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Isolation and screening of microalgae from agro-industrial wastewater (POME) for biomass and biodiesel sources

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ABSTRACT

Indigenous microalgae (KR349061) were isolated from the agro-industrial wastewater and screened in order to determine their potential for biomass and biodiesel sources. Bold's Basal Medium was used to isolate the colonies from sample. The nutrient contents were altered in order to optimize the growth of the microalgae. The isolates were identified based on the morphology and microscopic appearance of the isolated colonies. It was seen that the isolates comprised of several common green microalgae and cyanobacteria, among which the ideal candidate was selected and further investigated. Lipid content was determined for the strains that showed rapid growth. The results showed that *Chlamydomonas incerta* achieved highest lipid content of biomass (42.6%) after 2 d, also highest lipid productivity of 0.197 mg/L/d was observed when the initial COD concentration was 500 mg/L. *C. incerta* also showed higher growth rate and higher amount of lipid was examined for its potential as sustainable biodiesel feedstock along with its higher biomass yield.

Keywords: Microalgae; *Chlamydomonas incerta*; POME; Lipid; Biodiesel

1. Introduction

Microalgae have the potential to be the main source of biodiesel, which has the potential to replace fossil fuel [1,2]. It has also been proven to be one of the promising resources for producing biodiesel. Mainly because the use of microalgae to produce biodiesel will not compromise the production of products like fodder, food, and others derived from the crops [1,3]. The most important parameters in regulating algal growth by means of photosynthesis are the environmental conditions like light intensity, pH, temperature, turbulence, salinity [4,5], and nutrients [4].

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The range of optimal conditions as well as the tolerable range of operating conditions are species specific and various factors may be interdependent to each other [5]. The potential aspect of microalgae when compared to other advanced feedstock is that the variable number of species that are available for the production of biofuel [6]. Different species of microalgae can be selected to optimize its production for making biofuels. Microalgae, also provide a wide range of valuable by-products like omega-3 fatty acids, food, animal feed, nutritional compounds, jet fuel, bioethanol, biodiesel, medicines, fertilizers according to [7].

Widjaja et al. [8] have reported that many types of microalgal strains capable of producing a higher amount of lipid content have been screened and characterized, most of them belonging to the marine microalgae group. Microalgae produce many different types of lipids, hydrocarbons, and other complex oils which are mostly based on the species specificity [1]. Microalgae can utilize organic carbon sources (glucose, acetate, fructose, and others) and inorganic carbon (CO_2) for producing lipid [3]. Whereas, the quality and quantity of lipids produced inside the cell varies according to the growth conditions like temperature and light intensity or based on the nutrient content and its concentration such as iron, nitrogen, and phosphates [9,10]. The analysis of lipid from the sample traditionally is based on gravimetric determination and solvent extraction. The further characterization of lipid is performed using conventional techniques such as the HPLC or GC [11]. Normally, the lipid classes are divided into polar lipids (galactolipids, phospholipids) and neutral lipids (cholesterol, triglycerides). It is also known that triglycerides are the main sources for the production of biodiesel [3,12].

More than 30 microalgal strains have been screened for its potential of lipid production by determining the biomass productivity and lipid content [2,13]. The life cycle assessment on microalgae cultivation has shown that most of the energy use and emission of greenhouse gases are associated with fertilizer (nutrient) production [14]. However, the culturing of microalgae on a larger scale depends on the presence of huge amount of nitrogen and other chemical fertilizers, which drives the practice toward being non-ecofriendly in nature. At the same time, culturing of microalgae also plays an important role as for the purification of wastewater [15,16]. Nowadays, microalgae are getting considerable attention in terms of feedstock for the production of biodiesel. The aim of the research was to isolate and cultivate *Chlamydomonas incerta* from the sample containing Palm Oil Mill Effluent (POME) and to assess its impact using different concentrations of POME as a substrate for the growth of microalgae to produce more lipids, which was quantified using Nile red (NR) method.

2. Materials and methods

2.1. Sample collection

Water samples with visible microalgal population were collected from the facultative ponds and algae ponds located at Kahang, Johor, Malaysia before it is released into the river. Collections were carried out for the top and bottom layer of water at each location with the aim of obtaining the dominant microalgal species present in that particular area. Additional samples were obtained from the water bodies specifically located adjacent to the algal ponds. All the field samples were collected in sterile 50-mL tubes and maintained in refrigerator after transferring it to the laboratory.

2.2. Isolation of microalgae

In order to isolate a single microalgal species from the water sample, standard plating method was used in order to separate the mixed algal population. Different types of nutrients were used to isolate the colonies. The obtained field sample was first serial diluted as to aid the isolation process. Sterile petri plates containing approximately 15–20 mL of agar medium was used to plate these diluted samples. One millilitre of the diluted sample was transferred to the media plate and spread evenly across the surface. Inoculated plates were placed in a temperature-controlled greenhouse (20–25˚C), where the algae were allowed to grow for 14 d. Grown algal culture was streaked using sterile technique onto nutrient agar plates and placed back in the greenhouse for isolation. This streaking method was repeated until pure algal culture was obtained. The number of colonies that were transferred from each dilution plate onto other nutrient media plate depends on the amount of contamination and the identification of the present colonies, based on the colony morphology and the microscopic observation of each isolate. Following the isolation of individual microalgal colonies, each strain was labeled based on the sampling location and different nutrient requirements. The isolated algae were maintained as stock culture and maintained by re-plating each onto new nutrient media at least once a month or more frequently, depending on the nature of each isolated strain. The specifications of POME used in this study are shown in Table 1.

Table 1
Characteristic of the sampled POME

No	Parameter[a]	Concentration range (mg/L)	Average (mg/L)
1	pH	4.15–4.4.5	4.25
2	COD	1,350–2,120	1,600
3	Soluble COD	20,500–24,500	22,000
4	BOD	300–400	330
5	Total volatile solid	27,300–30,150	28,100
6	Total suspended solid	15,660–23,560	18,900
7	Total phosphorus	200–600	350
8	Total nitrogen	500–800	500

[a]All parameters are in units of mg/L except pH.

2.3. Media preparation

Microalgae were isolated from pond and cultured in modified Bold's Basal Medium (BBM) along with control. This medium was found to be effective for the growth of isolated microalgal strain. The media solution was sterilized by autoclaving at 121˚C for 15 min in order to eliminate the possibility of contamination. The pH of the medium was adjusted to 6.8 before autoclaving [17]. The microalgal culture was maintained in liquid medium and also for its maintenance as it is similar to the growth medium (modified BBM). Two millilitre of microalgal culture was inoculated into sterile conical flasks containing 18 mL of the culture medium. Now the flasks were incubated in the presence of light (100 μmol/s/m^2) at room temperature. Sterile BBM in presence of different types of vitamin was used for isolating maximum number of colonies from each of the taken sample. These nutrient contents were also modified for optimizing the isolation and growth of each colony [18].

2.4. Morphological identification

Microalgal cultures and contaminations were initially separated based on the morphological examination of colonies on the nutrient agar medium. The identification of the isolates was carried out based on the morphology of the individual cells under microscope. The strains were identified according to the method used by Wehr and Sheath [19]. Each isolate colony was labeled and photographed at magnifications (20×, 40×, 60×) using Canon PAXit microscope equipped (with DXM1200 digital camera and ACT-1 software program). A catalog of these isolates was

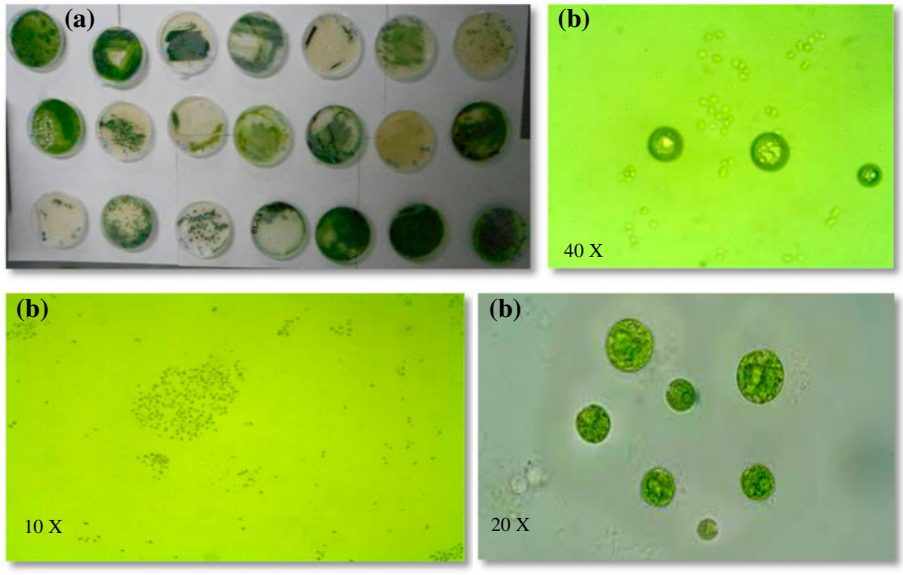

Fig. 1. (a) Isolation of microalgae from POME (b) microscopic observation of *C. incerta* under light microscope.

Fig. 2. *C. incerta* cultivation under different COD concentrations.
Notes: *a* = 0 mg/L, *b* = 250 mg/L, *c* = 500 mg/L, and *d* = 1,000 mg/L.

established for future reference. Photographic comparison of the original isolates along with the stock cultures was performed periodically as to ensure that contamination had not occurred.

2.5. Cultivation and harvesting

Preliminary study was done through the cultivation of microalgae *C. incerta* in POME with different concentrations at 0, 250, 500, and 1,000 (mg COD/L).The sample containing microalgal culture was monitored every day for its growth rate by measuring the optical density at 620 nm. The cultures were aerated using air pump and the sterile-specified media was added to each culture at the end of each week. Constant mixing of the algal culture in 2-L flask was provided by means of aeration. The temperature of the algal culture in the flask was found to be between 24 and 33°C. Harvesting of the microalgae was carried out using the centrifugation process [16].

2.6. NR staining for the determination of lipid

One millilitre of culture was taken and centrifuged at 2,000 rpm for 2 min [20]. Now, the supernatant was discarded and the pellet obtained was washed using phosphate buffer at pH 7.0. Further, 1 mL of phosphate buffer was added to the washed pellet in order to be used as stock solution. After that, 1 mL of the cell suspension diluted to 100 × 2.8 mL of the diluted cell suspension was transferred to a 5-mL cuvette. 1.8 µL of NR solution was added to the sample and it was vortexed well for 5 min. Now it was left under dark condition for 20 min before taking the reading of fluorescence intensity with chemiluminescence spectrophotometer [12].

3. Results and discussion

3.1. Isolation and identification

The samples obtained from the algal ponds located in Kahang, Johor, Malaysia were cultured in wastewater containing POME and allowed to grow by exposing it to direct sunlight. The characteristics and morphological features of the isolates showed similarity with *C. incerta*. The individual cells of the colony were found to be in the range of 10 µm, greenish, unicellular, and spherical in shape (Fig. 1).

3.2. Selection of suitable microalgal concentration

C. incerta during its exponential period was inoculated (2%, v/v) in the liquid medium.

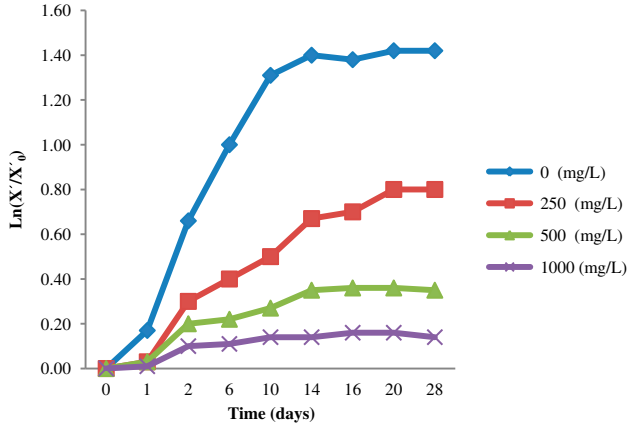

Fig. 3. Growth rate of *C. incerta* under different initial substrate concentrations of POME.

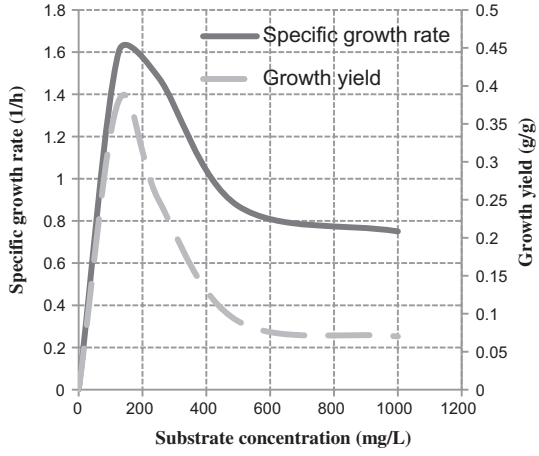

Fig. 4. Effect of COD concentration on the growth of *C. incerta*.

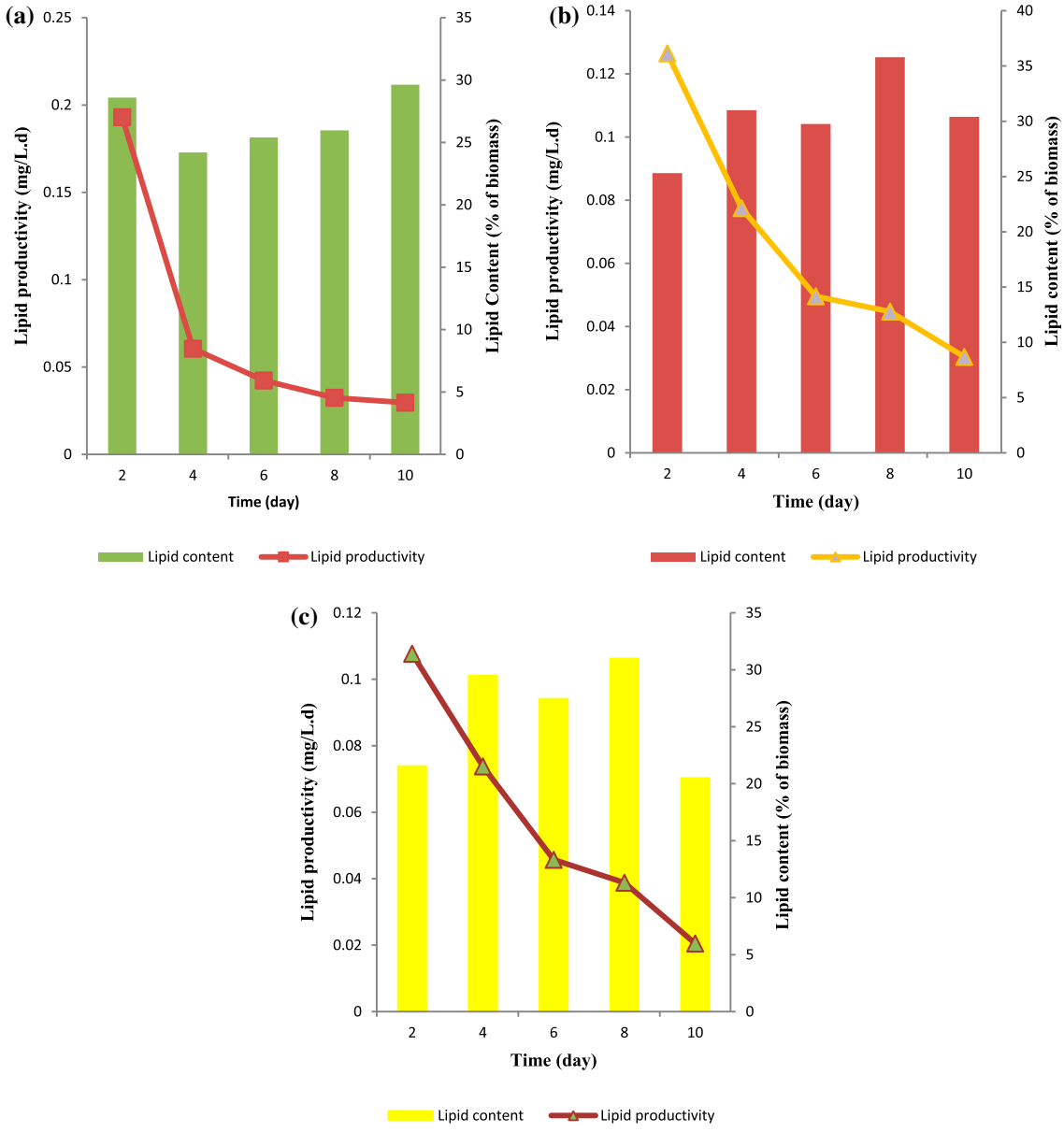

Fig. 5. Lipid productivity and lipid content (% of biomass) of *C. incerta* under different COD concentrations. (a) POME COD of 250 mg/L, (b) POME COD of 500 mg/L, and (c) POME COD of 1,000 mg/L.

The cultivation of *C. incerta* was initially carried out in a 250-mL conical flask containing 100 mL of liquid medium. POME used during the batch experiment was previously allowed to settle for 1 h and further it was diluted with BBM at the concentration of (250, 500, and 1,000 mg/L), also in the absence of POME to serve as control for the experiment (0 mg/L), as shown in Fig. 2. The substrate concentration was chosen based on the similar type of research conducted in the previous studies [4,21]. *C. incerta* was grown at room temperature under continuous illumination with the intensity of 1,033 Lux or ±14 μmol/m^2/s for 28 d. After that 1 mL of chloramphenicol (30 μg/mL) was added to each medium consisting of POME as to ensure that the culture will be free from contamination.

3.3. Optimization of microalgal growth rate using POME

It is seen from Fig. 3, cells obtained higher growth rate in presence of 250 mg/L POME, lower growth rate was observed with 1,000 mg/L of POME and

intermediate growth rate was found in presence of 500 mg/L POME. Variation in Napierian logarithm of (X´/X´0) ratio with the operation time for differential substrate concentrations is also shown in Fig. 3. X´ represents the growth of microalgal concentration (absorbance) during the experiment and X´0 represents the value of growth concentration (absorbance) at the beginning of the experiments It also clearly indicates that 250 mg/L is the most suitable substrate concentration needed for the growth of *C. incerta* growth, when compared to other concentrations, except for the substrate concentration of 0 mg/L.

3.4. Effect of POME on microalgal growth rate

The results obtained from Fig. 4 show that specific growth rate was found to decrease along with the increasing substrate concentration. This indicates that substrates can serve as inhibitors at higher concentration. The presence of organic substances may directly turn out to be the essential organic nutrient or can act as the accessory growth factor [22]. Growth was found to be slower with apparent COD inhibition at the concentration above 500 mg/L, which greatly affected the efficiency of biomass production.

3.5. Lipid production

The formation of lipid content is dependent on several factors like pH, substrate composition, light intensity according to the findings of [9,23,24]. In this study, in considering the different factors used for the production of lipid, *C. incerta* achieved highest lipid content (42.6%) of biomass after 2 d, also highest lipid productivity of 0.197 mg/L/d was obtained when the initial COD concentration was 500 mg/L (Fig. 5).

3.6. Effect of organic loading rate on biomass and lipid production using C. incerta in POME

The growth of *C. incerta* using POME was carried out using different organic loading rates ranging from 36 to 96 kg COD/m^3 d for 14 d. The obtained values were a result of regulating the influent flow rate. The biomass productivity and specific growth rate in all of the diluted POME wastewater sample was found to increase along with the increasing organic loading rate as shown in Fig. 6. It also shows that decrease in the lipid productivity of *C. incerta* at the end of the experiment was obtained with the organic loading rate (R^2 = 0.954), which in turn provides a good correlation between the biomass production (R^2 = 0.9421) and lipid production.

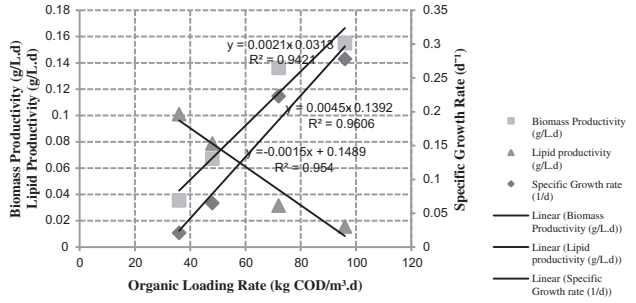

Fig. 6. Specific growth rate (μ), biomass productivity ($P_{biomass}$), and lipid productivity (P_{Lipid}) of *C. incerta* using different organic loading rates in diluted POME.

3.7. Effect of various concentrations of POME on cell density and lipid content of C. incerta

Four different concentrations of POME were tested: 0, 250, 500, and 1,000 mg/L. The readings were taken for lipid content and cell density (OD) at 620 nm. Whereas, the lipid content was analyzed using NR method.

It is seen from Fig. 7, that lowest lipid content was observed when the cells were cultivated in 500 mg/L of POME concentration. It can be concluded that microalgal cells do not make lipid as a storage product to survive. Instead, they can use these products as a result of protein biosynthesis for energy storage [3]. Therefore, the lipid content was found to be decreased.

Almost all of the microalgal cells showed decrease in the production of lipid from the initial day to until the 15th day. It was noticed that nutrient starvation increased the lipid content. However, the decrease in lipid content may be also due to the presence of more dead cells because the NR method used was unable to stain the cells. Therefore, lower observation of lipid content shows that the accuracy of the method can be affected due to the viability of cell [11]. Also, different sizes and shapes of cells contribute for the lower production of lipid [20]. The increase in biomass concentration does not always increase the storage of lipid

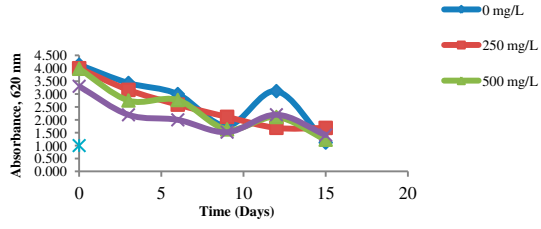

Fig. 7. Lipid content using different concentrations of POME.

content in the microalgal cell [25]. However, for the microalgae cells that were cultivated in 250 mg/L of POME concentration, the production of lipid was found to be much lower as reported by [20]. This clearly indicates the occurrence of independent relationship between the lipid content and biomass concentration in the microalgal cells.

4. Conclusion

The local habitat and weather are the major factors which determine the adequate growth of microalgal strains in the established area. In spite of having higher lipid content, microalgae cannot be used for the production of biodiesel if it does not grow well in the predetermined location or area. Moreover, local species have adapted for a longer time to the prevailing regional abiotic and biotic factors, which largely contribute to the higher production of biomass and lipid production. Throughout this research it is clearly seen that the local species like *C. incerta* have found the scope to be successfully used for the production of biodiesel feedstock at a large scale. The results of this study indicate that *C. incerta* is valuable for biofuel production. The finding also indicates that the cells are having higher growth rate in 250 mg/L of POME and is found to be suitable for the growth of *C. incerta* when compared to other concentrations. Moreover, COD concentration above 500 mg/L greatly affected biomass production. Also, *C. incerta* obtained highest lipid content of 42.6% after 2 d, as well as the highest lipid productivity of 0.197 mg/L/d was obtained when the initial COD concentration was 500 mg/L. It was found that the biomass productivity and specific growth rates in all of the diluted POME increased along with the increase in organic loading rate. This shows the occurrence of independent relationship between the biomass concentration and lipid content in the microalgal cells. It can be concluded that *C. incerta* has a greater potential for the production of biodiesel due to its faster growth and higher lipid production when compared to other strains.

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