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Optimization of culture conditions for lipid production by microalga *Asterarcys quadricellulare* and evaluation of lipids for biodiesel production

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Abstract
Culture conditions were optimized for lipid production in *Asterarcys quadricellulare,* an isolate from fresh water of Kedarnath, India. Under N limitation, biomass production decreased by 5% and the amount of lipids increased by 12% over control cultures (29% lipid content). Under continuous illumination and at pH 6.4 of the medium the biomass of the organism increased by 6% and 4%, respectively, while lipid content increased by 8% and 7%, respectively. The fatty acid profile of lipids of the organism under optimized conditions also changed. Under nitrogen limitation saturated fatty acid (SFA) content was 47%, monounsaturated fatty (MUFA) were 26% and polyunsaturated fatty acids (PUFA) were 27%; under continuous illumination SFA were 44%, MUFA were 30% and PUFA were 26% compared to control cultures with 40% SFA, 28% MUFA and 32% PUFA. This indicated that fatty acid profile of lipids under optimized conditions was better from biodiesel production point of view.

Keywords: Optimization, culture conditions, *Asterarcys quadricellulare*, lipids, biodiesel production

1. Introduction

An excessive use of fossil fuel due to large-scale energy demand has raised the threat of global energy crises. At present, the use of fossil fuels fulfills 90% of global energy demand, it is estimated that, by the year 2050, most of the fossil fuel reserves will be completely exhausted (Ho *et al*., 2013; Maity *et al*., 2014) [1, 2]. Biomass may be considered as a suitable alternative of fossil fuels as it is renewable. Various biomass sources such as energy crops, animal fat, agricultural residues and fungal or bacterial microbes have been used for the commercial production of biofuels (Ahmad *et al*; 2011] [3]. Worldwide, 10% of energy demand is fulfilled by the alternative sources of energy (biofuels) produced from biomass (Maity *et al*., 2014, (Ghosh *et al*. 2016, Ho *et al*., 2014) [2, 4, 5]. Microalgae, on the other hand, are one of the ideal sources for biofuel production (Hu *et al*., 2008) [6]. A number of suitable candidates of algae such as *Chlamydomonas reinhardtii, Dunaliella salina, Chlorella* spp., *Botryococcus braunii, Phaeodactylum tricornutum* and *Thalassiosira pseudonana* have been reported as promising biofuel candidates (Scott *et al*., 2010) [7]. Screening and selection of microalgae producing high amounts of neutral lipids is very crucial for the commercial success of algae-based biofuel production. Although some of naturally occurring algal strains have been screened but the criteria for the selection of algal strains as a feedstock for biofuel production have not been well defined (Rodolfi *et al*., 2009) [8]. The objective of the present study was to collect, isolate, purify and screen microalgal strains for lipid content, and to optimize the conditions for biomass productivity, oil content and better fatty acid composition of selected algal strains for biodiesel production. The present study was focused on optimization of culture conditions not only to increase biomass and lipid productivity but also to get better fatty acids composition from green microalga *Desmodesmus subspicatus*. Also an attempt was made to optimize 10 parameters; dry cell weight, net lipid productivity, lipid content (% of DCW), number of fatty acids in lipids, content of C16-C18 fatty acids of total lipid (TLC), content of fatty acids with C =C ≥ 4, content of linolenic acid, content of saturated, monounsaturated and polyunsaturated fatty acids in lipids of the test strain.

2. Material and Methods

2.1 Isolation, purification and cultural conditions

The isolate FKN45 was isolated from fresh water source of freshwater of Kedarnath (30º 44' 24.97" N; 79º 4' 40.67" E) of Uttarakhand state, India. Isolation and purification (Stanier 1977) [9] were performed on BG-11 medium (Rippka *et al*., 1979) [10] by serial dilution. Purified cultures were identified by following Prescott (1982) [11], Parsad and Misra (1992) [12]. Identified axenic algal strain is being maintained in culture room under culture code PUPMCC. The experimental cultures were grown in BG-11 medium (Rippka *et al*. 1979) [10] which contained the following ingredients (g L-1): Na2CO3 (0.02), NaNO3 (1.5), EDTA (0.001), Ferric ammonium citrate (0.006), Citric acid (0.006), CaCl2.2H2O (0.036), MgSO4.7H2O (0.075), K2HPO4 (0.04), H3BO3 (2.86), MnCl2.4H2O (1.81), ZnSO4.7H2O (0.22), CuSO4.5H2O (0.079), CoCl2.6H2O (0.049), NaMoO4.2H2O (0.39) Na2CO3 (0.02) with pH adjusted to 7.4. The cultures were incubated in a culture room at 28 °C ± 2 °C under 44.5 µmol photon flux density m-2 s-1 at the surface of culture vessels and illuminated for 14 h a

2.2 Identification of green algal isolate

The isolate was identified on morphological as well as partial 18S rRNA gene sequence basis. Purified microalgal strains were identified morphologically following Prescott (1982) [11], Philipose (1967) [12]. Identification was done on the basis of colony morphology, number of cells in colony, cell size, dimensions, chloroplast number, position and shape etc. The cell pellet was suspended in 500 µL of extraction buffer (200 mM Tris HCl, pH 8.5, 250 mM NaCl; 25 mM EDTA, 0.5% SDS) at 60 ºC in a water bath for 90 minutes and genomic DNA was extracted with 350 µL of ice cold phenol: chloroform: isoamyl alcohol (25:24:1 ratio) (Moncalvo *et al*. (1995) [13]. Extracted DNA was precipitated by adding 250 µL of cold isopropanol and centrifuged. The nucleic acid pellet was washed with 70% ethanol, air dried, resuspended in 50 µL of TE buffer (10 mM Tris HCl, pH 8.0 and 1 mM EDTA) and stored at -20 ºC. 18S rRNA gene fragment of 960 bp of the isolate was amplified by using primers: 18SU 467F 5′-ATCCAAGGAAGGCAGCAGGC -3′ and 18SU 1310R 5′-CTCCACCAACTAAGAACGGC -3′ (Matsunaga *et al*. 2009) [14]. Total PCR reaction mixture was comprised 2X buffer mixture, 10 µM forward primer, 10 µM reverse primer and 50 ng template DNA. Gene amplification was done by an initial denaturation at 94 °C for 4 min, followed by 35 cycles of 1 min at 94 °C, 1 min at 52 °C, and 2 min at 72 °C and final extension at 72 °C for 8 min. The gel-purified product was obtained using QIA quick PCR purification kit (Qiagen GmBh, Germany). The gene sequencing was done by using AB1310 automatic DNA sequencer (Applied Biosystem, USA). The sequence analyzed using the gapped BLASTn (http://www.ncbi.nlm.nih.gov/Blast) search algorithm and aligned to the near neighbours. The phylogenetic trees was constructed using MEGA5.05 software package (Tamura *et al*., 2011) [15].

2.3 Optimization of conditions for growth, lipid production and fatty acid composition

The exponentially growing stock cultures, after two washings with sterilized double distilled water, were inoculated in 100 mL basal medium in 250mL Erlenmeyer flasks to attain 0.1 absorbance at 720 nm. The cultures were incubated in the culture room and a known volume of culture was withdrawn at the desired time, washed with distilled water and centrifuged at 5,000 g for 15 min and suspended in distilled water to make a known volume. Absorbance at 720 nm, dry cell weight (DCW) and chlorophyll content of the washed cultures were determined. Chlorophyll was extracted with methanol at 60 ºC following Jeffrey and Humphrey (1975) [16]. Pelleted biomass was oven dried at 70 ºC for 24 h to determine its DCW. The effect of nitrogen limitation on growth and lipid content was studied by decreasing concentration of $NaNO_3$ from 17.6 mM to 2 mM through 8 mM and 4 mM. The effect of pH was studied by adjusting pH of medium at 6.4, 7.4, 8.4 and 9.4. The effect of continuous light was studied by incubated the experimental cultures under continuous light without any dark period.

2.3 Lipid Extraction

Total lipids were extracted from the biomass by slightly modifying the method of Bligh and Dyer (1959) [17]. The biomass was separated from the medium and washed by centrifugation at 5,000 g for 15 min. The harvested biomass was freeze dried under vacuum at -70 ºC. The freeze dried biomass was grounded in a mortar pestle, suspended in methanol and subjected to mild sonication. Then lipids were extracted with a mixture of chloroform: methanol: water (2:1:0.8 v/v). The organic layer was collected, the process was repeated twice and solvent evaporated in a rotary evaporator. The amount of lipids was determined gravimetrically.

2.4 Saponification

A known amount of the lipids (10 mg) was refluxed in a water bath at 70 °C with 50 mL alkaline methanol solution. After cooling the contents, 12 mL distilled water was added. The solution was transferred to a separating funnel and fatty acids were extracted with three washings with diethyl ether. Organic layer was collected and solvent was evaporated in a rotatory evaporator.

2.5 Fatty Acids Analysis

The fatty acid composition of saponified lipids was determined through HPLC (Waters, USA) with refractive index detector using C18W reversed-phase column (4.6 mm× 250mm ID, 5 µm particle size) and pure methanol (Sigma) as mobile phase. The sample was prepared by dissolving fatty acids in methanol to a final concentration of 1 mg mL^{-1}. Twenty microlitre samples were injected into the column and chromatographs obtained. The flow rate of mobile phase was 0.5 mL min^{-1} and the column temperature was maintained at 40 °C. Fatty acids were identified by comparing chromatograms with standards. Fatty acids were quantified by integrating HPLC chromatogram using external standard method.

2.7 Physical properties of lipids

Density of the lipids was measured with a pycnometer. The density of the lipids was calculated according to equation:

$$p = \frac{M_L/V_L}{M_W/V_W}$$

(Mw/ M_L = mass or weight of the water/Lipid, Vw/V_L=volume of water /lipid) Density was expressed in g per cubic meter (g m^{-3}).

The viscosity of the lipids was determined by a U-shaped glass capillary viscometer at 40 °C. The viscosity of the lipids was calculated by following equation:

$\eta_L = d_L t_L / d_w t_w \times \eta_w$

(dL= density of lipids, dw= density of distilled water, tL= flow rate of lipids, tw= flow rate of distilled water ηL= viscosity of lipids, ηw = viscosity of water)

2.8 Statistical Analysis
All experiments were performed thrice with three samples in each experiment and the results are expressed as mean value ± SD.

3. Results and Discussion
3.1 Identification of strain PUMCC 5.1.1
The isolate is being maintained in our culture collection with PUMCCC 5.1.1 code. The thallus of the organism is in the form of 4 celled spherical coenobium. Cells are ovoid in shape, single net-shaped chloroplast with pyrenoid present in each cell. Cells are 3-16 µm in diameter. The organism belongs to order Chlorococcales and was identified as *Asterarcys quadricellulare* on morphological basis (Fig. 1). The morphology based identity of organism was confirmed and its phylogenetic relationship was determined by 18S rDNA gene sequence analyses. The phylogenetic tree generated by aligning 18S rRNA gene sequence of the test strain with sequences of the microalgal strains obtained from NCBI GenBank) showed that strain *A. quadricellulare* PUMCC 5.1.1 showed 100% similarity with respective *A. quadricellulare* strains studied by other workers (Fig. 2). The nucleotide sequence obtained during the present study was deposited in NCBI GenBank database with accession number KT151952.

3.2 Effect of optimized conditions on biomass and lipid production
Accumulation of fatty acids in microalgae regulated by several environmental conditions such as temperature, nutrient availability and salinity (Salama *et al*., 2014) [18]. Under nutrient stress, lipid accumulation is favored, and TAG is formed as the dominant ingredient (Sibi *et al.* 2016) [19]. The selection of algal strains for the purpose of oil production must not be based only on the lipid content but biomass generation capacity should also be considered so as to define the net oil yield or productivity (Griffiths *et al*., 2012) [20]. Several strategies have been adopted to improve microalgal growth and lipid content. Lipid content and day growth rate was found to be inversely related in nitrogen deficient condition (Abomohra *et al.,* 2013) [21]. Nitrogen deficiency has been found to be the most suitable stimulant to raise lipid content of a number of microalgal species including Botryococcus sp. (Yeesang and Cheirsilp, 2011) [22], *Nannochloropsis oculata* (Converti *et al*., 2009) [23], *Nannochloropsis salina* (Fakhry and Maghraby, 2015) [24]. In the test strain, although biomass productivity decreased with decrease in nitrate concentration in the medium but lipid content increased (Msanne *et al*., 2012) [25]. When nitrate concentration in medium was decreased to 8 mM, biomass production decreased to 1.27 mg ml^{-1} than control (1.68 mg ml^{-1}) but the amount of lipids increased by 12% over control cultures (21% lipid content) (Fig. 2, 3).
Microalgal growth also depends on pH of the medium as it influences the nutrient availability, metabolism and biochemical composition of cells (Richmond, 2000, Bajhaiya *et al.* 2010) [26, 27]. Biomass productivity as well as lipid content of studied taxa was almost same in both control pH as well in medium with 8.4 pH of the medium (Fig. 4, 5). Results further revealed that pH of medium above 8.4 and below 6.4 did not support the growth of organism. Higher biomass productivity as well as lipid content in *Chlorella*, *Oedogonium*, *Spirogyra* and *S. obliquus* was observed within pH range of 7.0 to 8 (Munir *et al*. (2015), Zhu, (2010), Sassikant *et al*. (2014) [28, 29, 30].
Microalgal growth needs the input of light during the photosynthesis. As one of the key factors, light affects the performances of microalgal growth and the compositions (Zhu, 201; Hallenbeck *et al*; 2015) [30, 31]. Different species of microalgae respond differently to irradiance and photoperiod (Richmond 2004; Bouterfas *et al*., 2006) [32]. In studied taxa under continuous illumination of light biomass production increased to 2.8 mg ml^{-1} culture than control condition (1.6 mg ml-1). Net lipid productivity of the cultures incubated under continuous light increased to 0.594 mg ml-1 from 0.355 mg ml-1 of culture incubated under 14:10 h light: day cycle while lipid content increased by 7% as compared to control (Figs. 6, 7). Also the asexual life cycle of the test organism shortens by four days from 24 d. Although reports on effect of light intensity on biomass as well as lipid productivity of microalgae are available (Scott *et al*., 2010; Cheirsilip and Tropee, 2012; Mandotra 2016) [10, 33, 34]. Few studies have reported on effect of duration of light on lipid productivity in microalgae. Influence of the photoperiod on lipid productivity and growth rate of *C. vulgaris* (Khoeyi *et al*., 2012) [35], *Dunaliella* sp. (Janssen, 2002), *B. braunii* (Ruangsomboon *et al*., 2012) [36] and S. *obliquus* (Mata *et al*., 2012) [37] has been studied.

3.3 Composition of lipid under optimized conditions
Along with biomass, lipid productivity and fatty acid composition of lipids of test organisms grown under optimized conditions were also studied. Quantity as well as quality the of lipids within the algal biomass varies as a result of changes in growth conditions (temperature, pH and light intensity) or nutrient media characteristics (concentration of nitrogen, iron and phosphorus) (Liu *et al*., 2008) [38]. The content of monounsaturated and polyunsaturated fatty acids decreased while content of saturated fatty acids (SFA) in all the optimized conditions increased. The amount of saturated and monounsaturated fatty acids in cultures grown in medium with 8 mM NO_3 increased to 47% of total lipid content (TLC) and 26% of TLC, respectively, from 40% saturated and 28% monounsaturated fatty acids in lipids of control cultures. Most of algal oils possess high amounts of polyunsaturated fatty acids with four or five double bonds which are not desirable from biodiesel point of view (Damiani *et al*. 2010) [39]. In present study, the content of fatty acids with C=C ≥ 4 were less. Cultures grown in medium with 8 mM NO_3 produced C18:3, 8% of TLC and C20:4, 1% of TLC which was under the limits of EN1214 standard. Content of C16-C18 fatty acids in lipids of culture grown in 8 mM as well as of control culture did not varies significantly (Table 1).
With increase in duration of light amount of saturated fatty acids and monounsaturated fatty acids increased to 44% of TLC, 30% of TLC as compared to culture 40% of TLC and 26% of TLC grown under 14:10 light and dark regime. Polyunsaturated fatty acids were decreased to 26% of TLC from 32% polyunsaturated fatty acids in control culture. Also amount in polyunsaturated fatty acids amount of C18:3, C20:4 were 12% of TLC and 1% of TLC respectively. Lipids of culture incubated in continuous light produced maximum amount of C16-C18 fatty acids (68% of TLC) cultures to control culture (63% of TLC) (Table 1). Lipids of the cultures

grown in medium with 8.4 pH contained 62% C16-C18 fatty acids. Saturated, monounsaturated and polyunsaturated fatty acids in lipids of cultures with pH 8.4 were 42% of TLC, 24% of TLC and 34% of TLC compared to 40% of TLC, 28% of TLC and 32% of TLC, respectively in control cultures.

Lipids with maximum amount of saturated fatty acid and also fatty acids with C16-C18 increased under all optimized conditions, which are highly evaluated for biodiesel production. Lipids of *A. quadricellulare* PUMCC 5.1.1 produced maximum amount of saturated fatty acids (42% of TLC) and fatty acids with C16-C18 (70% of TLC) when incubate under continuous light as compared to control culture 38% of TLC, 63% of TLC, respectively (Table 1). Typically, the amount of polyunsaturated fatty acids (structural lipids) increases under low light conditions, whereas high light promotes the accumulation of saturated and mono-unsaturated fatty acids (storage lipids) (Khotimchenko and Yakovleva, 2005) [40].

3.4 Properties of lipid

Density and viscosity of lipids of test organism were also studied. Density and viscosity of lipids of test organisms obtained under optimized conditions were within the range prescribed by EN1214 standard for biodiesel (Density= 0.86-0.90 g m-3, viscosity= 3-5 mm-2 $^{s-1}$). Although the content of unsaturated fatty acids slightly decreased in test organism under optimized conditions, even then viscosity of lipid is in the range of EN1214 limits. High viscosity of biodiesel causes resistances to the injection pump of engines of vehicles (Mittelbach *et al*., 2004) [41].

So the best condition for the good quality and high quantity of *A. quadricellulare FKN45* was continuous light. Results obtained from the present study suggested that to determine the suitability of algal biomass as feedstock for lipid based algal biofuel; biomass yield, lipid content, its fatty acid composition and appropriate life cycle phase to harvest biomass should be criteria for selection of a particular microalgal strain. Consequently, studies on lipid content of *A. quadricellulare* are rare. Also lipids of *A. quadricellulare* FKN45 studied in the present study had significant amount of α-linolenic acid (C18:3 ω3, ALA) which is nutritionally important fatty acid. Thus this isolate may be a good alternative to fish oil as source of ω3 fatty acid for vegetarians. Secondly, genus *Asterarcys* studied presently has only one species recorded earlier i.e., *A. quadricellulare*. Consequently, studies on lipid content of *A. quadricellulare* are rare (Chaudhary *et al*., 2017) [42]. Thus *A. quadricellulare* FKN45 proves itself to be a good alternative to fish oil as source of ω3 fatty acid for vegetarians (Hong *et al*. 2012) [43].

Table 1: Fatty acid composition of A. *quadricellulare* PUMCC 5.1.1 under optimized conditions

S. No	Optimized Conditions	No. of fatty acid	SFA (% of TLC)	MUFA (% of TLC)	PUFA (% of TLC)	% of C16-C18	Linolenic acid (% of TLC)	% of ≥ =4 C bond (% of TLC)
1	Control	10	40±2.1	28± 1.4	32±1.6	63±3.5	0	1±0.09
2	8 mM NO3	10	47±2.2	26±1.4	27±1.6	66±2.8	8±0.4	1±0.09
3	pH 8.4	10	42±2	24±1.3	34±1.6	62±3.1	18±0.9	1±0.09
4	Continuous light	10	44±2	30±1.5	26±1.4	68±3.1	12±1	1±0.09

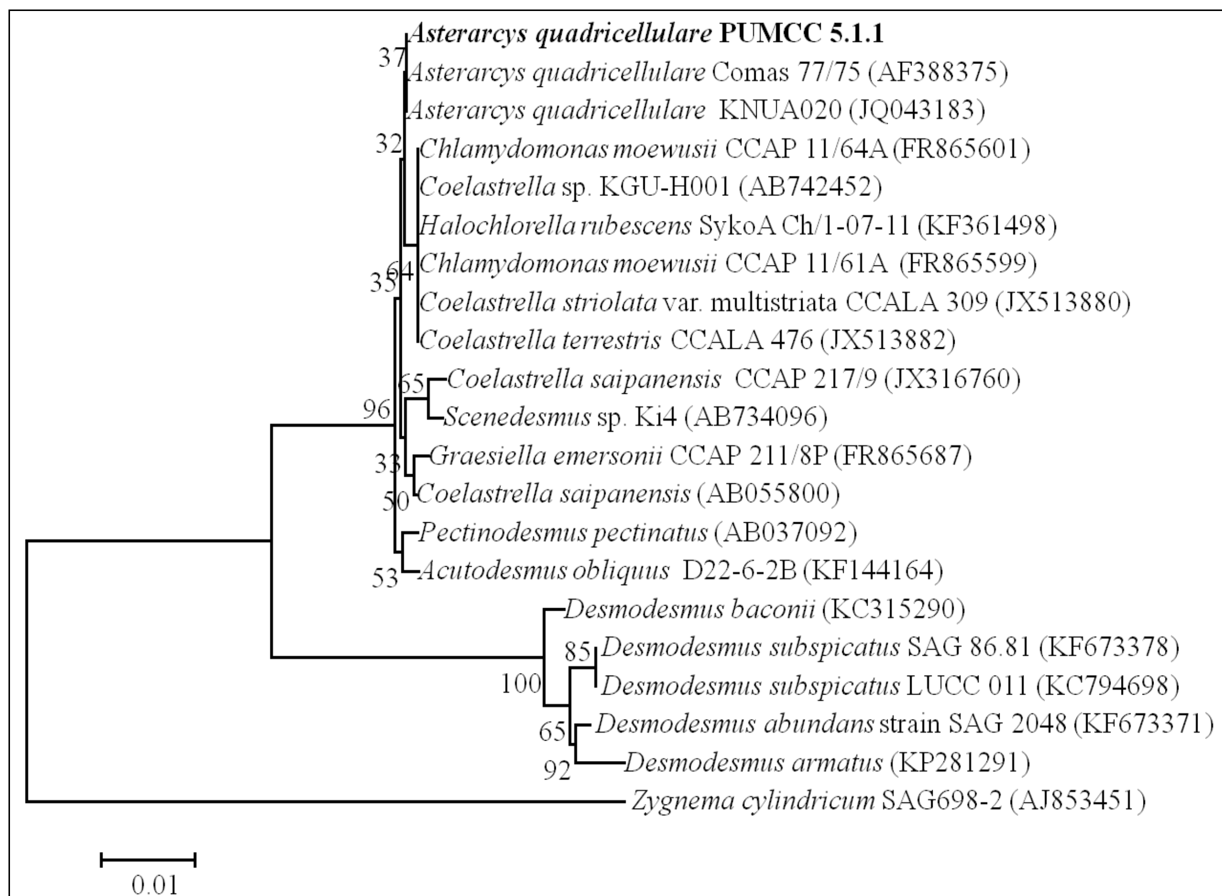

Fig 2: Phylogenetic tree showing relationship of *A. quadricellulare* PUMCC 5.1.1 with closely related taxa based on partial 18 S rRNA gene sequence

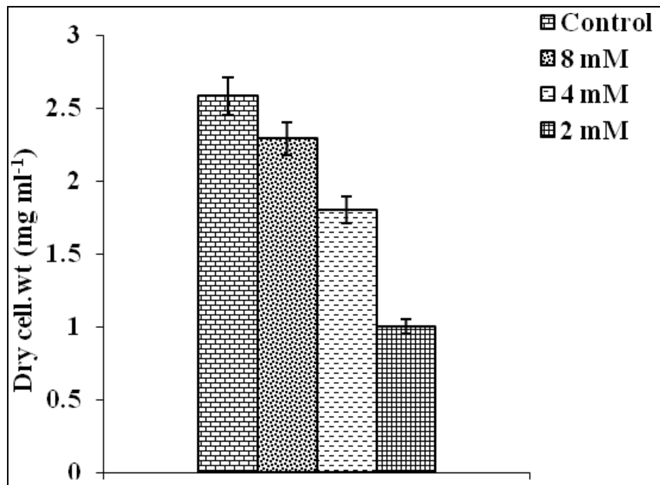

Fig 3: Growth of *A. quadricellulare* PUMCC in the different concentration of $NaNO_3$ in terms of dry cell weight of cultures

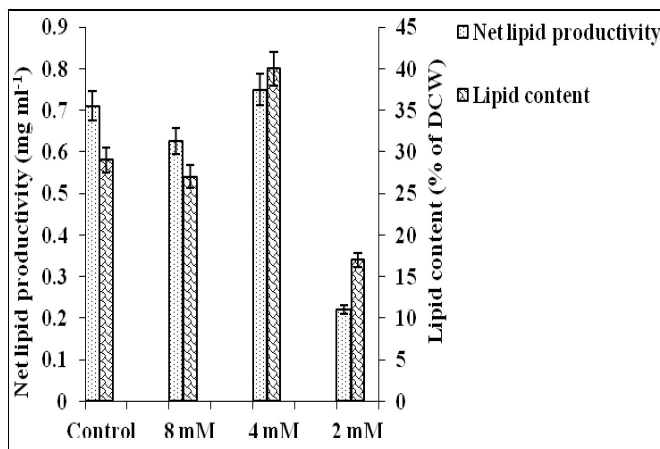

Fig 4: Net lipid productivity and lipid content of *A. quadricellulare* PUMCC 5.1.1 in different concentration of $NaNO_3$

Data on same day with same lower case alphabets are not significantly different from one another at 95% confidence level ($P_{cal<0.025}$)

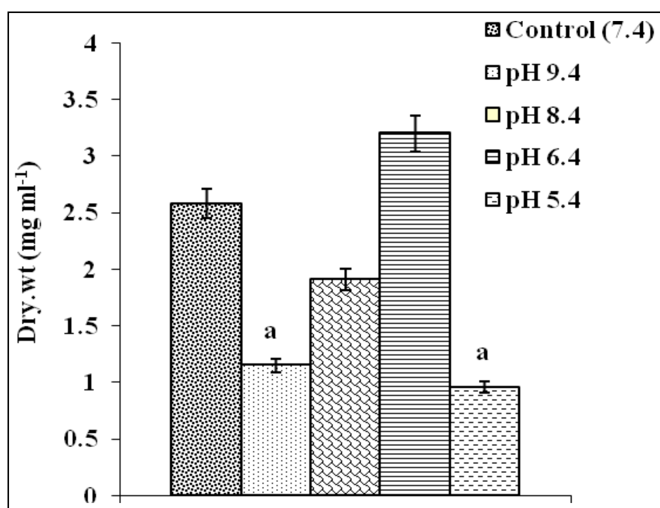

Fig 5: Growth of *A. quadricellulare* PUMCC 5.1.1 in different pH in terms of increase in dry cell weight cultures

Data on same day with same lower case alphabets are not significantly different from one another at 95% confidence level ($P_{cal<0.025}$)

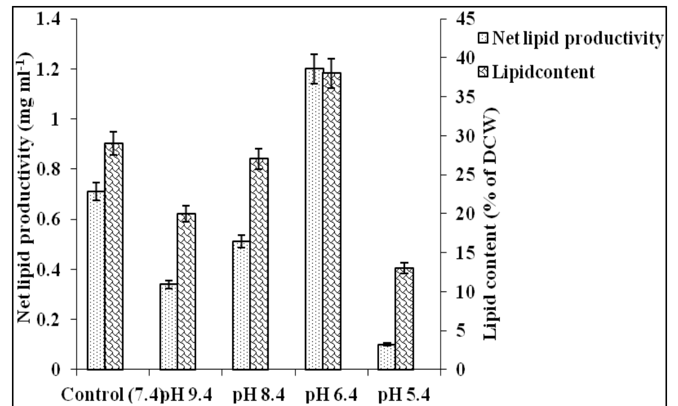

Fig 6: Net lipid productivity and lipid content of *A. quadricellulare* PUMCC 5.1.1 in different ph of medium

Data on same day with same lower case alphabets are not significantly different from one another at 95% confidence level ($P_{cal<0.025}$)

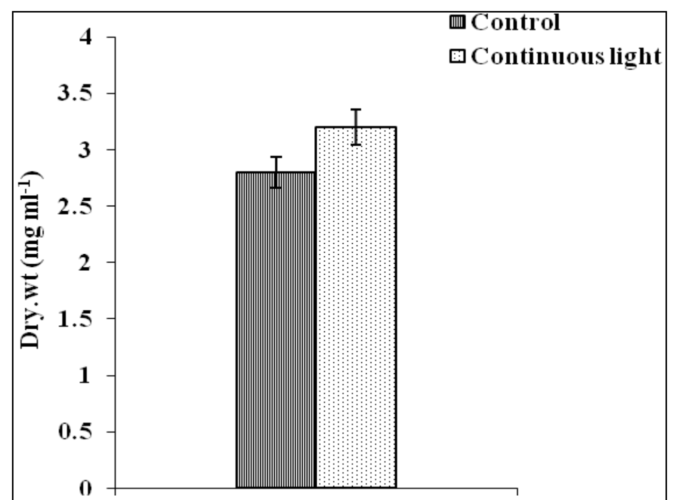

Fig 7: Growth of *A. quadricellulare* PUMCC 5.1.1 in terms of increase in dry cell weight of cultures

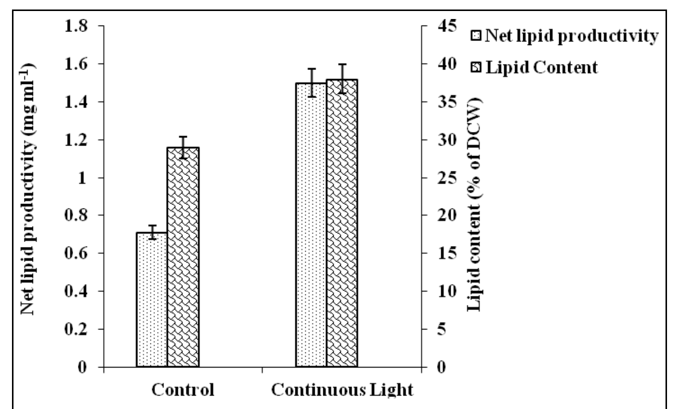

Fig 8: Net lipid productivity and lipid content of *A. quadricellulare* PUMCC 5.1.1 under continuous light

4. Conclusion

A. quadricellulare as algal good feedstock for biodiesel production. This species is a potential candidate for biofuel production at large scale when grown under continuous light. It is also observed that biomass, lipid content as well as fatty acid composition of lipids of studied microalgal strain are not positively correlated with one another.

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