2006), tobacco seeds (Usta, 2009) and palm (Al-Widyan, 2002).

Recently, algae has been selected as a new and effective source of fuel production due to the fact of many features including rapid biomass production, high capacity of photosynthesis and high lipids storage content that are suitable for biodiesel production (Johnson et al., 2008). Particularly, green unicellular microalgae are known to have higher biomass yield as compared to the plant crops due to the fact that they need less land for their cultivation (Brennan and Owende, 2010; Brune et al., 2009; Chisti, 2008).

Dried algae biomass can be used as a source of energy by direct combustion. However, this process does not seem to be attractive due to some disadvantages. More than that, the biomass can be converted to liquid fuel through a wide range of thermochemical reactions (Rittmann, 2008). Those reactions can include gasification, pyrolysis, hydrogenation and liquefaction of algae biomass to oil based biofuels (McKendry, 2002; Miao and Wu, 2004), another type of reaction named by biochemical conversion process. As an example: fermentation and aerobic digestion of biomass to produce bioethanol or methane (McKendry, 2002). In the same context, hydrogen can also be generated from algae through bio-photolysis (Melis, 2002). Finally, triglycerol lipids can be extracted from the harvested biomass that can be used to produce biodiesel by tranesterification process (Chisti, 2007; Hu et al., 2008; Miao and Wu, 2006; Brennan and Owende, 2010).

According to (Sheehan et al., 1998), the Department of Energy (US) analysed the total lipids productivity from many algae strains and compared it to the oil production capabilities of many oil seeds crops like soybean. The results showed that the algae have higher lipids yield production. This research was used as an insight in order to determine the microalgae strains which have great lipids amount. Moreover, identifying the growth cultivating factors can improve the lipids productivity (Hu et al., 2008). Several studies have proposed many ways on how to enhance the lipids productivity through manipulating the culture growth conditions. As an example of them is nutrient stress for the essential nutrients limitation (Converti et al., 2009; Li et al., 2008). A major down side of this method is the low biomass productivity. In other words, focusing on increasing the biomass productivity may be better solution on how to improve the energy production from microalgae (Griffiths and Harrison, 2009).

Another major advantage of microalgae cultivation is its ability to provide environmentally friendly technology through the combination of its growth with the treatment of waste water (de la Noue et al., 1992; Green et al., 1995; Oswald et al., 1957). This is due to the high amount of nutrients available in waste waters particularly the total N and total P concentrations in combination with the toxic matters which need more expensive chemical treatments (Gasperi et al., 2008). This feature makes the microalgae as sustainable candidates for low cost waste water treatment (Hoffmann, 1998; Mallick, 2002). Many microalgae strains have been tested for nutrients removal from waste water and they show a major positive effect in this field. However, this technology still used on an insignificant scale such as conventional oxidation (stabilization) ponds or the more developed suspended algal pond systems such as high-rate algal ponds which are shallow raceway-type oxidation ponds with mechanical mixing, and have been shown to be highly effective for wastewater treatment (Green et al., 1995; Hoffmann, 1998).

The main purpose of wastewater treatment is the reduction of high concentrations of pollutants specifically, N and P which can be dangerous to the environment due to the risks of eutrophication if these nutrients gather in the surface waters. The phosphorus can be more dangerous due to its difficulty of removing form the wastewater and in the industrial wastewater. Many research works have been conducted under wastewater conditions; mainly grown in agricultural manure waste water and municipal sewage waste water. All these studies showed the potential ability of algae removing N and P and in some cases metals. These primary works may be more beneficial if improving the biomass produced and the methods of biomass collection (Jon et al., 2011).

The main difference between the waste water media and other growth media is the concentration of the nutrients which can be lower or higher than the required media. These parameters will definitely vary from one wastewater treatment site to another and depending on the wastewater type (Jon et al., 2011). Besides, there was dissimilarity in the capacity of different algal species to bear with a particular wastewater state. Particularly, unicellular chlorophytic microalgae like *Chlorella* and *Botryoccocus* shows effective capacities of accumulating nutrients from waste water (Ruiz-Marin et al., 2010).

In Malaysia, the major sector leading to environmental pollution is the Palm oil industry; the country is considered to be a leader in the palm oil industry, producing 17.56 million tons of crude palm oil per year representing 15.87 tonnes of export (MPOB, 2009). A great amount of palm oil mill effluent generated from this industry reaching up to 15 million tonnes. This can be as major threat for public health.

Currently, the majority of palm oil mills in Malaysia have conventional methods of treatment using anaerobic and facultative digestion in pounds. These systems need large space of land and long treatment process (80 to 120 days). Furthermore, the microorganisms used need severe care (Ahmed and Chan, 2009). High organic matters are still available after being processed with the biological treatment. As results, it is necessary to find a way on how to reduce these nutrients for the good benefit of the environment and the public health.

1.2 PROBLEM STATEMENT AND RESEARCH SIGNIFICANCE

Today many problems are faced due to global warming because of the rise in the amount of CO_2 released to the air by industrial sectors. . This phenomenon may lead to major environmental problems like increase in the average surface temperature of the planet, which may be the reason of the modification in precipitation pattern, rise in level of oceans and accelerated glacial melting, etc. Furthermore, the shocking price of petroleum is now a great encouraging factor to reduce the dependence on its economy and to create alternative, sustainable and renewable source of energy.

Among the industrial sectors releasing huge amount of pollutants in Malaysia is the palm oil industry contributing with largest amounts of effluents emerging from the water used during the extraction of crude palm oil from the fresh fruit bunch. An average of 0.1 tonnes of raw POME is discharged for every tonne of fresh fruit bunch processed. It cannot be released unless it is treated due to an acidic characteristic and high amount of BOD (Ahmad et al., 2003). Over many years, the main treatment technology used for treating such a waste was anaerobic and facultative digestion (Biological method). Yet, this method is considered to be ineffective and the final liquid resulting is still containing high content of organic matters.

Presently, scientists keep trying to find the best source of biomass that can generate energy. Algae due to many characteristics such as rapid biomass production, high photosynthetic efficiency and ability to store a large amount of lipids (Xiong et al., 2010) are ideal source of biodiesel, This can be a solution to reduce the competition for agricultural land (Johnson et al., 2008). The major constituent of algae biomass is significant amounts of proteins (Becker., 2007) and the basic structure of these proteins is nitrogen (N) which is required as fertilizer, meaning that microalgae growth require large amount of fertilizers. Consequently, the use of these fertilizers may increase the cost of the process production in one hand and contribute to other environmental negative impacts from on the other hand (Sialve et al., 2009).

The combination of algae cultivation with waste waters can be a solution for many factors including: reducing the amount of waters used for the algae cultivation, reducing the pollutants load, providing the nutrients necessary for the growth of the biomass and serving as alternative solution for the waste waters treatment. Hence, the main objectives of this study are to identify the algae strain having the highest lipids content, investigating the growth nutrient limiting factor for the growth, identifying the optimum concentrations of wastewater nutrients for the selected strain grown in POME partially treated as medium, and to characterize the final lipids content of the biomass obtained from this cultivation. This study is also to determine the nutrients removal from the wastewater after the biomass harvesting. It is contributing with new theories in the field of microalgae biomass cultivation and the biodiesel production. This is considered to be safer source of energy, with preventive properties and without deliberating consequences to the environment.

1.3 RESEARCH OBJECTIVES

The main objective of this research is to produce a microalgae biomass from waste water, as the renewable raw material for biofuel production. Furthermore, this study is also to determine the different changes occurred in the wastewater after the biomass harvesting. The specific objectives based on the reasons discussed are as follow:

1. To characterize the POME before and after the cultivation of microalgae
2. To select the suitable strain for oil production and optimize the production of the biomass.
3. To extract the lipids and to identify the different fatty acids.

1.4 RESEARCH METHODOLOGY

The research was laboratory-based experimental works in a small scale photo bioreactor. The research has started with the screening of the strain for the highest lipids content by using Bold Basal Medium than transferring it to a synthetic medium based on the literature survey.

The characterization of the partially treated POME before starting the fermentation and after harvesting the biomass was based on the standard methods for water and waste water analysis by using Hach spectrophotometer available in the biotechnology environmental laboratory. For the next step, the selected microalgae strain was used in to identify the nutrients limiting factor based on screening. According to the results the significant nutrients were optimized by following Central Composite design given by DOE software.

Finally, the results from the optimization part were validated and the identification of fatty acids constituting the final lipid was done and the findings were compared to the literature.

1.5 SCOPE OF THE RESEARCH

This project was to study the growth parameters of microalgae grown in partially treated POME as medium and to address the optimum level of biomass used as potential source of biodiesel. In the cultivation process, first we select the suitable microalgae strain containing high lipids content, and then we characterize the different constituents of the POME partially treated. The essential nutrients for microalgae growth (C, N, and P) was optimized for the maximum algae biomass. The algae growth was measured daily by using spectrophotometer at 600nm, and the optimization part the central composite design was used. Moreover, there was characterization of the waste water used before the experiment and at the end after collecting the biomass by following Standards methods for the characterization of water and waste water. Finally, the lipids extraction method was done by using organic solvent (hexane). These lipids were characterized further by following oil and fats analysis methods including CG/MS to determine the different fatty acids contained in the resultant algae oil. It is expected that by these method and analysis will indicate the suitability of wastewater as medium for the cultivation of microalgae as a source of biodiesel.

1.6 THESIS OUTLINE

This thesis contains five chapters. Chapter one is an introduction and background of the research work. Chapter two presents literature survey and reviews related to the algae fuel, different factors affecting the growth, an overview on the characteristics of the two strains used in our work, the role of Palm oil industry in Malaysia and the process proposed for the treatment of the POME, currents studies and research done in the same field of our study, etc. Chapter three summarizes the detail of our methodology used in our work including the materials, equipment, the consumable items and the analytical and the experimental procedures followed. All the results obtained from our experimental work are well presented in chapter four followed by discussion based on the findings obtained and based on the results investigated by different scientific works related to our work. Finally this thesis is concluded by chapter five in combination with some recommendations for further studies.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

Different research studies in relation with the current study have been reviewed in this section. Chemical and physical character of the wastewater used in the same purpose, the prospects of using the waste water as culture medium for the algae growth and its growing conditions, essential factors to succeed the growth, and the potential strains which give great biomass yield as well as high lipids content were also discussed. Overview of the current algae fuel that can be converted from its biomass, and the process of converting the algae biomass to biodiesel were also presented in this chapter. Other than the above mentioned basic topics on microalgae applications, different cultivation system and other studies done by other researchers have been also illustrated.

2.2 MACRO AND MICROALGAE

Macro-algae or often called as seaweeds are multi cellular plants known by their ability of fast growing in salt of fresh water (Mc Hugh, 2003). Based on the color of pigmentation, they can be classified into brown, red and green seaweeds. They are mainly used for the production of food and the extraction of hydrocolloids (Anders et al., 2007).

Macro-algae can absorb the maximum available photons and grow on rocky substrates from stable multi-layered. This will help them to get a maximum productivity which can reach 10 times higher than plankton population (Lüning and

Pang, 2003). Among the two hundred species of macro-algae that are worldwide used, only ten species only are intensively cultivated. This is due to the fact that many species have specific requirement for their living environment (Lüning and Pang, 2003). Many Asian countries like China, the Philippines, North and South Korea, Japan and Indonesia, the USA, Canada and European countries such as France, Germany and the Netherlands are attempting to establish large-scale seaweed cultivation in order to convert it to value added products such as food, feed, chemicals, cosmetics and pharmaceutical products (Pérez 1997; Buck and Buchholz 2004; Reith et al., 2005).

Microalgae are prokaryotic or eukaryotic, unicellular or simple multicellular structured photosynthetic organisms, which can grow rapidly in suspension and live in difficult conditions (Li et al., 2008). Microalgae can be classified based on their abundance to three important classes which are diatoms (*Bacillariophyceae*), green algae (*Chlorophyceae*), and golden algae (*Chrysophyceae*) (Anders et al., 2007).

According to Richmond (2004), the total of microalgae species can reach 50,000. However, only 30,000 species have been studied and analyzed, He also stated that Microalgae are present in all existing earth ecosystems, not just aquatic but also terrestrial, representing a big variety of species living in a wide range of environmental conditions.

Microalgae have the ability of producing useful products such as foods, feeds, high-value bio-actives and potential biodiesel by the use of sunlight and carbon dioxide (Shimizu, 2003; Metzger and Largeau, 2005; Singh et al., 2005; Spolaore et al., 2006; Walter et al., 2005). For instance, the fresh water green algae *Haematococcus pluvialis* which is commercially important as a source for astaxanthin, *Chlorella vulgaris* as a supplementary food product, and the halophilic