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Integrated Biodiesel and Biogas Production from *Chlamydomonas reinhardtii*

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Abstract: In this study, we focus on the production of energy efficient Biodiesel and Biomass, whose principle is to extract the maximum amount of energy from biomass, the biodiesel production i.e. the lipid extraction from the biomass through the trans-esterification process and the residue is then subjected for the anaerobic process for biogas (methane) production and the leftover residue can be used for various other purposes. From this study we observed that cultivation of microalgae in open pond system using fertilizer as nutrients will reduce the most of biomass production since construction of and use of open ponds is cheaper than using photobioreactors thereby reducing the fixed capital investment. Using fertilizers as nutrient source will decrease the working capital , thus reduction in overall process but major problems like salt precipitation, evaporation and change in pH due to external environment were observed in open pond; these can be overcome by taking extra care and optimization. Gas chromatography-Mass spectroscopy (GC-MS) analysis of crude biodiesel sample confirmed the presence of fatty acid methyl ester (biodiesel). The green circle in GC-MS analysis ndicates presence of decanoic acid methyl ester. The maximum amount of biogas produced was 10 cm^3 in 27 days. The maximum amount of biogas produced from the algal biomass was 12 cm^3 for 18 – 27 days. The Gross calorific value was found to be 333 kcal/kg. The percentage of methane present was found to be 2.5% and the percentage of carbon dioxide present was found to be 2.59%.From the energy balance, the calorific value of the biogas produced from the residue after lipid extraction was found to be 0.81 kcal. From the feasibility study conducted, it was found that the cost of new media's formulated using fertilizers is approximately ten times less than High Salt Media thereby reducing the overall cost which improves the economics of biodiesel production.

Keywords: Biodiesel, Biogas, *C.reinhardtii*, Transesterification, Anaerobic digestion.

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1. INTRODUCTION

Algal population reaches its limits either due to nutrient reduction or high cell densities that limit light saturation a large number of algal cells death[1]. Under normal growth conditions most algal species have a lipid content of ~10–30% dry weight[2]. Most focus has been on the production of biodiesel, algae can also be a source of other fuels, such as bioethanol, biohydrogen, and biogas[12]. According to recent studies, Algae-based biofuels have been highlighted as one of the best current alternative source of renewable energy[13].Microalgae are a major focus of interest as the efficiency of biomass manufacture per hectare is estimated to reach 5–30 times that of crop plants. Lipids play a significant role, as the transformation capacity into biomethane is higher (1390 L biogas (72% CH_4, 28% CO_2) per kg organic dry substance) than that of proteins (800 Lbiogas (60% CH_4, 40% CO_2) per kg organic dry substance) and carbohydrates (746 L biogas (50% CH_4, 50% CO_2) per kg substance[3]. Biodiesel are currently produced from food crops such as sugar beet, sugar cane, soybean or rapeseed, thus rival with land use for food production and influence to find new feedstocks. Due to their high surface efficiency, microalgae are considered as a promising way of producing biofuels. Under typical growth conditions, microalgal biomass is mainly composed of proteins, cell wall carbohydrates and membrane lipids. Grouping of energy-rich reserve compounds such as starch and storage lipids (oil) occurs in many microalgae under conditions of nutrient shortage such as nitrogen (N) deficiency[4]. Microalgae produce the biomass two times much faster than terrestrial plants, and hence produce more biomass per hectare than higher plants.[9]The green unicellular alga *Chlamydomonas reinhardtii* is a widely established model organism to investigate numerous biological functions, including photosynthesis, starch metabolism or flagella. The latest sequencing of its whole genome, the accessibility of numerous molecular tools including transformation of the three (nuclear, plastid and mitochondrial) genomes, and the existence of a sexual cycle allowing genetic studies make *C. reinhardtii* an attractive model for molecular investigations. In addition to its well-known starch reserves, *C. reinhardtii* has also been observed to accumulate intracellular oil droplets under N limiting conditions[5].. The high density biomass leads to the maximum yield of algal biomass and accumulation of large amount of lipids for optimum growth conditions and survival of algal species.[2]. Microalgae are known to be rich in lipids; depending on the species, they produce many different kinds of lipids, tri- and diglycerides, phospholipids and glycolipids, hydrocarbons and others. Historically, much research has focused on the lipid (specifically the fatty acid) composition from either a taxonomic or a nutritional perspective. Cultured microalgae are more commonly used as feed for aquaculture applications due to the desirable fatty acid content of the algae, in particular servicing the need for essential polyunsaturated fatty acids as dietary supplements[6].An alternative motivation for microalgal culture was the production of high-value by-products such as pigments. The recent interest in the use of algal lipid-derived biofuels, biodiesel in particular, has progressed research on algal lipids and lipid metabolism[7]. The greatest advantage of these open ponds is their simplicity, leading to reduction in production costs and operating costs. While this is undeniably the easiest of all the growing techniques, it has some drawbacks owing to the fact that the environment in and around the pond is not completely under control. Bad weather can stunt algae growth. Contamination from the strains of bacteria and other organisms often result in undesirable species taking over the desired algae growing in the pond. The temperature of the water for growth of the algae must be retained at a particular temperature which could be hard to maintain. Another drawback is the uneven light intensity and distribution within the pond[8]. Developing renewable energy sources is critical to maintaining the economic growth of the planet while protecting the environment. First generation biofuels fixated on food crops like corn and sugarcane for ethanol production, and soybean and palm for biodiesel production and Second generation biofuels focused on cellulosic ethanol produced from terrestrial plants[8]. *Chlamydomonas reinhardtii* has been extensively used in the development of molecular tools for strain selection and engineering for green alga. According to the previous studies, more recombinant proteins have been expressed in *C. reinhardtii* than all other algal species[14]. *Chlamydomonas* is a model for biofuels and bioproducts production. They have highlighted the potential of algae as one of the most promising sources of sustainable liquid transportation fuels. Algae *Chlamydomonas reinhardtii's* have also been recognized and long established as unique biofactories for industrial, therapeutic, and nutraceutical co-products. The most extensively studied microalga *Chlamydomonas reinhardtii* has the ability to produce hydrogen via a photosynthetic water-splitting reaction.[12] *Chlamydomonas reinhardtii's* has long proven role in the field of basic research in green algae which has lined the way for realizing algal metabolism and developing genetic engineering protocols[8]. *C. reinhardtii* is also among the first of the engineered algal species to be studied in commercial settings, which allows academic researchers to understand the challenges of bringing transgenic algae to commercial-scale production[15]. In this context, our studies showed biodiesel and biogas production from single source algal biomass which extracted the maximum amount of energy using *Chlamydomonas reinhardtii*.

2. MATERIALS AND METHODS

2.1 Source algae and media preparation for master cultures

The algal culture of *C. reinhardtii* was obtained from 'Department of atomic energy, Mumbai'. The cultures were then grown in Sueoka's high salt media (HSM) in agar inoculated agar slant and conical flasks as shown in Figure 1and 2.

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2.2 *Preservation of cultures*

Fig 1. Agar slants and Agar flask inoculated with *C. reinhardtii*

Fig 2. Sub-Culturing of algae in conical flasks

2.3 *Mass cultivation of C. reihardtii*

Microalgae being photosynthetic, need a light source, carbon dioxide, water, and inorganic salts. The temperature range must be between 15 and 30°C (~60-80°F) for optimal growth.[10] The plastic containers were purchased online of brand Tablecraft with dimensions 21" x 16" x 5" made of Polyethylene were filled with required amount of water and were arranged according to the length and width of the pond to be created. The Tarpal sheet was placed in between the containers and was given a rectangular shape. The bricks were placed on the top of the container holding the sheet in place. The media was poured into the pond created and the culture was inoculated[10].

2.4 *Harvesting and Drying of Biomass*

Biomass from the media is separated by the process of flocculation. Ferric trichloride ($FeCl_3$) is a strong flocculant which is used for sedimenting biomass. 12.75mg/l of $FeCl_3$ is added and mixed well. Then it is allowed to settle down for half an hour. Then the spent media and settled biomass is separated. The wet biomass was spread on a tray and kept under sun for drying. Water content in biomass was evaporated over a period of 3 days. The dried biomass is collected and weighed[11].

2.5 *Production of Biodiesel*

The production of Biodiesel was carried out by the Trans-esterification process. The Transesterification process is the reaction of a triglyceride (fat/oil) with an alcohol to form esters and glycerol[10].

2.6 *The lipid extraction through In-situ Trans-esterification process*

The Lipid Extraction and Trans esterification was carried out in single step called *In-situ* Transesterification. About 10g of dried Algal Biomass was weighed into a clean conical flask. 164 ml of methanol was added to conical flask with biomass, care was taken to prevent the evaporation of methanol by covering with aluminum foil. Pre - treatment was done by allowing the mixture to stand at Room Temperature for one hour.1.4 ml of KOH (2%) was added and mixed well by shaking vigorously for 2min. Then it was incubated in water bath at 60°C for 4h s. After 4h, 0.3 ml of HCl (38%) was added to stop the reaction. 100 ml of petroleum ether was added & stirred well for 2 min. Crude biodiesel was purified by washing it with 20 ml of NaCl (0.9M) Solution. Centrifuge at 2000 rpm for 10 min at room temperature to obtain a two phase system is formed. The two layers were separated, and the upper aqueous layer was kept at room temperature for evaporation of solvent. The crude biodiesel obtained was analyzed by Gas Chromatography – Mass Spectrometry[10].

2.7 The Lipid Extraction from the Ex-situ Transesterification process

About 10g of dried algal biomass was subjected to cell disruption using mortar and pestle with petroleum ether. The biomass was then transferred into a separating funnel and soaked with petroleum ether for 1h. The upper layer was transferred into a separate test tube.1.4 ml of KOH (2%) was added and mixed well for 2 min. The sample test tubes were then incubated in hot water bath for 4hr at 60 ^{0} C. After 4h, 0.3 ml of HCl was added to stop the reaction. The mixture was then centrifuged at 3000rpm for 3 min. The upper biodiesel layer was separated and washed with water several times[10]. The yield of biodiesel was 2.6 ml for 20g of algal biomass.

2.8 Biogas Production

The biogas production was carried out using (i) algal biomass (ii) residual biomass after lipid extraction. Effluent from gobar gas plant was collected from Gandhi Krishi Vignana Kendra (GKVK), Bengaluru, India, and was used as inoculum. [7]. About 20g of the algal biomass was made into slurry using 20 ml water and then it was transferred to a digester containing 20ml effluent from biogas plant and set up was made such that biogas produced was collected and analyzed by gas chromatography[7].

3. STATISTICAL ANALYSIS

The data obtained were analyzed using SIGMA SOFTWARE (VERSION 12.0). Student's (paired) "t" test was used for analysis of comparison. The data were presented as mean standard deviation (SD). Probability value (P) of less than 0.05 was considered statistically significant.

4. RESULTS AND DISCUSSIONS

4.1 Growth pattern of C. reinhardtii HSM media

Optical density value in the table below gives the relation between the increase in biomass after each day (24 hrs).

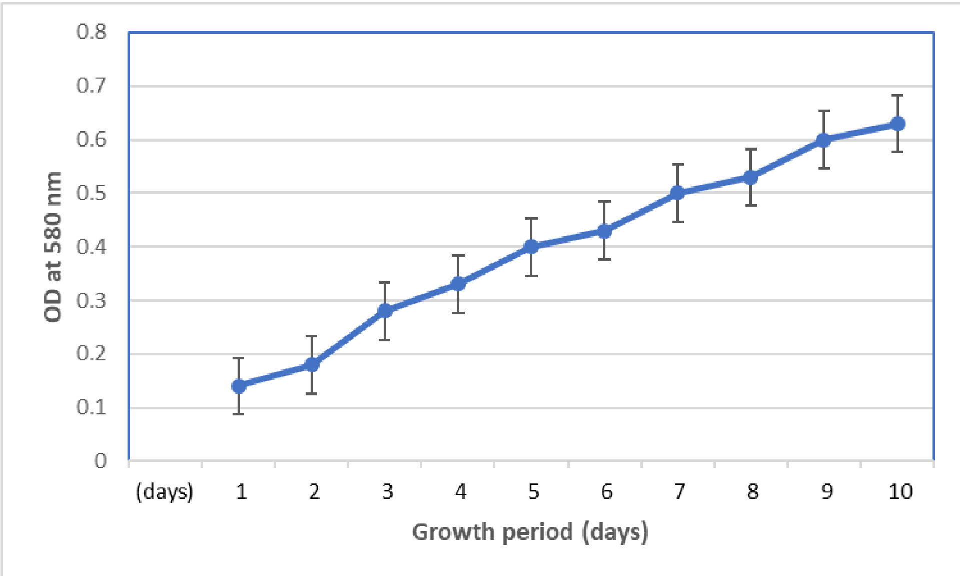

Fig 3. Time course of growth of *C. reinhardtii* HSM media

C.reinhardtii when grown in HSM media uniform growth rate was observed in 24hr light cycle (Fig 3). The cell concentration was determined using a hemocytometer. The cell count was in the range of 10^5cells per ml. The cell count was obtained after 10 days of culture.

4.2 Growth pattern of C. reinhardtii TAP media

Data tabulated below is optical density values in TAP media for growth period of 6 days.

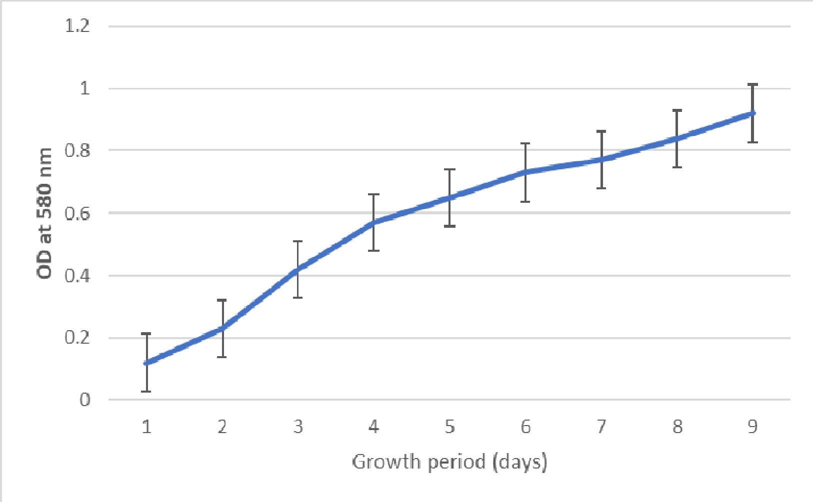

Fig 4. Time course of *C. reinhardtii* growth in TAP media

C.reinhardtii when grown in TAP media, uniform growth rate was observed in 24hr light cycle (Fig 4). The cell count was in the range of 10^4 cells per ml.

4.3 Growth pattern of C. reinhardtii in open pond

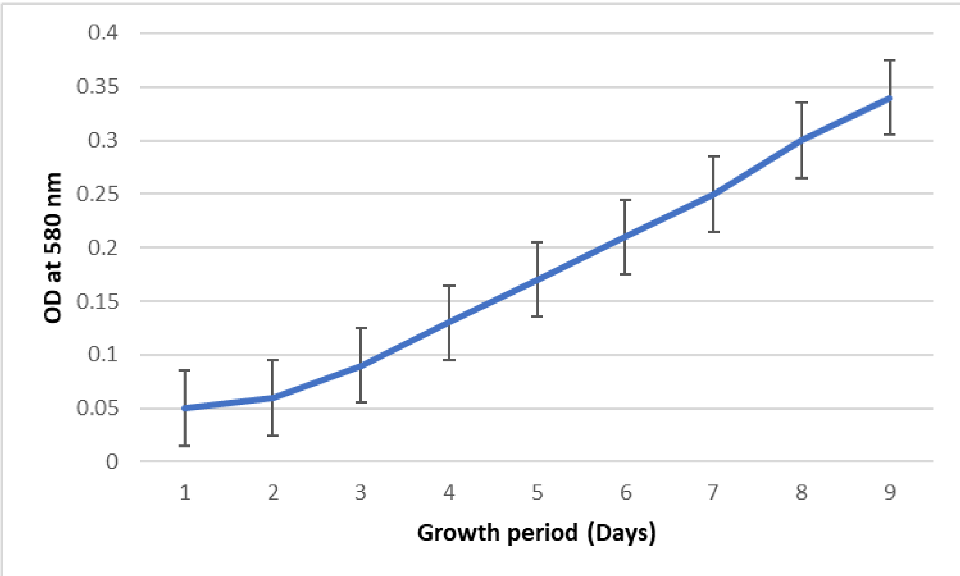

Fig 5. Graph showing the growth pattern of *C. reihardtii* in Open ponds

The above graph(Fig 5) shows the growth pattern of *Chlamydomonas* cells grown in open shallow pond. The cells count was in the range of 10^4cells per ml. Dry weight of biomass in open pond for one cycle of 600litres was 300g.

4.4 Oil Recovery

4.4.1 Oil extraction and Trans-esterification

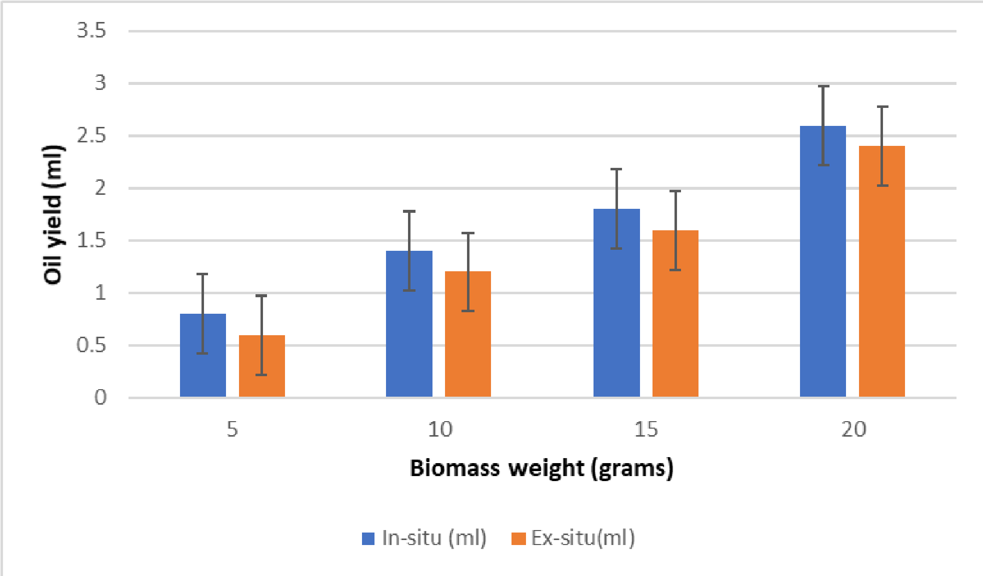

Fig 6. Comparison between the oil recovery from *In-situ* and *ex-situ* process

The graph (Fig 6) shows a comparison between the oil recovery from *In-situ* and *ex-situ* process. The maximum oil recovery was found for 20 g Biomass wherein oil recovery for the In-situ process was 2.6 ml and oil recovery for the ex-situ process was 2.4 ml.

4.4.2 Qualitative Analysis of Biodiesel

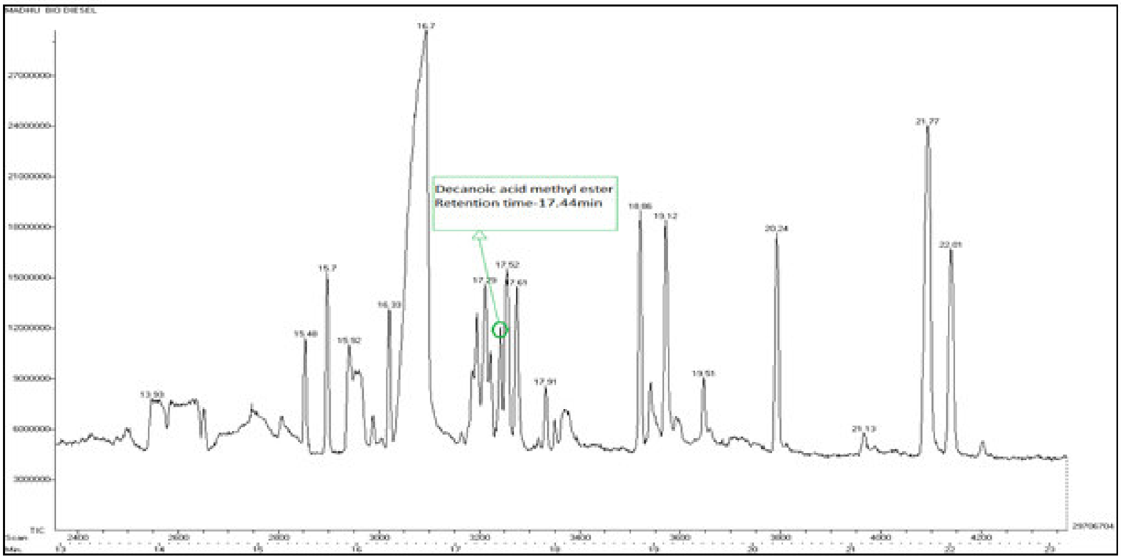

Fig 7. Analysis of the sample with Gas chromatography-Mass spectroscopy (GC-MS)

Gas chromatography-Mass spectroscopy (GC - MS)(Fig 7) analysis of crude biodiesel sample confirmed the presence of fatty acid methyl ester (biodiesel). The green circle in the above chart indicates presence of decanoic acid methyl ester.

4.5 Biogas production

4.5.1 Biogas from residue

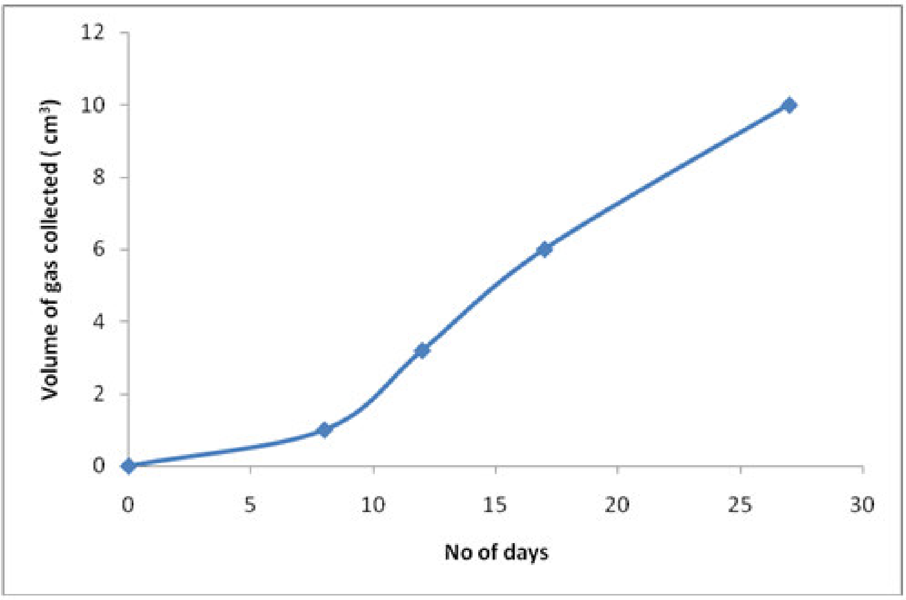

Fig 8. Graph representing the volume of gas collected vs no of days

From the above graph (Fig 8) it is clear that initially at zeroth day there was no gas production, gas production started at third day and around eighth day volume of gas collected was 1cm^3. After that the volume of gas collected increased, at twenty seventh day volume of gas collected was 10cm^3.

4.5.2 Biogas from algal biomass

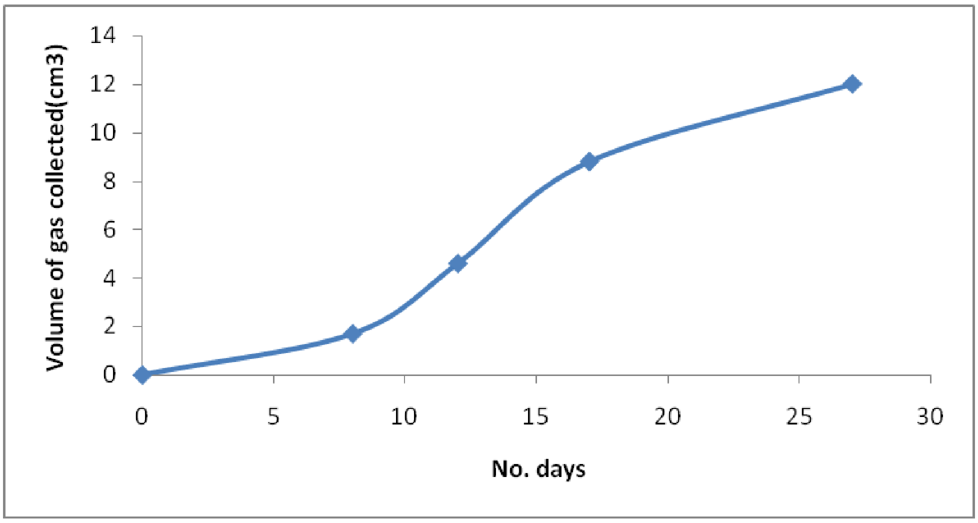

Fig 9. Graph representing the Volume of gas collected Vs. No. of days from the algal biomass.

The graph (Fig 9) shows the volume of biogas collected with respect to the number of days of collection of the algal biomass. The maximum amount of biogas produced from the algal biomass was 12 cm^{3}for 18 – 27 days.

4.6 Comparison of biogas production at different temperature

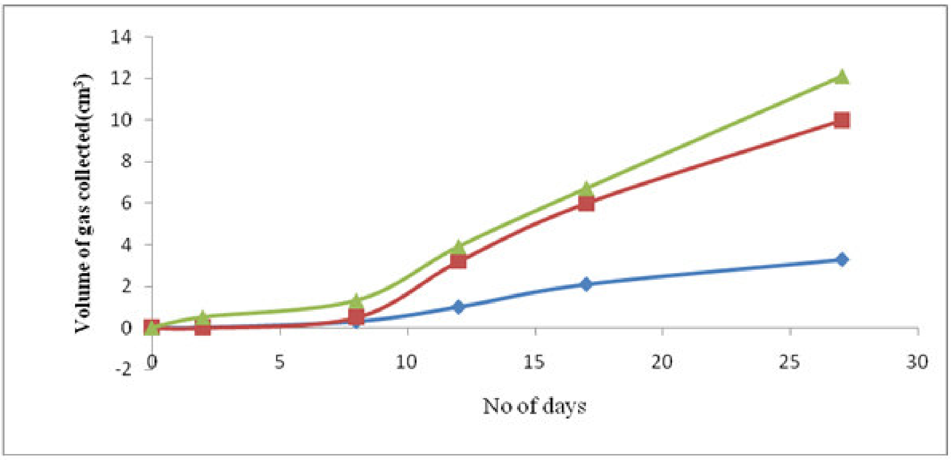

Fig 10. Graph showing the Effect of Temperature on the production of Biogas

The graph (Fig 10) shows the effect of temperature on the biogas production. At 26°C, the maximum amount of biogas produced was 3.3 cm^3, at 37°C the maximum amount of biogas produced was 10 cm^3 and at 42°C, the maximum amount of biogas produced was 12.1 cm^3. From our study, we found that cultivation of microalgae in open pond system using fertilizer as nutrients will reduce the cost of most of biomass production since construction of and use of open ponds is cheaper than using photo bioreactors there by reducing the fixed capital investment. Using fertilizers as nutrient source will decrease the working capital so reduction in overall process but major problems like salt precipitation, evaporation and change in pH due to external environment were observed in open pond; these can be overcome by taking extra care and optimization. *C. reinhardtii* when grown in HSM media uniform growth rate was observed in 24 hr light cycle. The cell count was in the range of 10^5cells per ml. The growth pattern of *Chlamydomonas* cells grown in open shallow pond showed that the cell count was in the range of 10^4cells per ml. Dry weight of biomass in open pond for one cycle of 600 liters was 300 g. The maximum oil recovery was found for 20 g Biomass wherein oil recovery for the *In-situ* process was 2.6 ml and oil recovery for the *ex-situ* process was 2.4 ml. Gas chromatography-Mass spectroscopy (GC - MS) analysis of crude biodiesel sample confirmed the presence of fatty acid methyl ester (biodiesel). The green circle in above chart indicates presence of decanoic acid methyl ester. The maximum amount of biogas produced was 10 cm^3. The maximum amount of biogas produced from the algal biomass was 12 cm^3 for 18 – 27 days. The sample of the biogas produced was analyzed at Shiva analytical (INDIA) limited, Bengaluru. The Gross calorific value was found to be 333 kcal/kg. The percentage of methane present was found to be 2.5% and the percentage of Carbon dioxide present was found to be 2.59%. From the energy balance, the Calorific value of the biogas produced from the residue after lipid extraction was found to be 0.81 Kcal. From the feasibility study conducted, it was found that the cost of new media's formulated using fertilizers is approximately ten times less than HSM media thereby reducing the overall cost which improves the economics of biodiesel production.

5. CONCLUSION

In conclusion, we found that growing of microalgae in open pond system using fertilizer as nutrients will reduce the production cost than photobioreactors thus promoting economic advantage and this can be further studied to promote optimizing the growth of microalgae for industrial scale production.

6. ACKNOWLEDGEMENT

Author would like to acknowledge to Shiva analytical (INDIA) limited, Bengaluru for helping to analyze our biogas sample.

7. AUTHORS CONTRIBUTION STATEMENT

Mr. Madhu H N devised the project, designed the model and the computational framework and analysed the data. Mrs. Manasa. V. Anand performed the numerical calculations for the suggested experiment. All authors discussed the methodology and results and contributed to the final manuscript.

8. CONFLICT OF INTEREST

Conflict of interest declared none

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