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Bioenergy from Algae: Growth and fatty oils production from *Chlorella pyrendoisa* and *Anabaena sp*.

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Abstract
The objective of this study was to analyze the use of two microalgae species as a potential feedstock for bio-diesel production. Fresh water, fast growing, green microalgae *Chlorella pyrenoidosa* and *Anabaena sp.* were selected for the study. Two suitable growth media; Fogg's and BG-11 were chosen for the cultivation of microalgae. *Chlorella pyrenoidosa* showed better growth in presence of Fogg`s medium and biomass production was achieved 0.3 g L^{-1} in 10 days. Maximum growth of *Anabaena sp* is observed in BG-11 medium and biomass yield is obtained 0.3028 g L^{-1} in 10 days. Biochemical estimation was performed in both the species for analyzing the protein, carbohydrate, lipid and chlorophyll content for their application. *Chlorella pyrendoisa* possess 38.46 % protein, 40.06 % carbohydrate, 5.65 % lipid and 11.17 % chlorophyll whereas *Anabaena sp.* showed presence of 41.51 % protein, 35.58 % carbohydrate, 12.58 % lipid and 7.63 % chlorophyll. 16.3 mg of lipid is extracted from 300 mg of dry *Chlorella pyrenoidosa* cells and converted to fatty acid methyl esters (FAME) by the method of acid based transesterification to yield 4.4 mg FAME. 13.5 mg of FAME was obtained from lipid extracted in *Anabaena sp.* Renewable energy has become one of the most important issue and increase in usage of fossil fuels will create instability in environment, economy and industry. One can indeed see the potential offered by algae as biofuel in terms of energy balance and carbon sequestration. To provide a clear picture regarding the use of use of algae as biodiesel or bio-ethanol at commercial use, R&D centres have to invest and give more focus to this area at large scale and to look for the ideal species.

Keywords: *Lipids, FAME, Bioenergy, Fogg`s, BG-11, Feedstock, Biomass.*

1. Introduction
Alternative feasible production of renewable energy is being world hot topic as first generation bio fuels, majorly produced from food crops and mostly oil seeds are limited in their ability to achieve targets for biofuel production at commercial scale. This has led us to look for an alternative source of biofuel (Gong *et al*., 2011). Microalgae feedstocks are gaining interest in the present day energy scenario and are the most promising source due to their fast growth potential coupled with relatively high oil content (Chisti, 2007). Microalgae have been known to survive under a wide range of conditions. Under optimal conditions microalgae have 5 and 20% dry weight while under unfavourable conditions lipid content increases between 20 and 50. (Hu *et al.*, 2008) Biodiesel derived from bio-oils is fully miscible with petroleum diesel and can be blended at any ratio. Biodiesel consistently shows reduced exhaust emissions compared to petroleum diesel; many studies have concluded that biodiesel use results in the reduction of unburned hydrocarbon, particulate, and CO emissions (Lackey *et al*., 2011). Biodiesel demand is constantly increasing due to increase in prices of fuel with gradual decrease of fossil fuels (Damiani et al., 2010). Unfortunately biodiesel produced from first generation biofuel from vegetable crops is not an efficient option because of competition with food supply. Microalgae as a source of biofuel is the best alternative because no high quality agricultural land is required to grow the biomass rather no land at all. Despite of their growth in aqueous media, microalgae need lower rates of water renewal than terrestrial crops need as irrigation water, so the load on freshwater sources is strongly reduced and microalgae may actually be cultivated in brackish water, requiring only sunlight and a few simple and non-expensive nutrients and on non-arable land. The ability of this microorganism to grow and survive under wide environment conditions and its ability to modify lipid metabolism under stress conditions, made it an interesting microorganism regarding the synthesis of non polar triacylglycerol which are the major substrate to produce biodiesel (Xu *et al*., 2006). Through transesterification biodiesel can be produced from lipids to give us FAME (Fatty acid methyl esters) along with glycerol as by-product.

(Knothe et. al., 2002) The technology and production of biodiesel from microalgae at large scale is still very expensive (Hu *et al*., 2006). Even with high advancement in algae biofuel technology the microalgae biofuel is still not viable for commercial use. The global cost associated with biodiesel production split into partial costs associated with biomass growth, harvesting, oil extraction and transesterification. Other operating and maintenance costs including nutrients, carbon dioxide supply and labour cost.

Several studies have been conducted regarding the biological activity of lipids as the lipid composition can easily manipulated by providing them different conditions like different media concentrations , light conditions etc. Under N and P-deficiency the lipid content was raised up to 14% in *C. vulgaris* and 30 % in *Nannochloropsis sp.* (Pruvost *et al*., 2011; Mandal *et al.*, 2009). Other than lipids microalgae are efficient in production of value added products such as DHA, EPA, asthaxanthin etc. The total lipid content of cells under high light intensity with nitrogen sufficient medium was less than the total lipid content of cells under high light intensity under nitrogen deprivation medium (Damiani *et al*., 2010). To meet the world needs there had been a lot of advancement of using microalgae to produce biodiesel. The major problems faced while growing the biomass in rich nutrient media which is major cost in biodiesel production and other is use of reactor design. Selecting the reactor design is the main decision in between closed photobioreactor or open ponds. Open ponds can use large space and relatively cheap to build. This study was undertaken to explore the basic nutrient and environments requirements and to do comparative analysis for growth and lipid production of *Chlorella sp.* and *Anabaena Sp.*

2. Biodiesel Production System

The system consist of two major areas: First section is the upstream processing section whose major function is sequestering the carbon dioxide i.e., harvesting the algae and extracting lipids. The other section includes the pre-treatment of lipids followed by transesterification to yield biodiesel. Making biodiesel from algae is a 6 step process that includes algae selection, cultivation, harvesting, drying, lipid extraction and lipid conversion.

3. Methodology

Organism and Culture Conditions

Fresh water green alga *Chlorella sp.* was grown in 1000 ml Erlenmeyer flasks containing 500 ml Fogg's media. Marine water alga *Anabaena sp.* was grown in 500 ml BG-11 media. The media were sterilized prior to inoculating with log phase fresh cells. The cultures were grown in laboratory conditions for 12-14 days under 24 h fluorescent illuminations (40 watt, white light) at 28 °C.

4. Estimation of Dry Weight

Dry cell weight of the given culture was calculated by electronic weighing balance. The excess medium was decanted and the known volume of culture was centrifuged at 3000 rpm for 10 min. The pellets collected were dried in Petri plate under vacuum conditions at 80 degree Celsius for 2-3 hours until it is dried completely.

5. Ouantification of Main Biochemical Components

Lipid

The lipid was extracted through Bligh and Dyer method (1959). A mixture of 2ml methanol and 1 ml chloroform was made and added to 1 g algal biomass. It was kept for 24 h at room temperature to dissolve the lipids properly. The mixture was centrifuged at 3000 rpm for 10 min. Supernatant was separated 2ml of chloroform was again added to the pellets and shaken properly. It was again centrifuged at 3000rpm for 5 mins and supernatant was separated. After adding 2 ml of 1% KCL to the supernatant separate layers will be formed. Lower layer will be pipette out and weighed.

6. Protein

The crude protein was determined by Lowry method by taking 0.5 ml of algal culture (Lowry et al., 1951). The absorbance of the sample was checked and the concentration was determined using standard curve.

7. Carbohydrate

The content of carbohydrate is estimated by the modified method of 3, 5- Dinitrosalicylic acid colorimetry using 100 mg of dry algal powder (Miao et al., 2003). The carbohydrate content was estimated using DNS reagent and optical density of the sample was determined against the blank at 540 nm in a UV-visible spectrophotometer.

Lipid Content (%) = wt. of lipid (g) × 100/ wt. of culture (g)

Total Protein Content = wt. of protein (from BSA curve) X 100/ dry cell mass (g)

Carbohydrate Content (%) = wt. of carbohydrate (from Glucose standard curve) X 100/ dry cell mass (g)

8. Conversion of Algal Lipid to Fame

Conversion of one molecule of triacylglyceride in the algal oil reacts with three water molecules of methanol to produce three molecules of methyl esters (FAME), the biodiesel product and one molecule of glycerol. According to the literature of alkali catalyzed transesterification, the reaction is carried out near the boiling point of the alcohol (60°C for methanol). The lipid extract was esterified under acidic condition by using standard method (Francisco *et al.*, 2010). 300mg dried oil was recovered with chloroform and 6 ml of NaOH (0.5 mol L^{-1}) in methanol was added. The mixture was then heated under reflux for 15 mins. After that, 18 ml of transesterification reagent (prepared from 2g ammonium chloride, 60 ml of methanol and 3 ml of sulphuric acid) was added and heated under reflux for another 15 mins and was subsequently transferred to the separating funnel. Separation of biodiesel was done using hexane and distilled water. A clear yellowish layer is recovered in the organic layer containing the FAMEs (biodiesel). After 2-3 washing of biodiesel with water, organic layer was collected and dried in rotary evaporator.

9. Results

Growth Curve Analysis

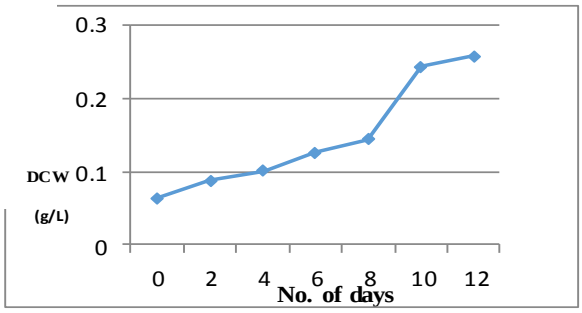

Fig 1: Growth curve of Anabaena sp. at 660 nm. From the figure we conclude that the log phase for Anabaena starts at day 4th of the culture and its stationary phase begins on 10th day.

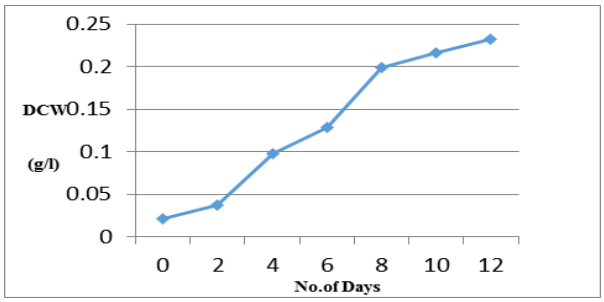

Fig 2: We conclude that the log phase for Chlorella starts at day 2nd day of the culture and its stationary phase begins on 11th day.

10. Determination of Main Biochemical Components

Microalgae	Biochemical components				
	Protein (%)	Chlorophyll (%)	Carbohydrate (%)	Lipid (%)	Others (%)
Chlorella sp.	38.46	11.17	40.06	5.65	4.66
Anabaena sp.	41.51	7.63	35.58	12.88	2.40

TABLE 3: Cell protein content was 41.51% *Anabaena sp.* grown in BG11 medium which is higher than the protein content of *Chlorella sp.* i.e. 38.46%. The carbohydrate content was more in *Chlorella Pyrenoidosa*. i.e. 40.06% where as it was 35.58% in case of Anabaena.sp. The lipid was much higher in case Anabaena i.e. 12.88% where as C.pyrenoidosa 5.65%. The chlorophyll content was also calculated as 11.17% in *Chlorella sp.* and 7.63% in *Anabaena sp.*

Lipid and FAME produced from Chlorella sp. and Anabaena sp

Microalgae	Dry Cell Mass (mg)	Mass of extracted Lipid (mg)	Lipid Content (%)	FAME (mg)
Chlorella sp.	300	16.3	5.43	4.4
Anabaena sp.	300	39.0	12.88	13.9

TABLE 4: The lipid production was much higher in case of Marine alga *Anabaena sp*. when grown in BG11 medium i.e.12.88% where as it was 5.43% lipid content in case of *Chlorella sp*. As the fame extracted will be more if total cell lipid is more. The FAME obtained from *Anabaena sp.* was 13.9mg whereas FAME obtained in *Chlorella sp*. was 4.4 mg

11. Discussion

Growing fuel demand due to depletion of non-renewable resources, mostly in developing nations is becoming one of the biggest issues globally. No new oil source or technology has been developed despite incredible development. Rising prices and depleting resources have encouraged researchers to look for an alternative resource and better technology. In recent years, a new approach has arisen towards producing biodiesel from micro algae due to their high oil content and lack of competition with food production for water and land. (Chisty., 2007). Apart from biodiesel production, algae have several applications like to enhance the food nutritional value, biofertilizers and phytoremediation. (Naoto., 2006) Media requirement is an

important factor since it is most expensive part of biodiesel production. We need to develop culturing technology and media composition for high biomass and desired lipid composition. Yanna Liang and his group showed that *Chlorella Vulgaris* provided 38% lipid content in autotrophic conditions and 33% lipid content in heterotrophic conditions but biomass was 6.8 times lesser in autotrophic conditions when compared with heterotrophic conditions. (Yanna et al., 2009). The underlying principle is that where there is insufficient nitrogen for protein production necessary for growth, excess carbon from photosynthesis is channelled into storage molecules, such as TAGs or starch, and protein content may be reduced (Scott et al., 2010). In one of the investigation done by Sherif H.Hasan et. al., that there is gradual increase in lipid content with increase in glucose concentration. (Sherif., 2012)Nitrogen and phosphorus deficiency, extreme environmental conditions, high light intensity or high temperature leads induces lipid accumulation in microalgae (Roessler., 1990, Zhukova et.al., 2006, Rodolfi et al., 2009)

In our study comparative study was carried out in different media to achieve high biomass and lipid content. It was observed that *Chlorella sp* was growing well in Fogg`s medium as compared to BG11 with 0.3g/l biomass whereas Anabaena sp showed better growth in BG11 i.e. 0.3028g/l. *Chlorella sp. showed better biomass in Fogg`s medium as compared to RM and MBM medium*. Medium rich with glucose (1% w/v) is the medium for Cyanobacteria. (Sherif., 2012). In our study Chlorella sp showed 5.43% lipid whereas Anabaena Sp showed excellent lipid accumulation of 12.88%. Major breakthroughs are still needed in developing bioreactor that could use industrial gases as main carbon substrate for harvesting and eventually meet with success. After extracting lipid the biomass residue should be used for fine chemical production. Novel researches are yet to be found to meet the requirements of richer countries due to increase in continuous demand of energy and food supplies.

12. Conclusion

In this study, the use of microalgae using microalgal oil from *chlorella sp. and Anabaena sp.* to produce biodiesel was done. *Chlorella sp* .showed better growth in Fogg`s medium as compared to BG11 medium whereas *Anabaena sp.* showed excellent growth in BG11 medium. Cellular lipid and protein was much greater in case of *Anabaena sp.* when grown in BG11 medium whereas there was increase in carbohydrate content of *Chlorella sp.* when grown in fogg`s medium as compared to *Anabaena sp*. when grown in BG11 medium. FAME obtained in *Anabaena sp.* was much higher than *Chlorella sp.* In future study the biomass and cellular content will be estimated using different types of media in different temperature conditions, light intensity and aeration conditions. These results show that the production of biodiesel from microalgal oil can indeed be use for commercial purpose. The production of biodiesel for microalgae will be profitable and it does not have competition with our food resources.

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