Aplinkos tyrimai, inžinerija ir vadyba, 2012. Nr. 3(61), P. 14-25 *ISSN 1392-1649 (print)*
Environmental Research, Engineering and Management, 2012. No. 3(61), P. 14-25 *ISSN 2029-2139 (online)*
http://erem.ktu.lt

Influence of Dissolved Inorganic Carbon and Nitrogen Sources on Growth, Total Lipid Content and Calorific Value of the Freshwater Oleaginous Microalgae *Ankistrodesmus falcatus* (Corda) Ralfs

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crossref http://dx.doi.org/10.5755/j01.erem.61.3.1423

(received in March, 2012, accepted in September, 2012)

Biofuel potentiality of a native strain of freshwater oleaginous microalgae *A.falcatus* was studied owing to its inherently high lipid content. The influence of media constituents, pH and nitrogen sources on growth (μ), total lipid (TL) content and calorific value (CV) was compared in the batch culture. The bold basal medium was superior (μ = 0.498 d^{-1}, TL = 43.4% and CV = 23.4 kJg^{-1}) to BG11 (μ = 0.385 d^{-1}, TL = 38.2% and CV = 19.2 kJg^{-1}). Initial culture pH < 7.0 and > 9.0 significantly affected the growth of microalgae. Medium pH (≈ 7.0) and $NaHCO_3$ (30 mgL^{-1}), were found suitable for the optimum growth at the light intensity of 35 μ mol photons $m^{-2}s^{-1}$, the temperature of 25 ± 2 ^0C and the photoperiod of 16:8 h light and dark diurnal cycles. NO_3^- instead of NH_4^+ was preferred in the range of KNO_3 > $NaNO_3$ > NH_4NO_3 > $(NH_2)_2CO$ for maximum biomass and lipid production. Maximum biomass, TL and CV (0.843gL^{-1}, 42.7% and 20.6 kJg^{-1} respectively) were determined in the medium containing KNO_3 (17.6 mM). A close linear correlation between an increase in TL and CV was observed (R^2 = 0.9461). With the further augmentation of TL and improved biomass yield the native strain of microalgae *A.falcatus* could be a potent candidate for biofuel production.

Key words: *Ankistrodesmus falcatus, biofuel, calorific value, microalgae, oleaginous.*

1. Introduction

Spectacular global economic development coupled with the increasing population growth and changing life styles since the dawn of the Industrial Revolution have caused ravenous appetite for fossil fuels resulting in a manifold increase in anthropogenic emissions of greenhouse gases (GHGs). The unprecedented rise of anthropogenic GHGs emissions, whose 75% constitute carbon dioxide (CO_2), is chiefly responsible for global warming (Ramanathan 1998). As the global energy consumption is raising with increasing demands, an abrupt rise in CO_2 and other GHGs is bound to take place, unless there is a replacement of current primary energy resources with a carbon neutral clean energy system. The quest for renewable sources of carbon neutral alternative energy has therefore become the center stage of current energy researches and policies.

Biomass is the only renewable source of alternative energy that can replace fossil fuels in all energy markets - in the production of heat, electricity and fuels for transport. Concerted efforts are now globally underway to harness alternative energy from renewable biomass sources generated via photosynthesis to move towards more sustainable bio-based economies, where biomass-derived products substitute petrochemical-derived products (Borowitzka and Moheimani 2011). Development of the first and second generations of biofuel, which are based on different categories of biomass feedstock and downstream processing typically obtained from starch, oil and cellulosic crops, require cultivated land

and water supplies that can compete with the land use for food production (Hill et al. 2006; Brennan and Owende 2010; Piccolo 2008). Extensive cropping of plants for biofuel production, which will certainly take place with the increasing fuel demand, raises a food vs fuel dilemma and also natural resources demand problems (Walkar 2010; Schenk et al. 2008; Searchinger et al. 2008; Singh et al. 2011). With such limitations of the first and second generation biofuels, microalgae based third and further generations of biofuel have gained an astonishing interest (Campbell 1996; Chisti 2007; Li et al. 2008; Rodolfi et al. 2009; Mata et al. 2010; Spolaore et al. 2006).

Microalgae are considered to represent the only current renewable biomass source to generate biofuel, which are likely to have a much lower adverse effect on the environment and on the world's food supply than conventional biofuel-producing crops. The unique diversity of microalgae and the spectrum of species available for amenability for biofuel production as compared to other advanced biomass feedstock have placed microalgae in the priority list. Various species may be selected to optimize the production of different biofuels. Microalgae biomass can be utilized to produce varieties of fuels, such as liquid fuels and gases, gas-or oil-based biofuels, bioethanol or methanol, biohydrogen and biodiesel (Hill et al. 2006; Brennan and Owende 2010; Walkar 2010; Chisti 2007; Li et al. 2008; Miao and Wu 2004; Pirt et al. 1983; Kosaric and Velikonja 1995; Illman et al. 2000; Banerjee et al. 2002; Pienkos and Darzins 2009; Gong and Jiang 2011; Pokoo-Aikins et al. 2010; Melis and Happe 2001; Clarens et al. 2010). Moreover, microalgae offer a diverse spectrum of valuable products and pollution solutions, such as food, nutritional compounds, omega-3 fatty acids, animal feed, organic fertilizers, biodegradable plastics, recombinant proteins, pigments, medicines, pharmaceuticals, and vaccines (Pulz 2004).

Production of liquid biofuels from microalgae involves to some extent extraction or conversion before the algae can be used as a biofuel. The direct uses of microalgae biomass do not involve such processing and thus minimize the cost. Illman et al. (2000) reported the use of dried powder of microalgae *Chlorella* as fuel together with a mixture of 85% cellulose powder and 15% diesel to run a diesel engine. A high calorific value of microalgae is essential for their use as a fuel, which chiefly depends on the lipid content besides carbohydrate and protein. An increasing calorific value is directly related to an increase in lipid content (Illman et al. 2000).

The economic feasibility of microalgae based biofuel production depends basically on two major factors viz. the cost of commercial scale cultivation (appropriate technology), and the selection of microalgae strain (appropriate strain). High lipid content, faster growth rate, ease of harvest, and dominance in nature possessing an ability to adapt to the prevalent climatic conditions are some of the desirable criteria for choosing the right species for biofuel production (Gong and Jiang 2011). Isolation of native strains from local water bodies can provide the best information regarding the selection of appropriate strain(s) in terms of biomass and lipid productivity, dominant in the native algal flora, and hence they are likely to be the most suitable for being selected as biomass feedstock (Abou-Shanab et al. 2011; Pérez et al. 2004; Odlare et al. 2011). In addition to the species selection, a nutritional requirement for culture optimization is another essential factor that significantly affects the growth rate and the yield of products (Sanchez et al. 2000; Li et al. 2008). Providing optimum conditions some microalgae can double their biomass in less than 24 hours (Chisti 2007). Such high yield and high density biomass is ideal for intensive cultivation and can provide an excellent biomass source of biofuels. Numerous studies have been conducted on enhancement of the lipid content in a number of microalgae by applying various stress condition during cultivation such as nitrogen deprivation (Illman 2000; Sanchez 2000), high light intensity (Khotimchenko and Yakovleva 2005), high salinity (Araujo et al. 2011; Rao et al. 2007), phosphate limitation (Reitan et al. 1994), or co-immobilizing in alginate beads with bacterium *Azospirilium brasilense* (Lebsky et al. 2001; de-Bashan 2002). Significant increase in lipid contents in microalgae was reported after being subjected to such stress conditions. Oil levels of 20-50% in dry weight biomass are quite common and some can exceed 80% (Day et al. 2011). However, stress conditions also have a negative influence on growth resulting in low overall biomass production. Therefore, it is more appropriate to apply stress condition in the later stage of the growth after the initial biomass production (Liu et al. 2008).

The inherently high lipid containing microalgae *A.falcatus* were previously extensively studied in the biodiesel production under the Aquatic Species Programme, USDOE (Sheehan et al. 1998). *A.falcatus* was also reported among very high lipid productivities (109 mg L^{-1} d^{-1}) with a high biomass productivity (0.46 g L^{-1} d^{-1}) (Griffiths and Harrison 2009). Kilham et al. (1997) reported the changes in biochemical contents and compositions of *A.falcatus* under various nutritional conditions. They showed increased lipid contents with increasing TGA under phosphorous limited conditions of both nitrogen limitation and non-limited conditions. Our preliminary screening of native oleaginous microalgae resources revealed the ubiquitous nature of *A.falcatus* strain in the region containing high total lipid content of around 40 ± 5% that could be utilized as a potential renewable feedstock of biofuel.

The present study was focused on growth, biomass productivity and total lipid content of a native Indian strain of freshwater microalgae A.falcatus. The principal objectives were: to investigate the influence of medium compositions, added nitrogen sources, dissolved inorganic carbon concentration and pH on growth with the aim to obtain optimum conditions for an enhanced growth rate and biomass productivity, improved lipid yield

and higher calorific value under laboratory conditions preceding the mass cultivation.

2. Methods

2.1. Microalgae strain isolation

A native strain of freshwater Chlorophyta - *A.falcatus* was isolated from the natural water samples collected from wetland Deepor Beel (Ramsar site), located (26.13°N 91.66°E) in Guwahati, Assam, India. Isolation of the individual algae was done after several dilutions followed by streaking into an agar plate containing the autoclaved BG11medium (Boussiba and Vonshak 1991) solidified with 1% agar (w/v) according to Kawai et al. (2005). Petri plates were allowed to stand for 4-7 days under controlled laboratory conditions. Individual algal cells were picked up by means of a sterilized Pasteur pipette and re-inoculated into 10 ml of the sterilized BG11 medium in screw-capped glass vials. Purity of the culture was periodically checked under microscope (LOBAMED, Model: ATC 2000) and by streaking into agar plates. Contamination free culture from other algal species was raised using BG11 medium. Subculture was done after every third week of inoculation.

2.2. Growth conditions and growth media

Isolated mono cultures of the tested microalgae were raised and maintained aseptically in the autoclaved BG11medium under controlled laboratory conditions. The pH of the medium was adjusted to 7.5 with either 1N HCl or 1N KOH solution prior to autoclaving. The growth conditions were: irradiance 35 µ mole photons. $m^{-2}s^{-1}$, temperature 25 ± 2 ^{0}C and 16:8 h light and dark diurnal cycles.

2.3. Effects of medium constituents and initial medium pH on growth

A triplicate study was conducted for 12 days of culture duration using 100 ml of BG11 and Bold Basal Medium (BBM) (Nichols and Bold 1969) in Erlenmeyer flasks to compare the growth responses of the studied microalgae species. The medium was adjusted to pH 7.5 with either 1N HCl or 1N KOH prior to autoclaving. Various constituents of both media are shown in Table 1.

Growth responses of the microalgae at various initial medium pH were investigated in a batch mode using the BG11 medium for 7 days of culture duration in order to observe the influence of initial medium pH on growth as well as dynamic changes of pH over the culture duration. The medium pH was adjusted to six levels of initial medium pH (from 5.0 to 10.0) in an increasing order with either 1N HCl or 1N KOH at the beginning of the experiment prior to autoclaving. The medium pH was not further adjusted to its initial value during the study period. Dynamic changes of the culture pH were studied in a batch mode for culture duration of 10 days. The initial culture pH was adjusted to pH 7.5 prior to autoclaving similarly as stated in the earlier experiment. Changes in the culture medium pH and an increase in cell numbers over culture duration were recorded after every 24 h using a standard laboratory pH meter (L1 120, Elico, India). Studies were conducted in triplicate under similar growth conditions as stated in earlier section.

Table 1. Culture media recipes

Constituents (gL^{-1})	BBM	BG11
$NaNO_3$	1.0	1.5
$CaCl_2.2H_2O$	0.025	0.036
$MgSO_47H_2O$	0.075	0.075
K_2HPO_4	0.075	0.4
NaCl	0.025	Nil
EDTA	Nil	0.001
Citric acid	Nil	0.006
Na_2CO_3	Nil	0.2
$FeCl_3\ 6H_2O$	0.005	Nil
$NaMoO_4$	4.2×10^{-5}	2.1×10^{-5}
$ZnSO_47H_2O$	4.4×10^{-4}	2.2×10^{-4}
$MnCl_24H_2O$	3.6×10^{-3}	1.8×10^{-3}
$CuSO_45H_2O$	1.6×10^{-5}	0.8×10^{-5}
H_3BO_4	5.7×10^{-3}	2.8×10^{-3}

2.4. Effects of dissolved inorganic carbon (DIC) on growth

Influence of DIC concentration on the growth responses of the tested microalgae was studied using five levels of sodium bicarbonate ($NaHCO_3$) salt concentration (10, 30, 50, 70 and 90 mg L^{-1}) in BG11 (Na_2CO_3 was excluded while preparing the $NaHCO_3$ enriched media). Normal BG11 medium was used as the control. Initial pH of each treatment was adjusted to pH 7.5 before autoclaving. The batch study was conducted in triplicate using 100 ml of the culture medium in 250 ml volume Erlenmeyer flasks, inoculated with 20% (v/v) of the exponentially growing culture. Growth conditions were the same as stated in earlier Sections.

2.5. Effects of added nitrogen (N) sources

Influence of various N-sources on the growth responses of the strain were studied in the batch culture in triplicate for 21 days. The BG11 medium was modified by substituting sodium nitrate ($NaNO_3$) as a regular N-source with equal molar concentration (17.6 mM) of potassium nitrate (KNO_3), urea $(NH_2)_2CO$ and ammonium nitrate (NH_4NO_3), respectively. The other medium constituents and pH were kept unchanged as those of the normal BG11 medium. For inoculation, 20% (v/v) of the two weeks old exponentially growing culture was used as inoculums for each of the treatment.

2.6. Growth evaluation

Growth characteristics were determined from the respective growth curves developed by plotting the observed number of cells (cells ml^{-1}) against the time of observations (days). Cell numbers were determined from a direct cell count using Neubour Haemocytometer. Cell density was determined by measuring optical density of the culture at 730 nm using a Spectrophotometer (SYSTRONICS, Model No.104). For each parameter, the average values were calculated from the data generated from three replicates of each study. Specific growth rate (μ) and doubling time (T_2) were calculated by using equations (1) and (2) according to Levasseur et al. (1993).

$$\mu = \ln (N_2/N_1) / t_2\text{-}t_1 \quad (1)$$

$$T_2 = 0.6931/\mu \quad (2)$$

where:
N_1 and N_2 – cell numbers at time t_1 and time t_2, respectively.

Biomass was determined as dry cell weight (DCW) and measured gravimetrically. A known volume of the culture was filtered through pre-weighed and pre-combusted GF/C filter paper. The filtered cell mass was oven dried at 80 °C for 6 h until the constant weight; cooled down to the room temperature in desiccators and dry weight of the sample was measured using an analytical balance with precision of 0.1 mg. Biomass was expressed in gram DCW per liter (gL^{-1}).

2.7. Determination of total cellular carbohydrate and protein content

Total cellular carbohydrate and protein content was determined from a known amount of the freeze dried cell biomass harvested at the end of a logarithmic phase of the growth. Protein content was determined according to Lowry et al. (1951) and carbohydrate content was determined according to Hedge et al. (1962).

2.8. Extraction and estimation of total cellular lipid content

Total cellular lipid content was determined quantitatively. A known volume of the culture was harvested during a late exponential phase of growth by centrifugation at 4000 rpm for 3-5 minutes, rinsed with 1% NaCl solution followed by rinsing with double distilled water twice. The biomass was lyophilized using a freeze-dryer (ALPHA 1-4, Germany) for 36-48 h and kept in a desiccator until its constant weight. Total lipids were extracted from a known amount of the finely grinded lyophilize cell biomass with 2:1 mixture of chloroform ($CHCl_3$) and methanol (CH_3OH) according to the method of Bligh and Dyer(1959). The residue was re-extracted (2-3 times) with $CHCl_3$. The filtrate was taken in a separating funnel and lipids were obtained in a bottom $CHCl_3$ layer formed upon adding 0.9% NaCl and collected in a cleaned pre-weighted glass vial. The solvent was evaporated at 55ºC in a water bath to near dryness and dried in desiccators under reduced pressure over anhydrous Na_2SO_4. The dried lipid extract was measured gravimetrically and expressed in percent (%) of total lipids per dry cell weight (DCW).

2.9. Determination of calorific value (CV)

Calorific value (CV) of studied microalgae was determined using an automatic adiabatic bomb calorimeter (Model: RBC 106/09) according to DIN 51900 T3 (testing of solid and liquid fuels, determination of gross calorific value by a bomb calorimeter and calculation of net calorific value; method with adiabatic jacket). A crucible with a pellet containing 1 g of dry algal biomass (moisture free) was inserted into the bomb and filled with oxygen (99.99% purity) under pressure of 30 bars. The bomb was placed in the adiabatic calorimetric equipment filled with water and the sample was ignited (Illman 2000). Calorific value was calculated from the resulting increase in the water temperature using equation (3).

$$CV = (W_E \times \Delta T/W_M) \times (4.18400 \times 10^{-3}) \text{ kJ } g^{-1} \quad (3)$$

where:
W_E – enthalpy of water equal to 2568.293 cal g;
ΔT – differences in temperature;
W_M - sample weight.

2.10. Statistical analysis

Statistical analysis such as, standard error of means (SE), two way analysis of variance (ANOVA), etc were done using the software GraphPad Prism version: 5.00.

3. Results

3.1. Effect of medium

The growth pattern of the native *A.falcatus* strain in two media, BG11 and BBM are depicted in Fig. 1(A & B). The principal compositional variation was the presence of 40 mM NaCl in BBM, while the BG11 medium was devoid of any NaCl in its composition. After an initial lag phase of about 2-3 days, the algae grew exponentially from the 4^{th} day onwards. The exponential growth phase in BBM lasted till the 8^{th} day with the maximum specific growth rate (μ) of 0.498 d^{-1} and the least doubling

time (T_2) of 1.39 days. On the other hand, the exponential growth phase in the BG11 medium lasted between the 4 - 6th days with a comparatively slower growth rate (μ = 0.385 d^{-1} and T_2 = 1.8 days) than that in BBM.

Total cellular lipid, carbohydrate and protein contents were estimated from the biomass at the end of the experiment (12 days of cultivation). Marginal increase in lipid (43.3%), carbohydrate (13.5%) and protein (5.1%) contents were recorded for the culture grown in the BBM compared to those of the BG11 medium (lipid 38.4%, carbohydrate 12.5% and protein 3.1%).

3.2. Effect of initial culture pH on growth

Growth responses of the studied *A.falcatus* strain under different initial culture pH (5.0 to 10.0) are shown in Table 2. The strain was found to grow in a wide spectrum of the initial culture pH, although the growth rates were significantly influenced by the initial culture medium pH value of < 7.0 and > 8.0 ($P < 0.0001$) (Fig. 2A). . The maximum growth (μ = 0.448d^{-1}) was observed with initial medium pH 8.0 till the 4th day from the day of inoculation, which slightly decreased afterwards producing an increase in total cell numbers of 26.5 × 10^5 ml^{-1}. Culture medium of pH 7.0, on the other hand, supported a constant growth of the strain with the highest specific growth rate of 0.445 d^{-1} producing the maximum cell numbers (27.6 × 10^5 ml^{-1}) after 7 days of cultivation (Table 2). Otherwise, an increase in cell numbers was the least in the culture grown with initial culture pH 10.0 (5.0 × 10^5 ml^{-1}) followed by pH 9.0 (11.2 × 10^5 ml^{-1}) and pH 5.0 (12.2 × 10^5 ml^{-1}). Results have clearly indicated that the initial culture pH has a significant effect on growth of the studied *A.falcatus* strain, which favors a neutral or nearly neutral pH (≈ pH 7.0 - 8.0) to sustain its optimum growth. Growth was retarded with pH of the medium below pH 7.0 and above pH 8.0.

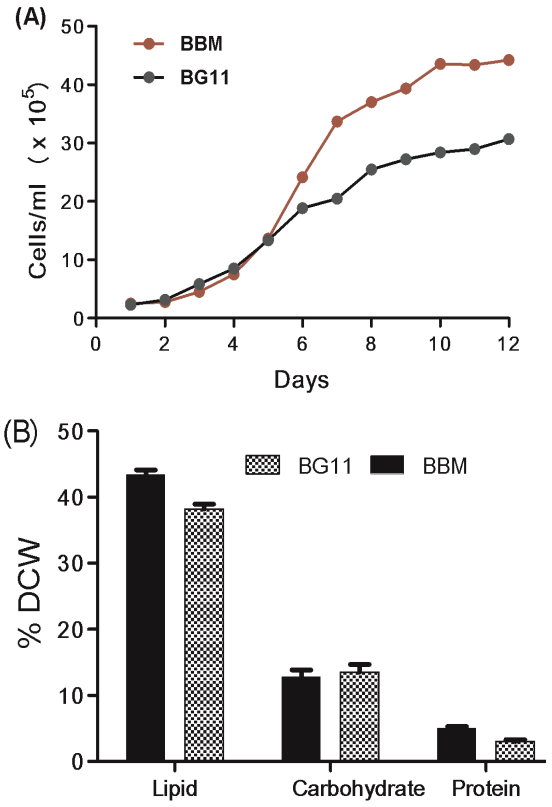

Fig. 1. Effect of medium constituents on (A) growth and (B) biochemical contents of A.falcatus. Error bars represent SE (n = 3).

Table 2. Effect of initial pH on growth

Days	Cells ml^{-1} (× 10^5)[a]					
	pH 5.0	**pH 6.0**	**pH 7.0**	**pH 8.0**	**pH 9.0**	**pH 10.0**
1	5.2 ± 0.14	5.3 ± 0.25	5.4 ± 0.08	5.3 ± 0.08	5.2 ± 0.05	5.3 ± 0.11
2	5.6 ± 0.11	8.0 ± 0.17	9.1 ± 0.15	11.1 ± 0.03	8.5 ± 0.21	5.3 ± 0.25
3	7.4 ± 0.17	11.3 ± 0.28	14.8 ± 0.25	18.5 ± 0.08	12.7 ± 0.31	6.0 ± 0.17
4	11.3 ± 0.25	16.2 ± 0.42	23.1 ± 0.15	23.3 ± 0.32	15.3 ± 0.35	7.7 ± 0.28
5	13.9 ± 0.15	20.7 ± 0.17	28.1 ± 0.28	27.2 ± 0.23	16.2 ± 0.21	8.8 ± 0.40
6	15.2 ± 0.23	22.6 ± 0.35	31.1 ± 0.17	29.6 ± 0.29	15.8 ± 0.46	10.1 ± 0.15
7	17.9 ± 0.13	25.2 ± 0.31	33.1 ± 0.26	31.8 ± 0.85	16.4 ± 0.40	10.3 ± 0.23
μd^{-1}	0.203 ± 0.002	0.353 ± 0.01	0.454 ± 0.02	**0.483 ± 0.01**	0.26 ± 0.003	0.098 ± 0.01
Increase in cells ml^{-1} (× 10^5)	12.7	19.9	**27.6**	26.5	11.2	**5.0**

[a]cell numbers are average of 10 independent counts; '±' represents standard error (SE) of means, n=3

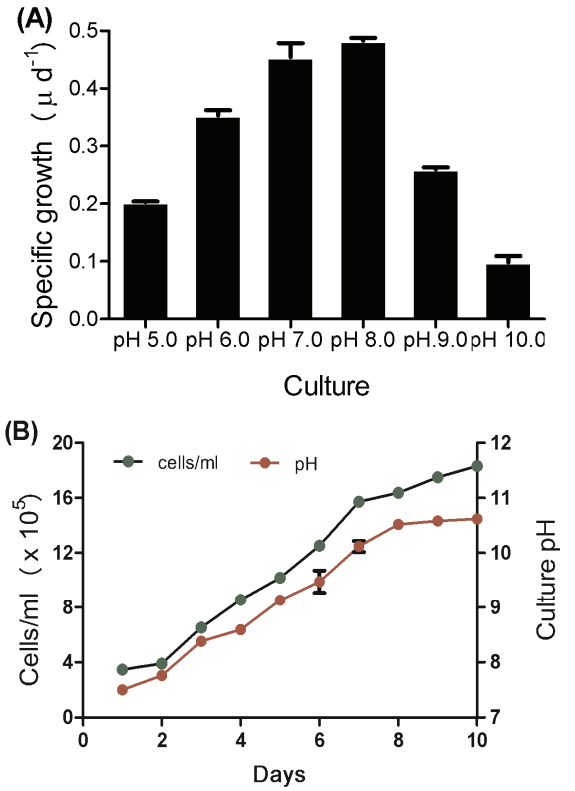

Fig. 2. Effect of initial culture pH on growth of A.falcatus strain (A). Dynamic change of culture pH and cell abundance during the experiment (B). Error bars represent SE (n = 3)

3.3. Dynamic change in culture pH over culture duration

A dynamic change of the culture pH in a studied batch mode, where the culture pH was not controlled intermediately is shown in Fig. 2(B). Irrespective of the initial culture pH (7.5) at the time of inoculation, the pH of the culture medium was found to increase gradually, reaching over 10.0 after 7 days of the culture period and then the increase in culture pH was nearly steady till the terminal 10^{th} day of the experiment (Fig. 2B). As seen from Fig 2(B) an increase in the culture pH as high as over 10.0 (day 7 onwards after inoculation) retarded the further growth and increase in cell numbers of the studied *A.falcatus* strain under the tested conditions.

3.4. Effect of dissolved inorganic carbon (DIC) on growth

DIC concentrations in terms of $NaHCO_3$ at various concentrations were found to influence growth of the studied *A.falcatus* strain (Fig. 3). $NaHCO_3$ at a concentration of 30 mg L^{-1} significantly ($P < 0.001$) stimulated the growth rate with the maximum specific growth rate of 0.368 d^{-1}. However, the concentration of bicarbonate salt beyond 50 mg L^{-1} retarded the growth rate. The maximum net increase in cell numbers 26.5 x 10^5 ml^{-1} was recorded in the medium supplemented with 30 mg L^{-1} of $NaHCO_3$, followed by 50 mg L^{-1} of $NaHCO_3$ (21.5 x 10^5 ml^{-1}) compared to 17.3 x10^5 ml^{-1} at the control on the terminal experiment day. No significant ($P > 0.05$) differences were found in other treatment with the control.

3.5. Effect of added N-sources on biomass and lipid productivity

Growth of the studied *A.falcatus* strain was influenced by the forms of N-sources contained in the culture medium, both in terms of biomass and lipid yield (Fig. 4A-B). Among the tested N-sources, KNO_3 was found to be the most suitable to grow the studied microalgae strain for biomass production (Fig. 4A). The strain yielded maximum 0.843 g L^{-1} biomass (DCW) containing 42.76% of total lipid content after 21 days of the culture period in the medium fed with KNO_3. The highest biomass and lipid productivity of 0.038 g $L^{-1}d^{-1}$ and 0.019 g $L^{-1}d^{-1}$, respectively, was also recorded in the culture fed with KNO_3; whereas both biomass and lipid productivity were the least in the cultures fed with urea (0.007 g $L^{-1}d^{-1}$ and 0.004 g $L^{-1}d^{-1}$, respectively) (Fig. 4B). The culture fed with $NaNO_3$ produced higher lipid content (41.3%) than that of NH_4NO_3 (37.7%), although the total biomass yield did not significantly differ (Fig. 4A). Based on the results revealed from our studies, KNO_3 was found to be the most suitable N-source for growing the studied *A.falcatus* strain considering its both high lipid and biomass productivity.

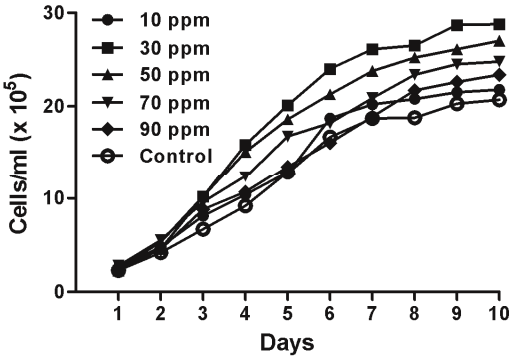

Fig.3. Effect of different concentrations of DIC on growth of A.falcatus strain.

3.6. Calorific value (CV)

The calorific value of biomass yield of the studied *A.falcatus* strain was found to vary with culture media compositions that directly influence the total lipid content of the biomass (Table 3). *A.falcatus* grown in the BG11 medium for a cultivation period of 12 days containing lipid 38.2% resulted in a calorific value 19.2 kJg^{-1}. Biomass produced in BBM for the same cultivation period containing a lipid content of 43.4% revealed an increased calorific value 23.4 kJg^{-1}. The lowest calorific value 10.46 kJg^{-1} was determined in biomass containing the least lipid content (23.16%) of

cultures grown in urea, while the biomass produced in KNO_3 supplemented culture containing the maximum lipid content (42.76%) revealed the highest calorific value (20.56 kJg^{-1}).

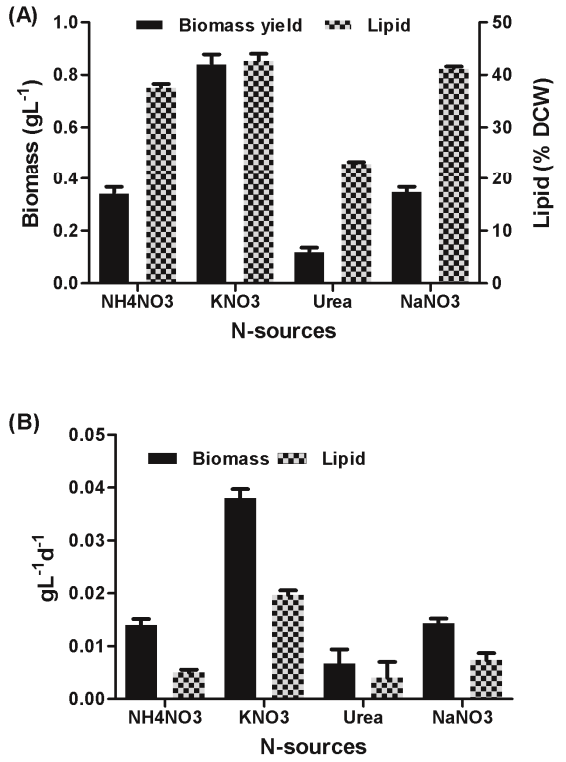

Fig.4. *Effect of added nitrogen sources on biomass and lipid production (A) and productivity (B) of A.falcatus strain. Error bars represent SE (n = 3).*

Table 3. *Comparison of calorific values of biomass under different treatments*

Treatments	**[a] Lipid (% DCW)**	**[a] Calorific value (kJg^{-1})**
Medium		
BG11	38.20 ± 0.737	19.20 ± 0.099
BBM	43.40 ± 0.700	23.43 ± 0.120
[c] N-sources		
KNO_3	42.76 ± 1.255	20.56 ± 0.433
$NaNO_3$	41.31 ± 0.289	20.06 ± 0.318
NH_4NO_3	37.70 ± 0.586	17.43 ± 0.120
Urea	23.16 ± 0.203	10.46 ± 0.145

[a] values are mean of three independent values; [b] values are mean of five independent values; [c] equal molar concentration of 17.6 mM was used for all N-sources in BG11 medium. '±' represents standard error of means

4. Discussion

The present studies on native strain of *A.falcatus* have revealed a high lipid content possessing potentialities to be used as renewable biomass feedstock for biofuel production. Our results have demonstrated that the strain favors BBM over BG11 for growth. In BBM the highest cell numbers (44.2 x 10^5 ml^{-1}) were recorded after 12 days of cultivation with an initial cell number of 2.5 x10^5 ml^{-1} at the time of inoculation. Marginal increase in biochemical contents such as lipid (43.4%), carbohydrate (12.8%) and protein (5.0%) at dry weight of biomass were also recorded in the cultures grown in BBM while in BG11 medium they were: lipid 38.2%, carbohydrate 13.5% and protein 3.0%. The growth stimulating activity of the BBM might be attributed to the presence of a low level of NaCl (0.025 g L^{-1}) in its constituents. Fodorpataki and Bartha (2004) also reported similar observations of such marginal increased in biochemical contents due to NaCl induced changes in growth conditions. BG11 medium on the other hand does not contain any NaCl. The results were in parity with an earlier report on *A.falcatus* strain (Kalita et al. 2011). Similar results on enhanced growth in BBM were also reported in *Haematococcus pluvialis* (Dominguez-Bocanegra et al. 2004; Imamoglu et al. 2009).

Freely available CO_2 and OH flux of the medium were reported to influence the nitrate uptake of *Ankistrodesmus* species (Eisele and Ullrich 1997) and growth of green algae species (Gardner et al. 2011). Medium pH is supposed to act mainly at the plasmalemma since pH within the cell and its compartments can be regarded as fairly stable. In the absence of CO_2, nitrate uptake, and thereby nitrate reduction in *Ankistrodesmus* were reported to be moderately inhibited, which is strongly pH dependent. Optimal uptake was reported in between pH 8.0 and about 9.0 (Eisele and Ullrich 1997). Our experimental results from the experiment on influence of initial culture pH have indicated that the native strain of *A.falcatus* can grow in a wide range of culture pH (5.0 - 10.0); however, its growth rate was significantly retarded in both acidic (< pH 7.0) and alkaline medium (> pH 9.0). The least number of cells (10.3 ml^{-1} x 10^5) was determined in the culture grown with initial pH 10.0. Culture medium pH between 7.0 and 9.0 was found to be suitable for growing the studied microalgae strain. The results were in accordance with the findings of Eisele and Ullrich (1997) in *A.braunii*. They further reported that at pH 8.2, the nitrate uptake was more rapid and almost the entire nitrate taken up was released as ammonia in the absence of CO_2, which might be the reason for an abrupt rise in medium alkalinity at the later stage of growth while no external CO_2 was supplied. Similar results were also found in our study, where a significant rise in the medium pH (< 10.0) was observed at the terminal day of the experiment. Such increase in culture pH significantly retarded further growth of the microalgae strain in the studied batch mode. An intermediate pH control mechanism is therefore essential in addition to nutrient addition for supporting continuous growth of the strain.

CO_2 is known to be one of the most important nutrients along with other essential minerals and optimum light condition for growing microalgae

photoautrophically. To sustain a stable growth and higher biomass production, CO_2 must be added into the culture medium from external sources. In this respect, many researchers (Powell et al. 2009) reported supply of pure CO_2 as a mixture of air plus CO_2 at different ratios. However, due to very low solubility of CO_2 in water (1.45 g L^{-1} at 25 ^{0}C, 100 kPa) (Wiebe et al. 1940), the majority of carbon dioxide tends to be lost in the air. Moreover, feasibility of appropriate technology, easy availability, and energy intensiveness hinder practical applicability of the technology. Alternatively, bicarbonate salts such as $NaHCO_3$ are highly soluble in water, which can serve as a better source of CO_2 for growing microalgae with minimum efforts. When $NaHCO_3$ dissolves in water, it dissociates to produce HCO_3^- ions that serve for microalgae as readily available dissolved inorganic carbon. However, the optimal upper limit of $NaHCO_3$ concentrations in the growth medium vary accordingly with varying microalgae species/strains (Gardner et al. 2011). Our results indicated that the medium containing 30 mg L^{-1} of $NaHCO_3$ stimulated the growth of the studied *A.falcatus* strain. The strain showed its best performance in the BG11 medium supplemented with 30 mg L^{-1} of $NaHCO_3$ with its highest specific growth (μ = 0.368 d^{-1}) and a maximum increase in cell numbers (26.5 x 10^5 ml^{-1}) in comparison to the control BG11 medium (μ = 0.278 d^{-1}; 17.3 x 10^5 ml^{-1}). At higher concentrations of $NaHCO_3$ (beyond 30 mg L^{-1}), the growth was retarded. Gardener et al. (2011) also reported similar observations in *Scenedesmus* sp strain WC-1 and Phaeodatylum tricornutum strain Pt-1.

Nitrogen, an essential component of protein and other constituents of protoplasm, is a major nutrient affecting the productivity of microalgae. Microalgae are known to be capable of utilizing various forms of nitrogenous sources (N) both a dissolved form of inorganic nitrogen (DIN) and a dissolved form of organic nitrogen (DON). Flynn and Butler (1986) reviewed the utilization of various forms of N-sources in marine microalgae. Selection of a suitable N-source for growing microalgae is another essential criterion for mass production, though the preferences for utilization vary from species to species (Tepe et al. 2006). In our studies, the native *A.falcatus* strain was found to favor nitrates (NO_3^-) over other forms of N-source for suitable growth. Lipid contents were also recorded higher in the cultures grown in the medium containing NO_3^- salts of potassium (K), sodium (Na) and ammonium (NH_4^+) than in the medium containing urea ($(NH_2)_2CO$) as the sole N-source. The results were in parity with similar observations reported by Chen et al. (2011) in green microalgae *Dunaliella tertiolecta*. The studied *A.falcatus* strain revealed the highest biomass (0.843 g L^{-1} DCW) and lipid (42.76% DCW) contents in the medium fed with KNO_3 (17.6 mM). Cultures fed with equal molar concentrations of NH_4NO_3 and $NaNO_3$ did not demonstrate any significant differences in total biomass production (0.35 g L^{-1} and 0.34 g L^{-1} respectively), while total lipid content was found to be higher in $NaNO_3$ (41.31% DCW) than that in NH_4NO_3 (37.7% DCW). Urea was found to be the least favored N-source for growing the studied microalge strain. The results were in accordance with the observations of Tepe et al. (2006) in *S.acuminatus* that inorganic N-sources are superior to organic N-sources. Organic N-sources need to be mineralized prior to be utilized by microalgae. Similar observation was also reported for *Scenedesmus* sp. LX1 (Xin et al. 2010), and *Chlorella protothecoids* (Shen et al. 2010). The order of preferences of different nitrogen sources of the studied *A.falcatus* strain was NO_3-N > NH_4-N > Urea. However, we strongly feel that more studies are needed to be carried out to find the optimum N-concentration; a much cheaper and better N-source in order to be utilized for mass cultivation of the native *A.falcatus* strain.

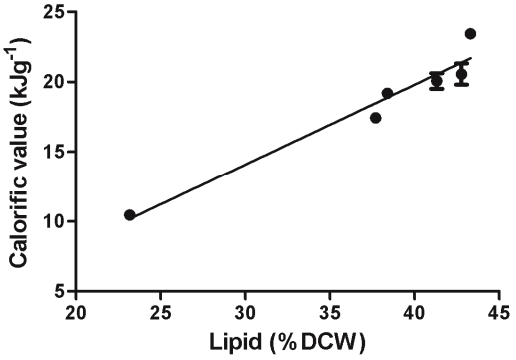

Fig. 5. Co-relation between increased lipid content and calorific value of A.falcatus strain; a close relationship of increasing calorific value with an increase of the total lipid content was observed (R^2 = 0.9461). SE were calculated from the mean, n = 3.

The cell biomass grown in normal BG11 medium has revealed calorific value in between 19.2 – 20.6 kJg^{-1} (Table 3), which is in a close agreement with Illman et al. (2000). The principal contribution to the calorific value of cell biomass comes from its total cellular carbohydrate, protein and lipid content (Illman et al. 2000; Scragg et al. 2002; 2003). Microalgae grown under normal conditions were reported to have calorific values between 18 - 21 kJg^{-1}, which is much lower than the calorific value of diesel (42 kJg^{-1}) (Illman et al. 2000). However, improved lipid content and thereby improved calorific values were also reported (Illman et al. 2000; Shen et al. 2010), which is also evident from our results (Table 3 and Fig. 5). The tested A. falcatus strain grown in BBM yielded an increased level of lipid content (43.4%) and revealed an increased calorific value (23.4 kJg^{-1}). Similar results were also reported by Ilmann et al. (2000) in *Chlorella vulgaris* and *C.emersonii*. The studied *A. falcatus* strain achieved a higher biomass yield (0.843 g L^{-1}) and productivity (0.038 g $L^{-1}d^{-1}$) in BG11 medium fed with KNO_3. The

lipid content (42.76%) and productivity (0.019 $gL^{-1}d^{-1}$) were also higher in the KNO_3 supplemented BG11 medium compared to the cultures fed with other N-sources resulting in an increased calorific value of 20.56 kJg^{-1} (Table 3, Fig. 4(A-B). A close relationship (R^2 = 0.9461) between the increasing lipid content and calorific value of *A.falcatus* was observed (Fig. 5). It is envisaged that microalgae biomass should be used as renewable biofuel for electricity generation using static diesel engines (Illman et al. 2000; Scragg et al. 2003) for which large microalgae biomass production with high productivity and high lipid content must be maintained.

Potential benefits of microalgae cultivation are enormous, and research into algae production using wastewater and power station emissions are inviting a highly prioritized proposition in sustainable and economic development. The ability to expand algal oil harvests depends mostly on: (a) isolation of new strains of algae that produce large amounts of desirable lipids, and (b) identification of environmental conditions that promote rapid growth of the oil-producing algae. Results of our present investigation have revealed the potentialities of the native microalgae strain *A. falcatus* to be utilized as a renewable biomass feedstock of biofuels. Our experimental results may be helpful towards a large-scale algae cultivation of the studied microalgae strain, which could be potentially used for sustainable biofuel production.

Acknowledgment

Partial financial support from the Defence Research Laboratory, Tezpur, Assam, DRDO, the Ministry of Defence, Govt. of India is gratefully acknowledged. Valuable suggestions from the reviewers are appreciated.

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Tirpių neorganinių anglies ir azoto junginių įtaka mikrodumblių *Ankistrodesmus falcatus (Corda)* Ralfs augimui, lipidų ir energetinės vertės nustatymas, auginimo švariame vandenyje ir vandenyje su priedais palyginimas

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(gauta 2012 m. kovo mėn., priimta spaudai 2012 m. rugsėjo mėn.)

Biologiniams degalams gaminti puikiai tinka švariame vandenyje augantys aliejiniai mikrodumbliai A. falcatus, kurie buvo anksčiau analizuoti dėl didelio lipidų kiekio juose. Siekiant nustatyti cheminę sudėtį, pH, azoto kiekį (μ), bendrą lipidų kiekį (TL) ir energetinę vertę (CV), laboratorijoje buvo atlikti tyrimai su mikrodumbliais. Duomenys rodo, jog dumblių, augintų laboratorijoje, rezultatai buvo geresni (μ = 0,498 d–1, TL = 43,4 % ir CV = 23,4 kJg–1) nei dumblių, augintų lauke (μ = 0,385 d–1, TL = 38,2 % ir CV = 19,2 kJg–1). Esant pH <7,0 ir >9,0, buvo stipriai neigiamai paveiktos dumblių kolonijos. Vidutinis pH (≈ 7,0) ir NaHCO3 koncentracija (30 mgL–1), šviesos intensyvumas 35 μ mol fotonų m–2s–1 , temperatūra 25 ± 2 0C ir 16:8 h šviesos ir tamsos fotoperiodinis ciklas – optimalios sąlygos dumbliams augti. Vietoj NH4+ buvo naudojamas NO3– jonas, kuris gaunamas iš junginių, naudojamų atitinkamai pagal eilę KNO3 > NaNO3 > NH4NO3 > (NH2)2CO, siekiant sukurti maksimalų biologinės masės ir lipidų koncentracijos prieaugį. Maksimalus biomasės kiekis, lipidų kiekis (TL) ir energetinė vertė (CV) (0,843gL–1, 42,7 % ir 20,6 kJg–1 atitinkamai) buvo nustatyta naudojant vidutinės koncentracijos KNO3 (17,6 mM) tirpalą. Taip pat buvo nustatyta teigiama koreliacija tarp lipidų kiekio ir biologinės masės prieaugio (R2 = 0,9461). Įvertinus šiuos argumentus ir padidinus biologinės masės bei lipidų prieaugį, būtų galima teigti, jog mikrodumbliai A. falcatus galėtų būtų viena iš alternatyvų biologiniams degalams gaminti.