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Cultivation Of *Chlorococcum oleofaciens* In Domestic Waste Water And Comparison Of Growth in Phototrophic And Heterotrophic Mode

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Abstract
Algae are versatile and one of the most important diversity of the microorganisms. They are aquatic and photosynthetic organisms well known for their ability to mitigate carbon di oxide. Besides, the rampant use of non renewable fuel resources such as coal, diesel for domestic and industrial purposes by the mankind has lead to the emission of green house gases (GHG) and the depletion of the available natural energy resources. Nature has offered various sources of renewable energy such as biomass, agricultural wastes which can be used to produce methane and ethanol. Algae prove to be a promising source of biodiesel. However, the major drawback in the algal system is the slow growth rate. It takes a longer time to produce sufficient biomass which hampers the process development for biodiesel production from algae. This paper aims at the cultivation of *Chlorococcum oleofaciens* in domestic waste water. The waste water parameters pH, total dissolved solids, chlorine, alkalinity and Chemical Oxygen Demand (COD) of the sewage water were also monitored. The quality of waste water was found to improve after algal cultivation proving that the algal cultivation in waste water can be an effective tool in waste water treatment. Also waste water serves as a valuable medium rather than other chemically defined medium. Heterotrophic cultivation of *C. oleofaciens* was also attempted. It was found that the quality of waste water improved. The heterotrophic cultivation using 1% w/v glucose, sodium acetate and a mixture of 1% glucose and 1% sodium thiosulphate was carried out in a chemically defined CFTRI medium. The biomass yield of *C. oleofaciens* cultivated in heterotrophic medium was found to be much greater than that of the algae grown in chemical medium**.**

Keywords— Biomass; Algae; Bioremediation; Heterotrophic cultivation

Introduction

The industrial revolution since 1800 has led to the utilization of tremendous amount of fossil fuels and the generation of energy related emissions of green house gases (GHG) like carbon dioxide, nitrogen di oxides, methane, sulphur dioxide and volatile organic compounds. Although nuclear, wind and tidal power are considered as recent options, the search for alternative energy resources has not ceased. It has also paved way for biofuels (EEA, 2007). Biofuels such as biodiesel can be obtained from the trans-esterification of lipid obtained from the biomass such as grains, oil seeds, lignocellulosic crops, sugarcane and straw wastes and jatropha (Tiwari *et al.,* 2007). Other important biofuels widely being used are methanol and ethanol. Apart from the above mentioned feed stock sources, microbial sources such as algae, diatoms also prove to be advantageous (Jones *et al.,* 1986). Microaglae are also more efficient than the conventional oleaginous plants in capturing solar energy. The biomass obtained from algae can be used for lipid extraction which can be trans-esterified to biodiesel (Yihe Gao *et al.,* 2009). Algae also serve as an effective feedstock. Hence, the biomass of algae is of more importance.

Algae has been cultivated in waste water as it plays a vital role in carbon mitigation. Yeoung *et al.* (1997) cultivated *Chlorella vulgaris* in effluent discharged from steel plant. Lee (1997) cultivated *Chlorella sp*. HA-1 and *Chlorococcum littorale* in thermal power plant effluent. The algae besides growing in the waste water also help in treating the waste water. Certain microalgae have the natural ability to metabolize sugars such as glucose as carbon source and can accumulate high proportions of lipid (Chunfang Gao *et al.,* 2010). Algae have been attempted with various carbon source and significant increase has been attained in biomass and lipid content. This paper aims at the cultivation of *C. oleofaciens* in sewage water and the heterotrophic cultivation of algae to enhance the biomass production.

Materials and Methods

Cultivation of algae

The algal cells were obtained from Vivekananda Institute of Algal Technology, Mylapore, Chennai.

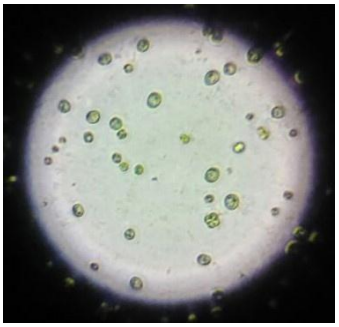

Fig: 1 Microscopic image of Live *C. oleofaciens* viewed under Light Microscope at 40× magnification

The alga was viewed under microscope and it appeared spherical. It were a unicellular cyanobacteria. It is widely present in freshwater. The cells were maintained in CFTRI medium (Venkataraman, 1985) containing, Sodium bicarbonate 4.5 g/L, Dipotassium hydrogen ortho phosphate 0.5 g/L, Sodium nitrate 1.5 g/L, Potassium sulphate 1.0 g/L, Sodium Chloride 1.0 g/L, Magnesium sulphate hepta hydrate 0.2 g/L, Calcium Chloride 0.04g/L, Ferrous sulphate 0.01 g/L. pH was adjusted to 10. The cultures were kept in a light chamber of light intensity 2000 lux at 26°C. Specific growth rate (μ) and doubling time (t_d) was calculated using the formula given below:

$$\mu = (\ln N_t - \ln N_0)/t \text{ Day}^{-1}$$

where, N_t – Number of cells after time t (cells/ml)

N_0 - Initial number of cells inoculated (cells/ml)

T – Number of days

The doubling time (td) is calculated by the formula,

$$t_d = \ln 2/ \mu \text{ day}$$

Analysis of waste water

The waste water collected from the waste water treatment plant of Kamaraj College of Engineering and Technology was tested for various parameters before and after cultivation of algae. Parameters like alkalinity, chlorine, total dissolved solids (TDS), Chemical oxygen demand (COD) were tested as per the protocol mentioned by Greenberg *et al.,* (1985).

Alkalinity is due to the presence of free hydroxyl ions through the hydrolysis of salts by weak acids and strong base. Amount of bicarbonate $(CO_3)^{2-}$ was determined by titration against 0.1N Hydrochloric acid using phenolphthalein as indicator. Similarly hydroxyl ions (OH^-) was determined by titration against 0.1N Hydrochloric acid using methyl orange as indicator.

$$\text{Amount of } (CO_3)^{2-} \text{ alkalinity (mg/ml)} = \frac{\text{(Volume of titrant×1000)}}{\text{Volume of sample}}$$

$$\text{Amount of } (OH)^- \text{ alkalinity (mg/ml)} = \frac{\text{(Volume of titrant×1000)}}{\text{Volume of sample}}$$

Total alkalinity is the sum of the alkalinity due to $(CO_3)^{2-}$ and $(OH)^-$ions.

Chlorine content was estimated by titrating 10ml of the waste water against 0.025N silver nitrate and 0.4ml of 1% potassium dichromate was used as the indicator.

$$\text{Chloride (mg/ml)} = \frac{\text{(Volume of } AgNO_3 \times 1000 \times 35.5)}{\text{Volume of sample}}$$

TDS was measured by TDS meter.

5 ml of 0.0417 M $K_2Cr_2O_7$ was diluted to 50ml. 15 ml con. Sulphuric acid was added and the flask was allowed to cool. Ferroin indicator was added to this and it was titrated against 0.1 N FAS. Molarity of FAS was calculated by the formula as follows:

$$\text{Molarity} = \frac{\text{(Volume of } K_2Cr_2O_7 \times 0.25)}{\text{Volume of FAS}}$$

10 ml of the water sample was taken in a conical flask. A blank sample was also set up as control in the similar manner. Ultra pure water was chosen as the blank. 5 ml of standard $K_2Cr_2O_7$ was added to it. 0.2 g of mercuric sulphate was added and it was stirred well. To this 15 ml of conc. H_2SO_4 was added little by little. The flasks were heated for 2 hours in a boiling water bath. Ferroin indicator was added and it was titrated against 0.1 N FAS solution. The endpoint was the appearance of brown colour. The COD was calculated as follows:

$$\text{COD (mg/ml)} = \frac{8000 \times M \times (V_b - V_a)}{V_s}$$

Where, M – Molarity of FAS
V_b – Volume of titrant for control
V_a - Volume of titrant for the water sample
V_s – Volume of the sample taken.

Cultivation of algae in waste water

100 ml of sewage water was taken in 250 ml sterile conical flasks. The parameters of the waste water like pH, temperature, total dissolved solids, conductivity, chloride, total alkalinity, chemical oxygen demand (COD) were observed initially. All the flasks were inoculated as to attain a final cell concentration of 10^6 cells/ml. Cell counting was performed by haemocytometer. The cells were suitably diluted (1:100) before counting using haemocytometer.

This was performed before inoculating cells into the medium and also used to monitor the growth on a daily basis. The parameters of the sewage water was monitored continuously upto 7^{th}, 13^{th} and 14^{th} day.

Heterotrophic Cultivation of algae

For the heterotrophic cultivation of algae, other carbon source like glucose, sodium acetate and a mixture of glucose and sodium acetate were used. 1% (w/v) carbon source was added to the 8 days old cultures grown in CFTRI medium (Fu-Ying Feng *et al.,* 2005).

Estimation of Biomass

Cellulose acetate membranes of pore size 0.2µ were used. The membranes were heated at 75°C for 5 hrs. The membranes were weighed and the empty weight was noted. 10 ml of the culture was allowed to pass through it and filtered at vaccum and were dried at 50°C overnight (Illman *et al.,* 2000). The biomass was calculated by the difference between the final and initial weight of the membrane.

Results and Discussion

Comparison of algal growth in CFTRI medium and Sewage

The growth curve of *C. oleofaciens* is shown in **Fig. 1** *C. oleofaciens* was inoculated in 100 ml of CFTRI medium and sewage waste water. In both the media, the algae was inoculated such that the final cell number was 10^6 cells/ml. In CFTRI medium, maximum cell number of 2.10×10^8 cells/ml was attained on the 9^{th} day. Similarly, in sewage water medium, on the 9^{th} day the maximum cell number attained was found to be 3.18×10^8 cells/ml. Specific growth rate µ and doubling tine td were also calculated. µ was found to decrease with time and whereas the doubling time increased with time in both the media. In CFTRI medium, the specific growth rate was found to be 0.042/hr and the doubling time which was observed as 16 hours 5 mins. Sewage water medium exhibited diauxic growth which was evident from the growth curve. Specific growth rate and doubling time on the second day was calculated as 0.06/hr and 4 hours 7 mins. Second log phase occurred after the 4^{th} day during which µ and t_d was found to be 0.64/hr and 11 hours and 6 mins respectively. The growth rate and the doubling time of sewage was found to be greater than that of the CFTRI medium. The diauxic growth suggested mixotrophic growth in sewage water where the algae simultaneously uses organic and inorganic carbon sources in the presence of light **(Kang *et al.,* 2004)**. Atmospheric CO_2 gets fixed during photosynthesis and organic carbon is assilmilated through aerobic respiration (Wang *et al.,* 2014). Therefore the growth rate in sewage water was also greater than the CFTRI medium and sewage water was found to serve as a better medium for the growth of algae.

Cultivation of C. oleofaciens in sewage water

Various parameters like pH, Total Dissolved Solids, chlorine content, alkalinity of the waste water was analyzed before being used for the cultivation of algae. These parameters were studied before and after the inoculation of algae and are tabulated as in the table: 1& 2. To understand the improvement or conditioning in water quality rendered by the algae, the above mentioned physical properties of the waste water were studied and analysed on the 7^{th}, 13^{th} and 14^{th} day of cultivation of algae. The results have been tabulated in table: 2 indicate the general improvement in parameters like TDS, Chloride, alkalinity and COD.

As the algal growth reached a log phase on the 7^{th} day, stationary phase on the 13^{th} and 14^{th} day, the samples were collected only on those days for the analysis of the above mentioned properties. The initial pH, TDS, chlorine, alkalinity and COD of the waste water was found to be 8.64, 567 ppm, 4615 ppm and 290 ppm respectively

There was a gradual increase in pH during cultivation and it was noted as 8.84 on the 7th, 9.5 on the 13th and 9.9 on the 14th day**.** Mohan *et al.,* (2010) reported increase in pH during cultivation of *C. oleofaciens* till 6th day and the pH remained constant thereafter. They also reported gradual increase in cell numbers till 2nd day followed by steep increase in cell number till 5th day of cultivation. Later a decreasing trend of cell number was reported.

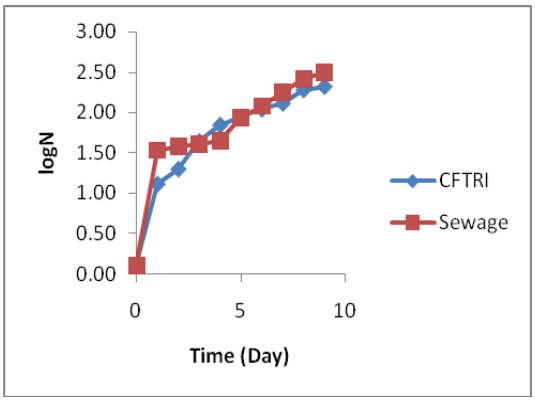

Fig. 2. Comparison of algal growth in CFTRI medium and Sewage

Table: 1 Physical Properties Of Sewage Water Before Inoculation Of C. oleofaciens

Parameters	**Sewage water**
pH	7.75
Temperature (°C)	32
TDS (ppm)	575
Chloride (ppm)	4,615
Alkalinity (ppm)	290
COD (ppm)	250

Table: 2 Effect Of Growth Of *C. oleofaciens* On Quality Of Sewage Water

	Day			
Parameter	0	7	13	14
pH	8.64	8.84	9.5	9.9
TDS (ppm)	567	508	343	336
Chlorine (ppm)	4615	3905	3905	3905
Alkalinity (ppm)	290	300	160	120
COD (ppm)	250	-	-	160

Total dissolved solids decreased gradually and reached to 336 ppm on the 14th day from 567 ppm. The drop in chlorine content was observed only until the 2nd day, thereafter it remained constant at 3905 ppm.

The alkalinity increased on the 4th day. The alkalinity is mainly due to the carbonate ions which are quantified using methyl orange indicator. The alkalinity contributed by the bicarbonate ions which is quantified by phenolphthalein indicator decreased throughout the experiment.

The COD dropped with the growth of algae from 250 ppm to 160 ppm. This shows that the algae is utilising the organic matter present in the waste water through the heterotrophic mode of metabolism. Similar results were reported by Liang Wang *et al.,* (2010) who cultivated the algae on the municipal waste water treatment plant. They found that the COD removal rate of the wastewater in the sludge tank cultivated with algae dropped so much compared against the wastewaters collected before primary settling, after primary settling and centrate respectively. This may be attributed to the alteration in the metabolism of the algae. They observed that when the organic substrate is not available, autotrophic growth uses CO_2 as the carbon source, excreting small molecular organic substances such as glycolic acid as a product of photosynthetic carbon reduction cycle (Merrett, & Lord,

2010). This might have contributed to the rise in pH. Thus the cultivation of algae in the domestic sewage water can serve as a effective method of waste water treatment.

Heterotrophic Cultivation

The algal cultures which were growing in CFTRI medium were transferred to the heterotrophic phase by adding sterile exogenous carbon source on the 8th day. The biomass was estimated on the 12th day Table: 3 shows the effect of carbon source on the algal growth.

Table: 3 Effect Of Exogenous Carbon Source On Biomass

Carbon Source	Biomass (g/L)
Control	0.5
1%Glucose	4.38
1%Sodiumacetate	1.52
1% Glucose & 1% sodium thiosulphate	4.12

Compared to sodium acetate, glucose and mixture of glucose and sodium thiosulphate, glucose was found to be a better organic carbon source as the biomass obtained by addition of 1% (w/v) glucose was 4.38g/L, which is 2.75 times more than that yielded by sodium acetate (1.52g/L). The biomass of a heterotrophic culture increased over 8 times compared to the photoautotrophic culture of *C. oleofaciens* (0.5g/L).

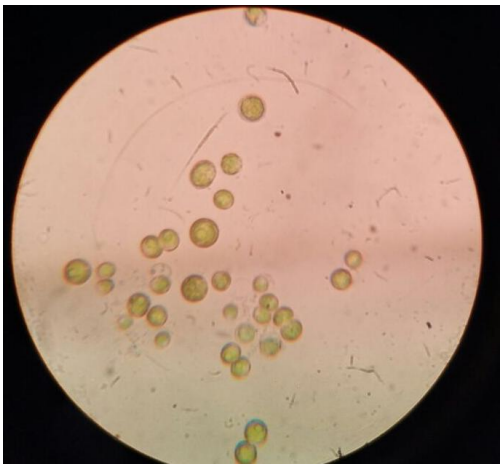

Fig: 3 Microscopic image of Live Heterotrophic *C. oleofaciens* cells viewed under Light Microscope at 100× magnification

During heterotrophic cultivation, aggregation of algal cells was observed and it settled at the bottom of the flask. This may be due to the increase in cell density. The culture also appeared bleached upon the addition of extraneous carbon source. This may be due to the destruction of chlorophyll by the formation of free radicals. In the process of degradation of glucose, plant cells produces reactive oxygen species (e.g.$^1O_2$ and O_2^-), which can heavily damage biomacromolecules (Wang *et al.,* 2002, Geckil *et al.,* 2003). Membranes and membrane proteins are most easily attacked by reactive oxygen. Photosynthesis inevitably produces reactive oxygen, but plants have elaborate systems to reduce the level of reactive oxygen. Sodium thiosulphate, acts as a reducing reagent. Hence it can scavenge reactive oxygen effectively and protect cells against the damage of reactive oxygen produced by the biodegradation of exogenous organic carbon. Fu-Ying Feng *et al.,* (2005) reported the effect of the addition of glucose and sodium thiosulphate to the culture medium. They found that the algal growth was inhibited by the presence of sodium thiosulphate and the degree of inhibition increased sharply with an increase of sodium thiosulphate concentration. However, the inhibition of growth by addition of sodium thiosulphate was almost completely eliminated the addtion glucose in the medium.

The heterotrophic cells when visualized under light microscope, appeared larger in size. Wei Xiong *et al.,* (2010) also compared the appearance of autotrophic and heterotrophic cells of *C. protothecoides* when viewed through electron microscope. They found that the chloroplasts, were clearly visible in photosynthetic cells and

membranes were abundantly accumulated in these chloroplasts and a number of starch granules could also be seen. They also proposed that the thylakoid membranes disappeared rapidly within 48 h of heterotrophic cultivation suggesting the degeneration of chloroplasts. Chlorophyll breakdown and chloroplast degeneration was found to be associated with lipogenesis during the fermentation stage of the Photosynthesis-fermentation model.

After the addition of sugar, the growth of the bacteria on the walls of flasks was visible after the 12th day. It may be due to the free availability of carbon source. These bacteria may be symbiotic in nature utilizing the sugar secreted by the algae. Hiroyuki Ueda *et al.,* (2009) observed that some bacteria detected in the cultures might be associated with algae and/or can be hemi-selectively enriched with algae. Watanabe *et al.,* (2008) illustrated the high proportion of *Rhizobiales* bacteria in alga-associated populations. However, it must be noted that *Rhizobiales* bacteria have been isolated from algae other than *Chlorella*; for instance, *Ochrobacterium sp.* was isolated from brown algae samples (Zhou *et al.,*2008).

The cultivation of *C. oleofaciens* in domestic waste water can be an effective and economically viable bioremediation tool. The heterotrophic cultivation of algae increases the biomass by the addition of an extraneous carbon source in the medium. The biomass obtained can be used for the various applications. Heterotrophic cultivation can be attempted in waste water as well. This may reduce the problem of contamination. The change in the morphology of heterotrophic cells suggest that metabolic change in the organism which should be further investigated.

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