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Research Article

Biomass and Lipid Productivity by Four Fresh Water Microalgae in Photoreactor

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Abstract

The unicellular microalgae isolated from environment were investigated for their potential for biodiesel production by characterizing their productivity in terms of biomass and lipid. The step involved includes determination of biological needs for micronutrients (nitrate), pH on biomass productivity. In this study four potential algal strains *Chlorella* sp., *Coelastrum microporum, Chlorococcum echinozygotum* and *Scenedesmus pectinatus* were screened for biomass and lipid production. The four fresh water algae showing high potential for fatty acid production were identified using 18S rDNA technologies. The fatty acid productivity for the strain *Scenedemus pectinate* depicted 202 mg/g of dry wt/L, in presence of nitrate (250 mg/L) as nitrogen source; optimum pH was 6.4 at 1600 μmol/m^2/s light intensity.

Keywords**:** Fresh water algae, fatty acid productivity, 18S rDNA technologies

INTRODUCTION

The accumulation of green house gases in the environment have already exceed the 'dangerously high' thresh hole of 450 ppm. This is due to wide use of fossil fuels. More over it has been accepted that fossil fuels are unsustainable due to depleting resources. To achieve environmental and economic sustainability, fuel production should be not only renewable, but also capable of requesting atmospheric CO_2. Several renewable energy sources such as solar, wind, hydel and biomass system are in various stages of development and their applications are steadily increasing. However, area which has attracted considerable attention in recent years is the biofuel such as bioethanol and biodiesel.

First generations of biofuels, which have attained economic levels of production, have been mainly extracted from food, oil crops and animal fats using conventional technology (Nigam & Singh, 2011). Second generation of biofuel have the potential to use waste residues and make use of waste land thereby promoting rural development and improve the economic conditions of developing countries.

The most promising second generation bio-fuel is algae which are capable of using CO_2 and sunlight to produce a variety of organic molecules, particularly, carbohydrates and lipids. These photosynthetic algae are known to produce high biomass yields with high oil content and can be cultivated in fresh water or wastewater (Hanon *et al*., 2010). Microalgae are already reported to produce 15-300 times more oil for biodiesel production than traditional crops on area basis (Schenek *et al*., 2008). Since the CO_2 fixation rates of microalgae/cyanobacteria are about 10-50 times faster than terrestrial plants, the use of these biological agents is considered as one of the effective approaches to control global warming. Chisty (2007) has estimated that 1 kg algal dry biomass requires 1.83 kg CO_2 for growth. The utilization of atmospheric CO_2 can have a significant benefit in the context of reducing global warming. However, algal water demand is as high as 11-13 million liters/ha/day for cultivation in open pond (Chinnaswamy *et al*., 2010). Their ability to grow in fresh water, municipal, industrial wastewaters and sea water not only overcomes this hurdle but also provides treated wastewater for other uses.

Microalgae are group of unicellular, tiny photosynthetic microorganisms that fix CO_2 from atmosphere and been used recently for CO_2 reduction (Ge *et al*., 2011). Microalgae can be grown in photo bioreactor that may be illuminated naturally (outdoor) or artificially (indoor). Consequently, microalgae can grow rapidly and convert solar energy to chemical energy in CO_2 fixation (Mata *et al*., 2010). Under suitable culture conditions, some microalgae species are able to accumulate up to 50-70% of oil/lipid per dry weight (Chisty, 2007). Coupling the cultivation of photosynthetic microorganisms with the biofixation of CO_2, algae have the potential not only to reduce the costs of culture media for growth on industrial scale but also have capability to reduce carbon emission (Beneman and Hughes, 1997).

Simultaneously recognition of the effect of omega 3 fatty acid, eicosapentaenoic (EPA) and dodehexaenoic acid (DHA), in human health have prompted to identify alternate source for it. Although the current conventional source of omega 3 fatty acids are fish and fish oils, but to fulfill the demand, alternative sources of EPA, DHA are being sought especially from algae (Tonon *et al*., 2002). Microalgae are prokaryotic or eukaryotic, sunlight driven cell factories that converts CO_2 and water to potential biofuel, foods, feeds and high value production (Tonon *et al*., 2002). The study investigated fresh water algae isolated from environment been compared for fatty acid production at optimal growth conditions of pH, nitrogen.

MATERIAL AND METHODS

Micro algal sample collection and isolation

The microalgae were isolated from different ponds, irrigation water. The samples were cultured in Bold Basal Media**.**

Growth alkalinity and pH measurement

Growth, alkalinity and pH measurements were made in triplicate every twenty four hours. The growth was measured by monitoring the optical density at 670 nm using UV-vis spectrophotometer. The dry weight was also calculated to determine biomass concentration, pH, and alkalinity measurements were made using standard methods (APHA, 1998).

Extraction of lipid from biomass and fatty acid analysis

Algal cells were harvested by centrifugation (10000 rpm) for 10 min. The cell pellets separated from the supernatant were washed with distilled water and dried. Fifty mg of dried algal biomass was taken in 15 ml of test tube, 1.6 ml of double distilled water, 4 ml methanol and 2 ml of chloroform were added and mixed thoroughly for 30 Seconds. Thereafter, an additional 2 ml of chloroform and 2 ml of double distilled water were added and solution was mixed for 30 Seconds. Following this, the mixture was centrifuged, at 5000 rpm for 10 min. The upper layer was decanted and the lower chloroform layer containing the extracted lipids was collected in another test tube. The extraction procedure was repeated again with the residual pellet and both the chloroform extracts were mixed together and evaporated till it gets dried. The dried total lipids were measured gravimetrically and lipid content was calculated as percentage of algal biomass.

DNA isolation, PCR amplification

During CO_2 sequestration, algae samples were processed for DNA extraction as method. 18S rDNA gene was amplified using universal eukaryotic primers F5'-GTCAGAGGTGAAATTCTTGGATTTA-3' and R5'-AAGGGCAGGGACGTAATCAACG-3' (Gross *et al*., 2001). The PCR conditions were 30 cycles of denaturation at 95°C for 2 min. followed by annealing at 55°C for 2 min. and final extension at 72°C for 10 min. The reaction mixture content 5µl DNA template, 1X PCR buffer and 5U Taq DNA polymerase made up to a final volume of 50µl. The amplified product was resolved on 1.2% (w/v) agarose.

Phylogenetic analysis

Nucleotide sequences obtained in this study were deposited in Gene Bank and their accession numbers are available in the online addendum and can be viewed at http//www.ncbi.nlm.nih.gov. Phylogenic tree was constructed using Bootstrap Tree Method from Clustal X version 2.0.11 multiprocessor and tree view 32 version 1.66 software. The method involves aligning sequences using the neighbor-joining (N-J) method. First the sequence were aligned and distances were calculated (percentage divergence) between all pairs of samples for multiple alignment. Finally the N-J method was applied to the distance matrix. The tree was created using the Boot strap N-J Tree method using 1,000 titrations.

Reactor details

Algal cultivation was done in photoreactors consisting of 2L borosilicate glass bottles fitted with rubber stopper. CO_2 from a gas cylinder was mixed with air to get desired concentration and bubbled through fine diffuser. The schematic of the experimental set up is shown in Figure 1. The flow rate (20 mL/min) of gas was measured using a rotameter. The photoreactor was irradiated using standard fluorescent lamps (40w) placed on both the sides. The excess gas was discharged through an outlet tube. The inlet and outlet gas samples were sampled at regular intervals and analyzed for CO_2. The algal samples were collected from an outlet at regular intervals and analyzed for various parameters.

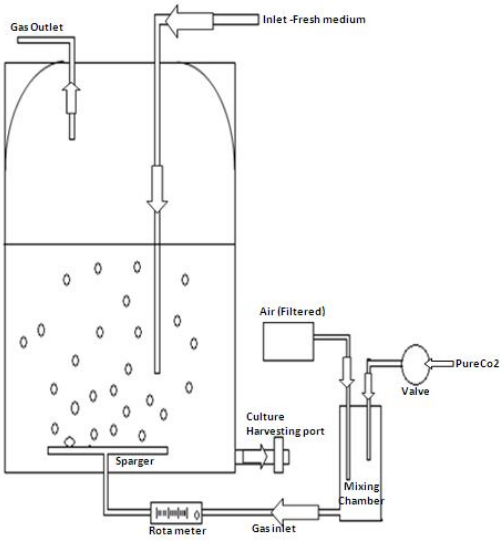

Figure 1: Schematic diagram of lab scale 2L Photo bioreactor

RESULTS AND DISCUSSION

Characterization and growth of microalgae

The algae were identified as *Chlorella, Coelastrum microporum, Chlorococcum echinozygotum* and *Scenedesmus pectinatus* by 18S rDNA technologies and its GenBank accession no were given in Table 1. A phylogenetic study based on 18S rDNA sequences, is one of the most useful methods for inferring the relationships between genera or between species belonging to a genus (Hayashimoto *et al*., 2005). Similarly, the biodiversity of the strains *Chlorella, Coelastrum microporum, Chlorococcum echinozygotum* and *Scenedesmus pectinatus* was demonstrated by constructing a phylogenetic tree. The nearest phylogenetic neighbor of all the sixteen strains from NCBI and present study were used. They were identified following the BLAST analysis of the 18S rRNA gene sequence and based on >97 % 18S rRNA gene sequence similarity, the seventeen isolates were categorized to two groups comprising of two sub groups. The strains that were reported for CO_2 sequestration were collected from NCBI and were used as reference strains for the construction of a dendrogram (Figure 2) that can explain the biodiversity pattern of the isolates algae with that of the reported strains. Based on the phylogeny, it was found that these reference strains mainly belonged to the phylum *Chlorophyceae*. Among the thirteen strains, five strains belonged to the family chlamydomonadaeceae and chlorococcaceae showing close similarity (64.7 %) with that of the neighbour *Chlorella* sp. (KC166137) that was reported to grow under heterotrophic condition.

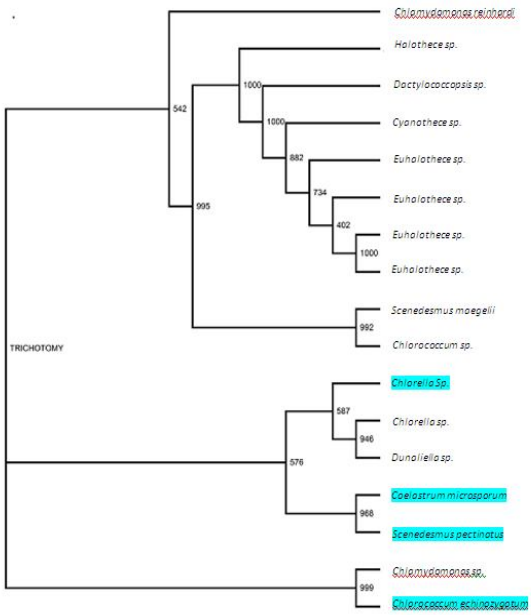

Figure 2: Phylogeny relationship for fresh water algae

KC492078 – *Chlorococcum*, KC492079 - *Chlamydomonas* sp., KC492080 - *Chlorella* sp., KC492081 - *Chlamydomonas* sp., KC218498 - *Chlorella* sp., KC166137 - *Chlamydomonas reinhardtii*, KC218488 - *Scenedesmus maegelii* , KC218482 - *Chlorococcum* sp.,KC218500 - *Dunaliella* sp., JN934686 - *Chlamydomonas* sp., AJ000708 - *Cyanothece* sp., AJ000709 - *Euhalothece* sp.,AJ000710 - *Euhalothece* sp.,AJ000711 - *Dactylococcopsis* sp.,AJ000712 - *Euhalothece* sp. ,AJ000713 - *Euhalothece* sp. ,AJ000724 - *Halothece* sp.

Growth parameters and lipid accumulation characteristics of some of the algal isolates were determined in order to screen them for fatty acid production. The specific growth rate and lipid productivity of algal strains isolated from environment were given in Table 1. The time course of growth of all the four microalgae strains is presented in Figure 3. Cell number of the algae increased steadily with a lag phase of two days followed by logarithmic phase for four days. After fifteen days of cultivation average biomass growth was in the range of 126 - 768 mg/L and fatty acid productivities of 38-198 mg/L/day (Figure 4) for *Chlorella* sp, *Coelastrum microporum*, *Chlorococcum echinozygotum* and *Scenedesmus pectinatus* respectively. After thirteen days the cell growth was observed after nitrogen sources were depleted, was likely due to utilization of nitrogen previously incorporated into cellular constituents (Li *et al.*, 2008). The pH during the growth increased from 6.4 to 7.16 -7.34 after fifteen days. This may be due to utilization of carbon dioxide from atmosphere which leads to accumulation of free OH^- ions. The alkalinity during operation was steadily increased from 180 to 290 mg/L on fifteen days of incubation (Figure 5). This increased in alkalinity levels with the time may be due to formation of bicarbonates.

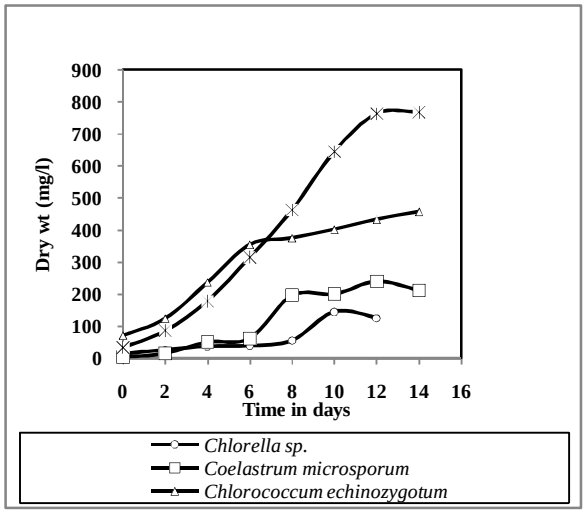

Figure 3: Growth of fresh water algae in bold basal medium

(Medium has pH 6.4, 1600 μmol/m^2/s, 250 mg/L of nitrate, 25 mg/L sodium Chloride)

Table 1: Identification, growth rate, lipid productivity for fresh water algae

Culture ID	Source of isolation	Identification	Gene bank ccession Number	Specific growth rate, μ/day	Lipid content (%)
A1	NEERI Terrace Tank	*Chlorella* sp	KC49074	0.18	13
A3	Irrigation water	*Coelastrum microporum*	KC49076	0.29	29
A8	Irrigation water	*Chlorococcum echinozygotum*	KC49075	0.13	21
A11	Lake water	*Scenedesmus pectinatus*	KC49077	0.23	16

All the results are average of three values

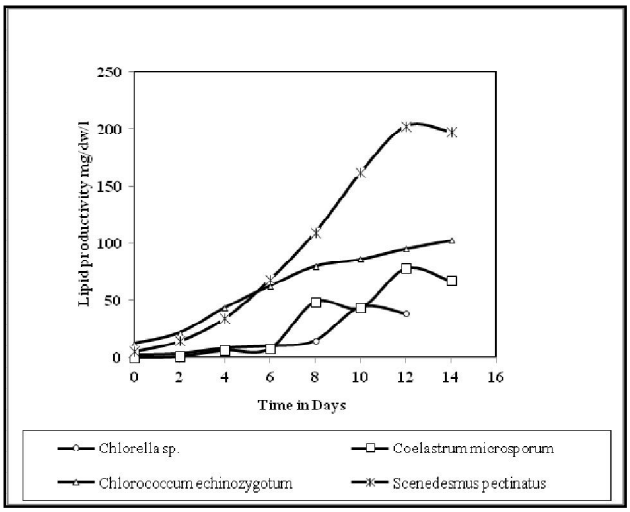

Figure 4: Fatty acid productivity by fresh water algae

The high alkalinity and alkaline pH are indicative of CO_2 fixation. A microalga when cultured under autotrophic condition, the growth is always affected by lower penetrating light intensity (Ceron Gracia *et al.*, 2006).

Several studies have reported that the quantity and quality of lipids within the cell can vary as a result of changes in growth conditions, such as pH, light intensity, or nutrient media characteristics, concentration of nitrogen (Provost *et al.*, 2011; Wang & Lan, 2011). Wang and Lan (2011) have obtained biomass productivity of 350 mg DCW/l/d with a biomass concentration of 3.15 g DW/l with *N. oleoabundans* grown in artificial wastewater at sodium nitrate and phosphate concentration of 140 mg/L and 47 mg/L.

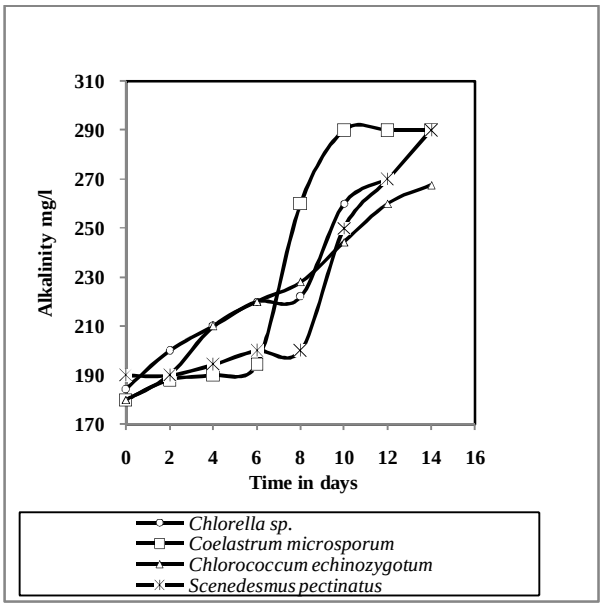

Figure 5 Monitoring of alkalinity during growth of algae

Effect of variation in pH

It has been suggested that pH can affect the lipid biosynthesis of microalgae. The effect of pH from 5.5, 6.4, 7.5 & 8.5 were studied for biomass production and fatty acid productivity. In acidic as well as alkaline condition, all the algal cultures were able to grow, but growth in terms of biomass at acidic pH was comparatively less. The effect of variation of pH on fatty acid productivity for fresh water algae is given in Table 2. At pH 6.4 in case of *Chlorococcum echinozygotum* and *Scenedesmus pectinatus*, fatty acid productivity were maximum and with 103, 202 mg/L/day respectively. However at pH 7.5 *Chlorella* sp (86 mg/L/day), and *Coelastrum microporum* (116 mg/L/day) showed better production, where as the production of other two were reduced. It seems that neutral pH may be favoring for growth and lipid productivity of fresh water algae. Dayananda *et al* (2007) reported that pH had no significant effect on biomass yield and hydrocarbon production of *B.braunii*, when it ranged from 6.0 to 8.5. Cell density was decreased at pH 5.5 due to acidity of medium.

Effect of Nitrogen

All the algal cultures were grown in absence and in presence of sodium nitrate as source of nitrogen. The concentration of sodium nitrate varied from (100 mg/L to 500 mg/L). In absence of nitrogen source all algae were able to grow, but growth was limited up to 91-172 mg/L (dry wt. basis) and the maximum was for *Coelastrum microporum.* Indicating algae have ability to fix up atmospheric nitrogen (Table 3). Provost *et al*. (2011) have reported biomass growth in *N. oleoabundans* in photobioreactor even though the cells were grown in nitrate free medium. A major shift in cell composition was also observed by him, with progression lipid accumulation. But increase in growth with increase in nitrate concentration was obtained till 250 mg/L, but further increase did not show significant impact on growth. When peptone (250 mg/L) was used as organic nitrogen source, the growth was in the range of 100 mg/L to 793 mg/L. Algal isolate *Scenedesmus pectinatus* has shown the maximum growth to 793 mg/L on peptone (Table 4). Absence of nitrogen has shown decreased in fatty acid productivity in *Chlorella* sp, and *Coelastrum microsporum Chlorococcum echinozygotum* and *Scenedesmus pectinatus* (Table 4).The optimum nitrate concentration has shown direct effect on fatty acid production in all the four algae. Mutlu *et al.*(2011) have also observed decrease in fatty acid production with increased in nitrate source in case of *Chlorella* sp. Widjaja (2009) cultured *C. vulgaris* in the nitrogen deficient medium for a period of 7 and 17 days. At the end of 17 days, the total lipid was found to be higher. However, Lv *et al*. (2010) studied effect of nitrate concentration on lipid level. When concentration of nitrate (0.2 to 5.0 m M equivalent to 17 to 425 mg/L) was incorporated in medium for *C. vulgaris*, they observed decrease in lipid with increase in nitrate concentration. Park *et al*. (2012) observed suppression of lipid synthesis in *N. oculata* indicating the conserved mechanism (s) associated with nitrogen metabolism might be involved in lipid biosynthesis across the microalgae species.

Fatty acid profile of microalgae

Fatty acids are primary metabolites of acetyl COA pathways, which is genetically determined evolutionary very old and therefore conservative (Petkov *et al*., 2007). The compositions of fatty acids have known to determine the importance for byproduct extraction. The chemical features of fatty acids, such as carbon chain length and the extent of unsaturation contributes in further use. Therefore we investigated fatty acid biosynthesis from fresh water algae under optimal environmental conditions. The total fat content of the algae varied in the range of 24.85- 36.45% (w/w). Among the identified fatty acids, the proportion of

Table 2: Effect of variation in pH on fatty acid productivity by fresh water algae

Parameters	***Chlorella* sp**			***Coelastrum microsporum***			***Chlorococcum echinozygotum***			***Scenedesmus pectinatus***		
	Dry wt, mg/L	**Fatty acid,%**	**Fatty acid, productivitymg/g/L**	**Dry wt, mg/L**	**Fatty acid, %**	**Fatty acid, productivity, mg/g/L**	**Dry wt, mg/L**	**Fatty acid,%**	**Fatty acid, productivity, mg/g/L mg**	**Dry wt, mg/L**	**Fatty acid,%**	**Fatty acid, productivity, mg/g/L**
pH 5.5	26	24	6	34	31	11	44	31	12	29	27	7
pH 6.4	146	30	44	241	32	78	457	22	103	763	27	202
pH 7.5	269	30	86	353	33	116	394	34	107	270	38	106
pH 8.5	156	32	68	257	36	94	284	32	90	183	32	90

Table 3: Effect of nitrogen source on fatty acid productivity by fresh water algae

Parameters	***Chlorella* sp**			***Coelastrum microsporum***			***Chlorococcum echinozygotum***			***Scenedesmus pectinatus***		
	Dry wt, mg/L	**Fatty acid,%**	**Fatty acid, productivity, mg/g/L**	**Dry wt, mg/L**	**Fatty acid, %**	**Fatty acid, productivity, mg/g/L**	**Dry wt, mg/L**	**Fatty acid,%**	**Fatty acid, productivity, mg/g/L**	**Dry wt, mg/L**	**Fatty acid,%**	**Fatty acid, productivity, mg/g/L**
Absence of nitrogen	91	44	40	172	50	97	156	37	58	170	60	102
Nitrate, 100 mg/L	112	26	29	219	32	69	371	23	86	469	29	137
Nitrate, 250 mg/L	146	30	44	241	32	78	457	22	103	763	26	202
Nitrate, 500 mg/L	136	16	22	137	28	39	317	21	67	769	24	181
Peptone	172	26	45	229	32	54	431	21	92	793	29	227

Table 4: Fatty acid profile of fresh water algae

Fatty acid name	**Lipid number**	**Type**	**Fatty acid Concentration µg/mg of dry wt**			
			***Chlorella* sp.**	***Coelastrum microporum***	***Chlorococcum echinozygotum***	***Scenedesmus pectinatus***
Linoelidic acid ME	C18:2T	PUFA	0.00	0.00	0.00	0.00
Linoleic ME	C18:2C	PUFA	34.86	32.55	28.83	28.30
Linolenic ME	C18:3	PUFA	6.37	15.70	10.24	7.88
8,11,14-Eicosatrienoate	C20:3c	PUFA	0.00	0.00	0.00	0.49
4,7,10,13,15,19-Decosahexanoic acid	C22:6	PUFA	0.46	0.34	0.00	0.00

C18:2, C18:3, C20:2, C20:2A were identified (Table 5) which ranged from 0.34 to 34.86 % of the total identified fatty acids-polyunsaturated fatty acids (PUFA). But C18:2 and C18:3 was the main component and therefore algae can be good candidate for the production of high quality PUFA and biodiesel.

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