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Regular article

# Cultivation of micro-algae for Production of Biodiesel: An optimized Process

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**Microalgae are considered as one of the potential source of biodiesel for the future. The search to obtain the potential strains from the algal diversity capable of producing oil is critical for sustainable production of biodiesel. In the present study, microalgae biomass with oil/lipid accumulation capability and their morphological features was isolated from Lake Abaya and Chamo. The algal biomass was cultivated *in vitro* and media optimization for maximum biomass was done using different basal media, BG-11 medium, and Chu -10. In addition the various carbon sources, nitrogen sources, pH and temperature were considered in this study for optimization. Green algae *Oedogonium*, *Chlorella* and *Cladophora* species were observed to be dominant species and the maximum oil per dry algal biomass was found to be from *Oedogonium* sp. Thus from the present study for the cultivation of the selected algae, BG-11 medium supplemented with tryptone (0.2%) sucrose (2%) and pH- 6 with incubation temperature of 30$^0$C was found to be suitable. These results suggest that *Oedogonium* sp. has several desirable features that make it a potential candidate for biodiesel production.**

**Key words:** Microalgae, basal media, biodiesel production, algal biomass, *Oedogonium*.

The transportation and energy sectors are the major anthropogenic sources, responsible in European Union (EU) for more than 20% and 60% of green house gas emissions, respectively (EEA, 2004). [illegible] source, representing about 9% of green house gas emissions, where the most important gases are nitrous oxide and methane (EEA, 2007). It is expected that with the development of new growing economies, such as India and China, the global consumption of energy will raise and lead to more environmental damage (IEA, 2007). Green house gas contributes not only to global warming but also to other impacts on the environment and human life. Oceans absorb approximately one-third of the CO2 emitted each year by human activities and as its levels increase in the atmosphere, the [illegible] will also increase [illegible] gradually to more acidic. This pH decrease may cause the quick loss of coral reefs and of marine ecosystem biodiversity with huge implications in ocean life and consequently in earth life (Ormerod *et al.*, 2002).

In marine ecosystem, algae assume the role of plants and most belongs to the phytoplankton, both in terms of biomass and

diversity. Among the algae, more than 50,000 of microalgae species exist today, but only 30,000 of approximately 50% are identified as global primary potential species for the biodiesel production (Falkowski *et al.*, 1998; Rajvanshi and Sharma, 2012).

Many research reports and articles described many advantages of using microalgae for biodiesel production in comparison with other available feedstock (Tsukahara and Sawayama, 2005; Chisti, 2007; Rosenberg *et al.*, 2008; Schenk *et al.*, 2008; Li *et al.*, 2008a; Li *et al.*, 2008b; Hossain *et al.*, 2008; Hu *et al.*, 2008; Rodolfi *et al.*, 2009; Georgianna and Mayfield, 2012; Wu *et al.*, 2012; Menetrez, 2012; Kumar and Sharma, 2014). From practical point of view, they are easy to cultivate, can grow with little or even no attention, using water unsuitable for human consumption and easy to obtain nutrients.

Microalgae reproduce themselves using photosynthesis to convert sun energy into chemical energy, completing an entire growth cycle every few days (Sheehan *et al.*, 1998). Moreover they can grow almost anywhere, requiring sunlight and some simple nutrients, although the growth rates can be accelerated by the addition of specific nutrients and sufficient aeration (Aslan and Kapdan, 2006; Pratoomyot *et al.*, 2005; Renaud *et al.*, 1999).

Different microalgae species can be adapted to live in a variety of environmental conditions. Thus, it is possible to identify the species best suited to local environments or specific growth characteristics, which is not possible to do with other current biodiesel feedstock (e.g. soybean, rapeseed, sunflower and palm oil). They have much higher growth rates and productivity when compared to conventional forestry, agricultural crops, and other aquatic plants, requiring much less land area than other biodiesel feedstock of agricultural origin, up to 49 or 132 times less when compared to rapeseed or soybean crops, for a 30% (w/w) of oil content in algae biomass (Chisti, 2007). Harvesting of microalgal biomass is usually difficult due to its small size (2-20 µm) and it constitutes a major part of the cost of algae production of 20-30 % (Chen *et al.*, 2011; Suali and Sarbatly, 2012). Therefore, the competition for arable soil with other crops, in particular for human consumption, is greatly reduced.

Naturally, algae can grow extremely rapidly unlike terrestrial sources. Ethiopia in general and the Arba Minch area in particular, has precious aquatic biodiversity representing a valuable resource for the development of an indigenous biodiesel feedstock. Isolation of such oleaginous microalgae is critical step in developing oil-rich strains for the production of biodiesel feedstock. Hence, this study describes the outcomes of experiments carried out to optimize the process parameters using appropriate statistical tools for media optimization and select potential algal strain as a potential biodiesel feedstock.

## Materials and Methods

### Sample collection site:

Arba Minch is found in Southern Nations, Nationalities, and Peoples' Region (SNNPR). It located at 30°56'N of the equator and 37°44'E with surface area of 2184 hectares of land with altitude ranged from 1200-1400 masl with average temperature 30.6°C, annual rainfall 575mm, situated in 505 km away towards south of Addis Ababa in Great Rift Valley of lake Abaya and Chamo. Water samples used to isolate microalgae were collected from different sites that appeared to contain algal growth in a freshwater lake, Abaya and Chamo, Arba Minch **(Figure 1)**.

### Isolation and purification of microalgae

Bold basal medium (BBM) was used in this study for first phase screening. The media was autoclaved for 20 min before use and the antibiotics was added after filtering through 22µl filter, 0.2ml of serially diluted

water sample was inoculated into 20 mL media on a petri plate containing BBM solidified with 1.5% (w/v) of bacteriological agar, and then incubated at 27°C for two weeks.


**Figure 1:** Locations of sample collection areas from the Greater Rift Valley lakes of Abaya and Chamo. Site 1: Arba Minch Airport area; Site 2: Merab area; Site 3: Shele mazoria area; Site 4: Nechsar National Park area. Source: Edited from Google Map 2015. Further details are given in Table 2.

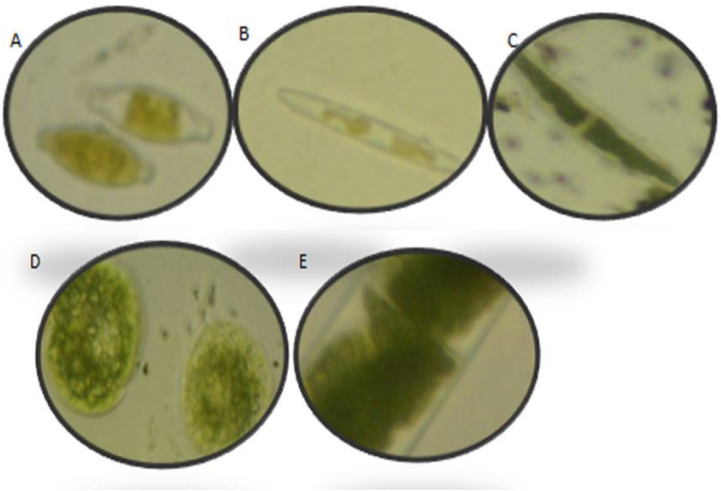


**Figure 2:** Microscopic images of some common algal species found from the sample collection areas of site 1 to 4. A. Diatoms sp., B. *Cymbella* sp., C. *Cladophoro* sp., D. *Chlorella* sp,. E. *Oedogonium* sp. Further details are given in Table 2.

Every two days, the flasks were examined for algal growth using an optical microscope. Then the colony grown was transferred to liquid BBM media (Bischoff and Bold, 1963). 50 mL BBM in a 250 mL Erlenmeyer flask was incubated at 27°C with

shaking at 150 rpm for three weeks. Algal growth was monitored by measuring daily changes in optical density at 680 nm with a spectrophotometer. Then after the 1ml sample was taken for identification. The purities of the culture were ensured by repeated plating and regular observation under a microscope. Microscopic identification was performed **(Figure 2)**. The purified algal samples were observed under microscope and the morphological properties of the isolates were identified based on the manuals (Benson, 1998; Janse van Vuuren *et al*., 2006).

**Media composition and culture environment**

Different basal media, BG-11 medium (Allen and Stanier, 1968), and Chu #10 Medium (Chu, 1942) were used for cultivation of the algae. In addition, these basal media were supplemented with 3% sucrose and 1.5-2.0% bacteriological agar for solid phase culture system and the pH of the media was adjusted to 5.8±0.2 before autoclaving. All chemicals were purchased from Hi Media, India unless and otherwise mentioned.

**Determination of growth kinetics parameters**

*Settled cell volume (SCV) and Packed cell volume (PCV)*

Both parameters were chosen for fast estimation of culture growth *in vitro*. Settled cell volume was determined by allowing 10ml of cell suspension to sediment in pre-autoclaved 15ml graduated conical centrifuge tubes every half an hour interval for 4hrs. It was calculated as the percentage of the volume of sediment cell mass to total volume of suspension. On the other hand, the PCV were determined in terms of the volume of packed cell after centrifuging at 1000 rpm for 10 min to the total volume of the suspension. Thereafter, the culture collected back to respective flasks aseptically.

*Fresh cell weight (FCW) and Dry cell weight (DCW)*

The manipulation of samples was performed in non-sterile conditions taking 3ml of the sample every day for a week and every four days for a month. Fresh cell weights were estimated collecting the cell suspension in a laminar hood using 3ml eppendorf tube and repeatedly washing the cell pack with about same volume of distilled water. Microalgae cells were harvested by centrifugation and washed twice with deionizer water. The FCW were determined thereafter. Microalgae pellets were dried overnight at $105^0$C for dry weight measurement (Takagi *et al*., 2006). Microalgae dry weight was measured according to a method previously reported. Experiments were carried out in triplicate, and data are expressed as mean SD.

**Methods for oil extraction**

Extraction of total lipids from the algal biomass was performed according to the modified procedure of Bligh and Dyer, (1959). Briefly, the microalgal cells were harvested by centrifugation and washed with double distilled water and the lipids were exhaustively extracted with hexane, filtered and concentrated by rotator evaporator (Buchan, Swiss) at $50^0$C. For phase separation 20ml 0.5% NaCl solution was added and gently shaken and kept until a clear separations observed. The NaCl solution was added to prevent the formation of a stable emulsion. The organic layer was successfully taken and concentrated using once again in rotary evaporator.

**Replications and Statistical Analysis**

All experiments were repeated 3 to 5 times on different days. The average values ±SE are presented. Statistical analysis of the data was done using the software Sigma plot (version 10.0).

## Results

### Cell Cultivation and Culture Maintenance

*The effect of Media on the biomass*

In the present study, the growth kinetics parameters were considered such as fresh cell weight, dry cell weight, settled cell volume and packed cell volume on *Oedogonium* sp. cultivated in BG-11and Chu-10 media supplemented with 2% sucrose and 0.2% tryptone. The isolate had grown maximum and in 15 days of culture time maximum biomass was obtained. Increased fresh cell weight and settled cell volume was obtained from BG-11 media which was compared to Chu-10 media (**Figure 3A and C**). Whereas, the dry cell weight and packed cell volume was higher on Chu-10 media compared to BG-11 media (**Figure 3B and D**).

*Effect of various factors on biomass productivity*

Different pH and temperature can significantly control the growth of micro algae. The growth of the *Oedogonium* species is very sensitive to pH and temperature. Effect of different pH was tested. An initial acid/base titration of the medium was conducted and appropriate acid and base additions were made to 1 L volumes of culture medium to attain the desired pH levels. Generally, 5 or more pH levels were tested simultaneously in this experiment. Maximum dry weight was observed at pH 6 compared to acidic and above neutral pH (**Figure 4**). Similarly, the isolates were grown at different temperature ranging from $25^0$C to $45^0$C in temperature controlled water bath, where highest dry weight was observed at $30^0$C compared to sub-optimal and supra-optimal temperatures (**Figure 5**).

Various nitrogen sources were supplemented in BG-11 for *Oedogonium* sp. growth. The isolate was grown in the presence of tryptone and compared to other nitrogen sources (**Figure 6**).

Maximum growth was obtained at 0.2% of tryptone when compared to other nitrogenous sources. Outcome of different carbon sources such as sorbitol, sucrose, glucose, fructose and maltose on *Oedogonium* sp growth were also tested. Higher growth was obtained at 2% of sucrose compared to other carbon sources (**Figure 7**).

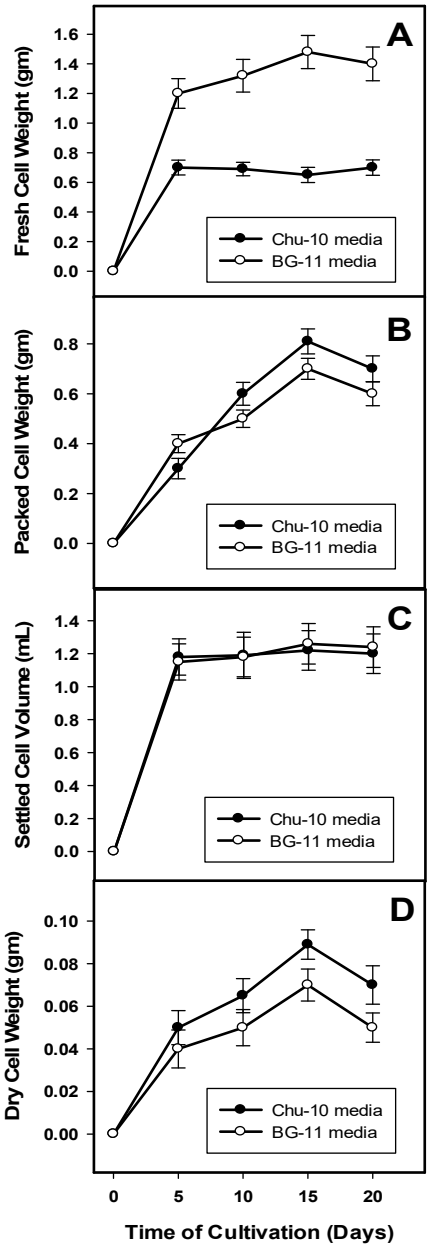


**Figure 3:** Effect of different media compositions on various growth parameters (A) Fresh cell weight (B) Packed cell weight (C) Settled cell volume (D) Dry cell weight of *Oedogonium* spp.

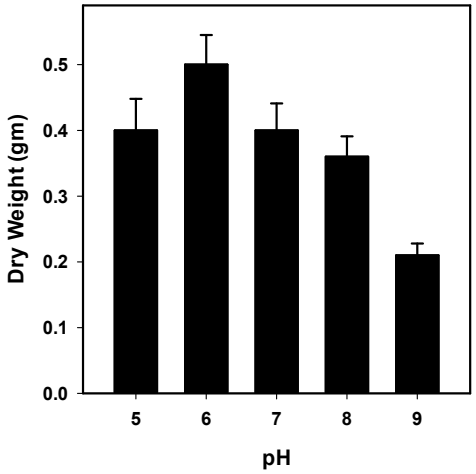


**Figure 4:** Effect of different pH on dry weight (gm) of *Oedogonium* spp. on 15th day of growth.

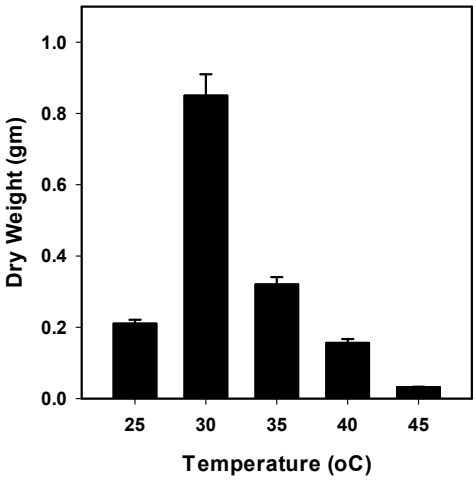


**Figure 5:** Effect of different incubation temperatures on dry weight (gm) of *Oedogonium* spp. on 15th day of growth.

**Isolation and screening of the microalgae**

The isolation and screening of the microalgae collected from both lakes Abaya and Chamo was performed using identification manual. Different genus was observed from the collections **(Figure 2 and Table 1).** Further screening was done on the view of the biomass productivity and oil recovery. The oil and biomass recovery of the three dominant species is shown in (**Table 1**).

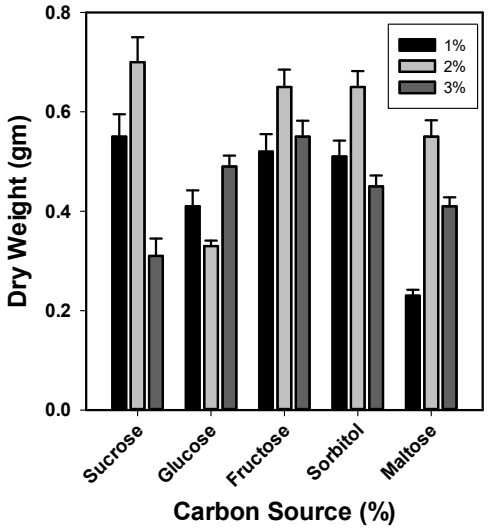


**Figure 6:** Effect of different carbon sources on dry weight (gm) of *Oedogonium* spp. on 15th day of growth.

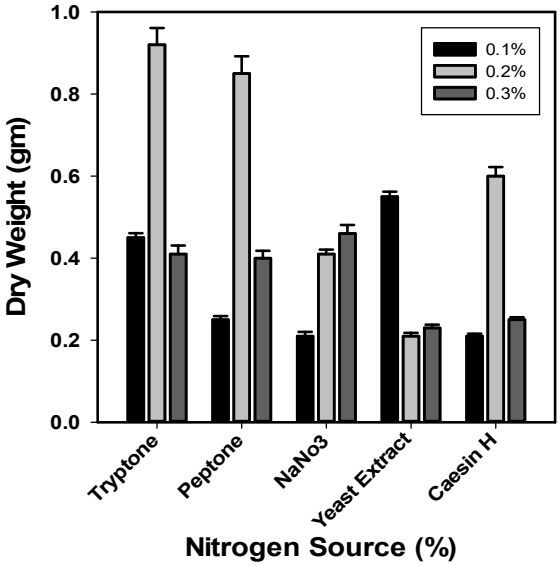


**Figure 7:** Effect of different nitrogenous sources on dry weight (gm) of *Oedogonium* spp. on 15th day of growth.

Solvent extraction proved to be successful in order to extract lipids from microalgae. In this approach, organic solvents, such as petroleum ether, hexane, acetone, chloroform are added to algae paste. There was a significant increase in oil

extracted from *Oedogonium* sp. compared with similar dry weight. However, the changes in the diesel production among the three genera were not significant (**Table 2**).

**Table 1:** Various genus of micro-algae found at different location of Great rift valley of lake abaya and chamo.

| Location | | Number of strains | Dominant genus |
|---|---|---|---|
| Abaya lake | Air port | 11 | *Oedogonium* sp. |
| | Merab | 9 | *Cladophora* sp. |
| Chamo lake | Shele mazoria | 8 | *Chlorella* sp. |
| | Nechsar park | 15 | *Oedogonium* sp. |

**Table 2:** Oil extracted from different species of biomass.

| Algal isolates | Dry weight (gm) | Oil extracted (gm) | Diesel produced (gm) |
|---|---|---|---|
| *Chlorella* sp. | 10 | 2.16 | 0.38 |
| *Oedogonium* sp. | 10 | 2.93 | 0.92 |
| *Cladophora* sp. | 10 | 1.78 | 0.41 |

**Discussion**

Microalgae have long been a topic of interest for biodiesel production. This is due to many factors, including their high lipid content, the ability to grow on non arable land and/or salt water, their fast rate of growth, and the fact that they do not compete with food crops (Baum, 1994). According to various reports, biodiesel has many benefits over petroleum diesel. Not only is it renewable, but it is a sustainable resource. It is a biodegradable and nontoxic substance (Zhang *et al*., 2003), and so spills are not nearly the concern as when dealing with petroleum fuels.

In the present study total of 23 isolates were identified in which green algae and diatoms occupy the major algal population in the area **(Figure 2 and Table 1).** From green algae *Oedogonium*, *Chlorella* and *Cladophora* species were dominant species observed in this study. The algal biomass and blooming was observed however to be reduced year after year. Actually, algalization is dependent on a number of factors that include flooding due to rains, simultaneously use of inorganic fertilizers, animal manures, pesticides and amount of light available (Nayak and Prasanna, 2007). Hence *Oedogonium* sp. was chosen from further study.

In this study, the oil extracted from 10gm of dry algal biomass was higher in Oedogonium sp. Then after, the production of biodiesel was also evaluated, *Oedogonium* sp gave the maximum biodiesel per dry weight **(Table 2)**. This result was in agreement to the work of Hossain *et al*., (2008). Therefore our results indicated that biodiesel can be produced from *Oedogonium* sp.

In this study the various growth kinetics parameters mentioned like PCV, SCV, FCW and DCW were evaluated**.** Upon the cultivation of the selected algae, the cultivation media was BG-11. For various growth kinetics parameter, complex biochemical and physiological networks of algal cell have been manipulated in a small place of operation and state of art. For instance, algal cell suspension culture for biochemical studies has been reported as advance cell suspension culture techniques. At the early stage of algal cell, different biological and physiological process has been reported working in

harmony. These shows that reliable and reproducible growth kinetics determination strategies can play a significant role to understand biophysiochemical process at any time of the cell cycle.

Conductivity measurement has been reported as a method of choice to determine plant growth kinetics and nitrogen uptake (Hahlbroeck and Kuhlen 1972). The early exponential phase and beginning of stationary phase has been rapidly distinguished by conductivity measurement. However, the relationship between conductivity change and cell growth has not been associated (Kwok et al., 1992). Hence, other cell growth kinetics estimation has been demonstrated to study the growth kinetics in liquid system.

Volumetric estimation like packed cell volume (PCV) and settled cell volume (SCV) to determine growth kinetics of *Solanum tuberosum* and *Haplopappus gracilis* have been reported (Gilissen *et al.,* 1983). Though the growth of cells can be measured in aseptic condition, this volumetric estimation has been misleading to examine growth kinetic of heterogeneous cellular displays of suspended cells and hairy root cultures (Kwok *et al.,* 1992). Fresh cell weight (FCW) and dry cell weight (DCW) have been reported to solve the aforementioned hurdles (Ryu *et al.,* 1990).

This can be solved by taking small aliquot of the suspended cells during the study time for analysis since the operation is in non-sterile conditions. Though rapid estimation of the sample has been possible in fresh cell over dry cell weight; the actual biomass gain per culture time misrepresented because of water uptake. Hence, dry cell weights were considered using the above factors to measure reliable and reproducible growth kinetics. Up to date, there is no or limited report for the determination of growth kinetic using the dry cell weight of algal cell in liquid system. The cultivation cycle found to be around two weeks **(Figure 3 (A-D))**.

Mass cultivation of algal cells in a short period with minimal microbial contamination is common practices in many research laboratories through liquid system. Two types of basal media Chu-10 and BG-11 were considered for mass cultivation of the isolated algae for better biomass recovery. It was observed that *Oedogonium* species grew better in BG-11 supplemented with various carbon and nitrogen sources.

The various growth parameters like pH, Temperature, carbon sources and nitrogen sources were considered. From the result pH- 6 and Temperature 30$^{o}$C – found to be optimal **(Figure 4 and 5)**. Various carbohydrates used as carbon source also play a significant role for algal biomass recovery. The commonly used carbon sources are maltose, sucrose, glucose, sorbitol, and fructose. Among these major carbohydrates, sucrose at 2% found to be the best carbon source **(Figure 6)**.

The other component is the nitrate used as a nitrogen source. The $NO^{-3}/NH^{+4}$ ratios played a significant role biomass recovery. $NO^{-3}/NH^{+4}$ usually can be used either singly or in combination in standard culture media. The role of nitrogen $NO^{-3}/NH^{+4}$ on biomass recovery is significant. The nitrogen supply has been found to have effects on biomass yield and on the growth rate of cells, and production of biologically active molecules. The ratio of $NO^{-3}/NH^{+4}$ present in the culture medium can affect the activity of cell growth substances. The relative proportion of these ions also affects the response of cells to growth regulators in terms of both cell division and morphogenesis. In this study, various organic and inorganic nitrogen sources were considered Tryptone, Peptone, NaNO3, Yeast extract, and Caesin Hydrolysate. Of which tryptone (0.2%) gave the maximum biomass yield **(Figure 7).**

A wide variety of nitrogen sources, such as ammonia, nitrate, nitrite and urea, have been used for growing microalgae (Becker, 1994). In these experimental data, urea and NaNO3 have been used to investigate the effect of Nitrogen sources and concentration of NaNO3 on the growth of biomass. The Nitrogen source and concentration on the *Chlorella* sp. biomass growth have been reported.

Decrease in nitrogen sources concentrations in the medium and increase in algal metabolism products led to a slight decrease in the biomass amount. However, the lipid content in Chlorella species can, be increased to 53–66% by nitrogen deprivation (Xiong *et al.,* 2008; Hsieh and Wu, 2009). Similarly, nitrogen deprivation or limitation the microalgal cell proliferation is prevented, but lipid accumulation of oleaginous microalgae begins when an excess carbon substrate is still assimilated by the cells and is converted to triacylglycerols (Meng *et al.,* 2009). In conclusion, the media supplements for this algal population could be the relevant for biomass recovery thereby high production of biodiesel.

**Conclusion**

Biodiesel derived from microalgae appear to be the only current renewable source that can potentially substitute petroleum fuels. Nonetheless, there are many challenges remained in biodiesel production. The biggest one is that microalgal biodiesel are not economically competitive with fossil fuels at today's energy prices. Many efforts have been done to reduce the production costs by governments, researchers and entrepreneurs. Our results proved that biodiesel can be produced from microalgae. The results suggested that *Oedogonium* sp. is more appropriate for producing biodiesel. Further understanding on the influence of cultural conditions on biodiesel production, the algae can be exploited for using BG-11 media supplemented with 2% sucrose, 0.2% tryptone at pH-6 incubated at $30^0$C. The results of this study indicate that the naturally isolated microalgal strain *Oedogonium* sp. may be a valuable candidate for biodiesel production.

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**Conflict of Interest:**
"The authors declare that we have no affiliations with or financial involvement with any organization or entity with a direct financial or any other interest in the subject matter or materials discussed in the manuscript".

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