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Optimization of growth of two microalgal isolates for biofuel feedstock

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Abstract

A research involving random isolation and characterization of naturally occurring microalgae in Bangladesh was carried out for assessing their potential for biofuel feedstock and other uses. Among the isolates, one identified as a green alga *Chlamydomonas noctigama* and another one as blue-green alga *Nostoc spongiaeforme* were grown in modified Chu-10D medium. The optimization of their growth was performed following incubation of the isolates under different levels of media concentration, temperature, pH, light intensity and aeration. Both the species showed optimum growth in terms of total chlorophyll at a temperature of 25^{o}C. However, significant differences (at 5% level) in growth were observed for the isolates under other conditions. The optimum growth of *Chlamydomonas noctigama* was observed for the vitamin B1+B6, whereas there was no significant difference on growth of *Nostoc spongiaeforme* for any vitamin supplement. The optimum pH for the growth of *Chlamydomonas noctigama* and *Nostoc spongiaeforme* were 6.5 and 7.5, light intensity 110 μEm^{-2} s^{-1} and 70 μEm^{-2} s^{-1}, and media concentration 2x and 1x of normal concentration, respectively.

Keywords: Algae; Green alga; Blue-green alga; *Chlamydomonas noctigama; Nostoc spongiaeforme;* Optimization

Introduction

Algae are emerging to be one of the most promising long-term, sustainable sources of biomass and oils for fuel, food, feed, and other co-products. Algae efficiently use CO_2, and are responsible for more than 40% of the global carbon fixation, with the majority of this productivity coming from marine microalgae. Algae can produce biomass very rapidly, with some species doubling in as few as 6 hrs, and many exhibiting two doublings per day (Hannon *et al.,* 2010). All algae have the capacity to produce energy-rich oils, and a number of microalgal species have been found to naturally accumulate high oil levels in total dry biomass (Rodolfi *et al.,* 2009).

Microalgae biofuels may provide a viable alternative to fossil fuels; however, this technology must overcome a number of hurdles before it can compete in the fuel market and be broadly deployed. These challenges include native strain identification and improvement, both in terms of oil productivity and crop protection, nutrient and resource allocation and use, and the production of co-products to improve the economics of the entire system (Pankaj and Awasthi, 2015).

Algae are easy to grow and cultivate anywhere with less energy requirements and using very few of the nutrients. The ideal growth conditions for microalgal cultures are strain specific and microalgae cultivation requires specific environmental conditions including temperature ranges, light intensities, mixing conditions, nutrient composition, and gas exchange. It is known that microalgae respond with physiological alterations to the environmental conditions where they grow (Schenk *et al.,* 2008). This behavior can be viewed as a biotechnological attribute that can be manipulated in order to control the algal biochemical composition and growth, focusing on specific compounds and higher productivity. The need for clean and low-cost algae production demands for investigations on algal physiological response under different growth conditions.

A few thousand algal species are reported to occur in fresh water and marine environment in Bangladesh (Ahmed *et al.*, 2008). However, the potential for these natural resources as biofuel feedstock and other probable uses are not assessed to that extent. Despite of its importance related to biodiesel production as reported in many countries of the world, quite a few studies have been conducted in Bangladesh with a very insignificant number of species such as *Spirulina, Cholorococcum, Spirogyra* sp. (Qudus and Halim, 2012) thus requiring a comprehensive work involving isolation,

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characterization and optimization of their growth and selection of algal strains as a potential feedstock. Therefore, the present investigation was undertaken in order to evaluate *in vitro* growth of some of the isolates under different conditions and their potential in terms of biomass production was assessed.

Materials and methods

Isolation and Identification

For isolation, water samples were randomly collected in plastic water bottles and also by dropper in glass vials containing modified Chu-10D medium from different natural and artificial water bodies (e.g. ponds, ditches, sprinklers etc.) located at Dhaka University campus area and Khulna region, during 01 March 2014 to 20 December 2014. Direct isolations were done by picking up single filament or single cell using Platinum wire loop or sterile Pasture pipette. In some cases, series of dilutions were made in sterile medium using homogenized suspension of natural algal samples.

Identification of both blue-green and green algae was carried out following cross matching of morphological characteristics as described by Siddiqui *et al.* (2007) and Ahmed *et al.* (2008).

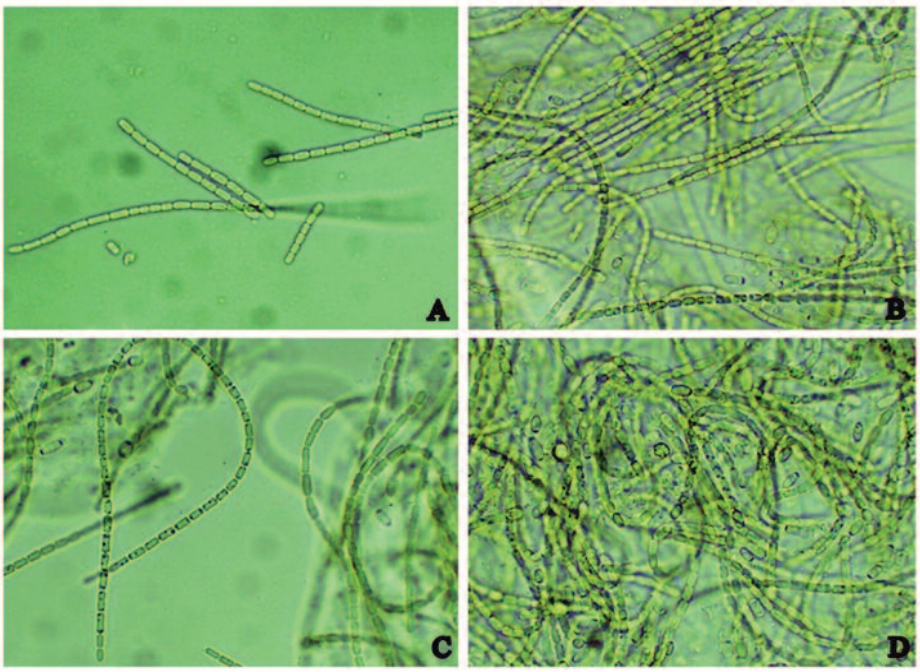
Characteristics of blue-green alga (Nostoc spongiaeforme)

Class: Cyanophyceae
Order: Nostocales
Family: Nostocacae
Genus: *Nostoc*
Species: *Nostoc spongiaeforme*

Description

Colony globose when young, later expanding into an irregular gelatinous ball, 2-3 cm in diameter, blue-green in colour, dense peripherally with much contorted trichomes, central portion watery with less contorted trichomes. Trichomes 2.8-8.7 µm wide. Cells 2.8-8.7 µm long. Heterocysts sub-spherical or oblong 4.2-5.8 µm wide, 4.8-8.4 µm long. Akinetes sub-spherical or ellipsoidal occurring in chains of 3-15, 45-8.0 µm broad and 5.0-9.0 µm long, wall smooth.

Habitat: Rice field



Figs. 1. A-D. *Nostoc spongiaeforme*, A. Hormogonia of various length, B. Fully developed filaments with low cyanophycin granules, C. Several developed filaments with intercalary heterocyst, D. Mature filaments with numerous granules

Characteristics of green alga (Chlamydomonas noctigama)

Class: Chlorophyceae
Order: Volvocales
Family: Chlamydomonadaceae
Genus: *Chlamydomonas*
Species: *Chlamydomonas noctigama*

Description

Cells spherical with true cell wall, flagella present. Chloroplasts parietal, massive cup-shaped, longitudinally ridged, pyrenoid usually one, in some cases two or three.

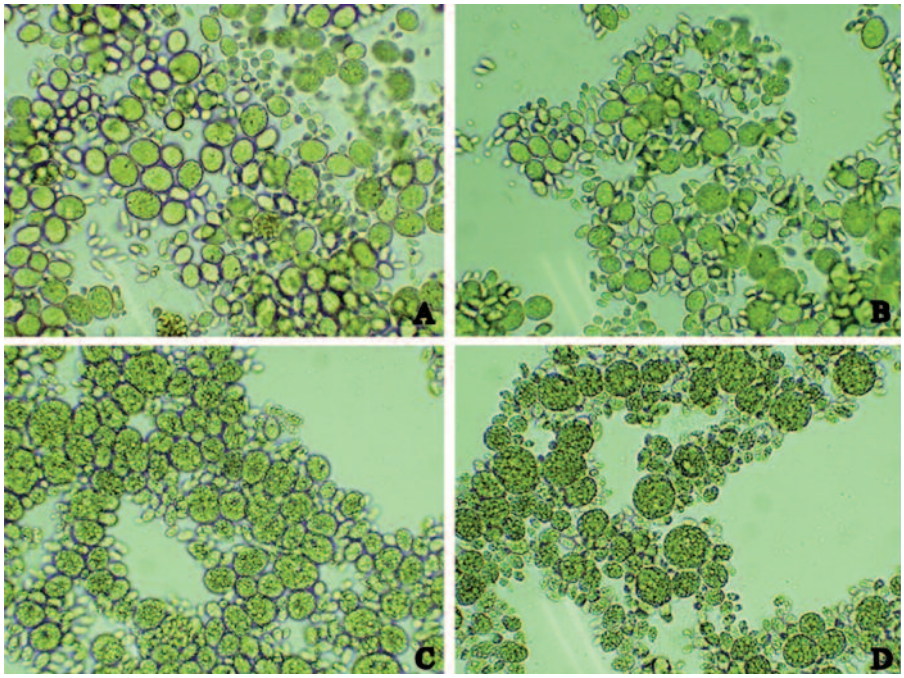
Habitat: Pond, fountain, planktonic.

Media Composition

The liquid medium used in this study is not an absolute inorganic medium but with two organic compounds, the EDTA as a chelating agent and HEPES as a buffer.

Sterilization

All the required instruments were sterilized at 120°C temperature and 15 lb/sq. inch (10.35 K Pa) pressure for 15 minutes.



Figs. 2. A-D. *Chlamydomonas noctigama*, A-B. Early stage of mature non-granular cells, developing cells and zoospores, C-D. Mature cells and granular zoospores and with one distinct pyronoid in each cell

Culturing of algae

After identification, isolated microalgae were used as inoculants for algal monoculture. Identification of species followed by the genus was done with the 4, 8 and 15 days old monoculture. *In vitro* algal culture was done in modified Chu-10D culture medium (Sinclair and Whitton, 1977) incubated in the controlled growth room, at 23 to 28ºC temperature in an average light flux of 71 $\mu Em^{-2}s^{-1}$.

Maintenance and subculturing

Stock cultures were maintained in 60 ml liquid medium incubated standing in an average 25°C growth room under continuous light (*ca* 40 $\mu Em^{-2}s^{-1}$). Subcultures to fresh medium were made after about three months. Stocks for experimental purposes were maintained at 32°C under continuous light of average 71 $\mu Em^{-2}s^{-1}$.

Table I. Composition of modified Chu-10D medium (mg l^{-1} of salts) used in the present study with Chu-10D of Sinclair and Whitton (1977)

Salts		Modified Chu-10D medium (Aziz and Whitton 1987)	Chu-10D
$Ca(NO_3)_2.4H_2O$ (for GA)		57.6	57.6
KH_2PO_4		7.8	7.8
$MgSO_4.7H_2O$		25	25
$Na_2SiO_3.5H_2O$		-	10.88
$NaHCO_3$		23.73	15.85
$CaCl_2.2H_2O$ (for BGA)		35.84	-
Fe-EDTA	$FeCl_3.6H_2O$	-	2.42
	Na EDTA.$2H_2O$	-	3.18
Micronutrients	$MnCl_2.4H_2O$	0.045	0.045
	$NaMoO_4.2H_2O$	0.007	0.007
	$ZnSO_4.7H_2O$	0.056	0.056
	$CuSO_4.5H_2O$	0.02	0.02
	$CoSO_4.7H_2O$	0.01	0.01
	H_3BO_4	0.72	0.72
NaOH*		*ca* 59.0	-

*added as 1M NaOH solution (*ca* 1.48 ml l^{-1} medium) during buffering with HEPES to pH 7.2

Table II. Composition of modified Chu-10D medium (mg l^{-1} of elements) of Aziz and Whitton (1987) used in the present study and comparison with Chu-10D of Sinclair and Whitton (1977)

Elements	Modified Chu-10D medium	Chu-10D
N (only for GA)	6.83	6.83
P	1.78	1.78
K	2.24	2.24
Na	ca 40.0*	6.69
Ca	9.78	9.78
Mg	2.47	2.47
S	3.25	3.25
Fe	0.5	0.5
Si	-	1.44
Cl	17.26	0.016
Mn	0.012	0.012
Mo	0.0028	0.0028
Zn	0.013	0.013
Cu	0.005	0.005
Co	0.002	0.002
B	0.125	0.125

*Na concentration increased from 6.69 to 40.0 mg l^{-1} due to the addition of 1M NaOH solution (*ca* 1.48 ml l^{-1}) during buffering with HEPES to pH 7.2

Estimation of growth

Total chlorophyll and optical density (O.D.) have been used to estimate growth. During early stage of growth, the whole contents of each flask were used for either total chlorophyll or O.D.

Chlorophyll *a* (for blue-green alga) was estimated by following the procedure based on the recommendations of Marker *et al.* (1980).

Chlorophyll *a* and *b* can be calculated by following formulae according to APHA (American Public Health Association), 1985.

By measuring the optical density at 750 nm growth was estimated using a spectrophotometer as described by Rodolfi *et al.*, 2009. During early stage of growth, the whole contents of each flask were used for O.D.

Optimization of growth conditions

The need for clean and low-cost algae production demands for investigations on algal physiological response under different growth conditions. Experiments were carried out in batch culture under continuous light. In the growth room

illumination was provided by white fluorescent tubes (average 71 $\mu Em^{-2}s^{-1.}$) above and below the alga. The flasks were usually randomly rearranged at 24 h interval. The temperature was set at a range of 24 to 26 ºC.

pH

Seven pH values (i.e. 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0) for GA and five pH values (i.e. 6.5, 7.0, 7.5, 8.0 and 8.5) for BGA had been used to find out the optimum pH value for respective algal growth in the medium.

Temperature

The growth of both GA and BGA was observed at four different temperature (i.e. 20, 25, 30 and 35 ºC) to find out optimum temperature for respective algal growth in the medium.

Light intensity

Five light intensities (i.e. 30, 50, 70, 90 and 110 $\mu Em^{-2}s^{-1.}$) had been used to find out the optimum light intensity for the growth of selected algal strains.

Vitamin supplements

Four vitamin solutions, individually (i.e. B1, B6, B7, B12) and in six combinations (i.e. B1+B6, B1+B7, B1+B12, B7+B12, B1+B7+B12 and B1+B6+B7+B12) had been used to find out the effect of vitamin for two algal growths in the medium.

Media concentration

Three concentrations of media, i.e. 1x, 1.5x and 2x had been used to find out the optimum concentration of media for two algal growths.

Air bubbling

The effect of air bubbling on the growth of the selected microalgae had been observed by supplying air-bubbles in the culture-flasks with control at the flux of 8 $Lmin^{-1}$ and the pressure of 0.0067 M Pa using a SOBO aquarium air pump (SB-348 A).

Results and discussion

The effect of different growth conditions, namely pH, light intensity, temperature, nutrient concentration in growth medium, vitamins and air-bubbles supply were evaluated to optimize the conditions of growth as well as the production of biomass for selected microalgal samples (*Chlamydomonas noctigama* and *Nostoc spongiaeforme*). The standard deviation was done to measure the sample fluctuation, analysis of variance (ANOVA) of the data was computed to determine the F-value and the test of significance was computed by Duncan's Multiple Range Test (DMRT), using IBM SPSS Statistics V.22.

pH

The pH can affect the growth of microorganisms and each species has a definite pH growth range and pH growth opti-

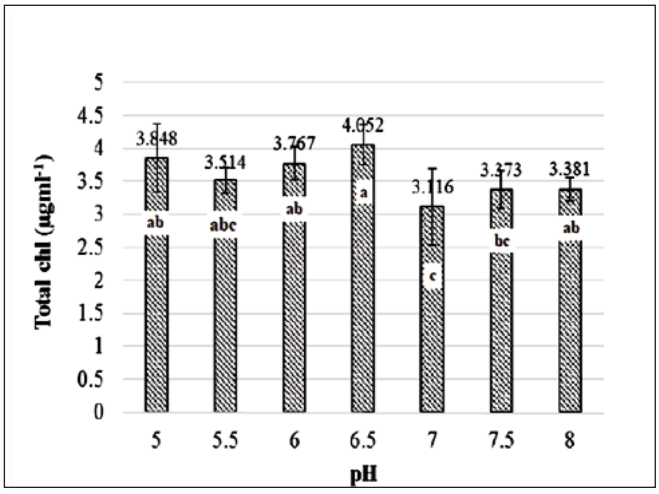


Fig. 3. Effect of pH on the growth as total chlorophyll (chl *a* and *b*) of *Chlamydomonas noctigama*

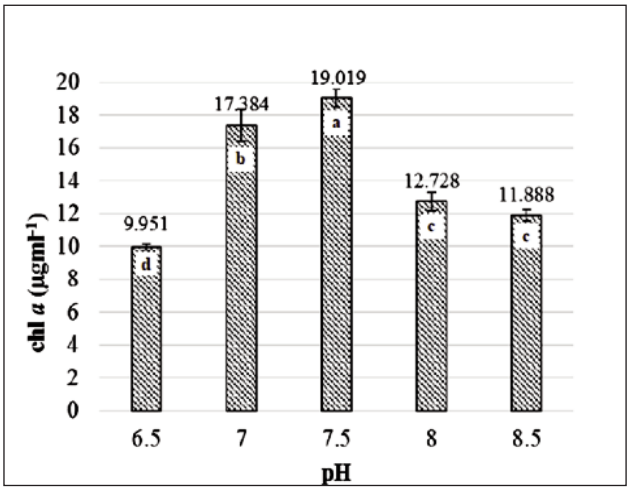


Fig. 4. Effect of pH on the growth as chlorophyll (chl *a*) of *Nostoc spongiaeforme*

mum. Extreme pH conditions influence photosynthesis, growth and nutrient assimilation in algae (Gensemer *et al.*, 1993). The effect of different pH values on growth of Chlamydomonas noctigama (as total chlorophyll) and *Nostoc spongiaeforme* (as chlorophyll a) was presented in Fig. 3 and 4, respectively. The result was statistically significant at 5% level. This means pH had significant influence on the growth of both algal species.

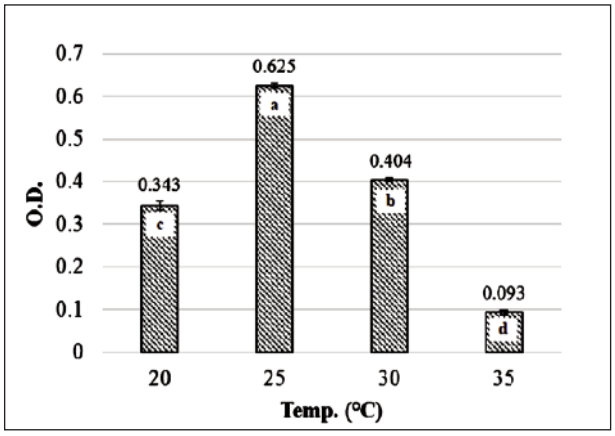


Fig. 5. Effect of temperature on the growth as O.D. of *Chlamydomonas noctigama*

The highest growth (4.052 $\mu gml^{-1.}$) and the lowest growth (3.116 $\mu gml^{-1.}$) as total chlorophyll (chlorophyll a and b) per ml of *Chlamydomonas noctigama* were found at pH 6.5 and pH 7.0, respectively. The highest growth was slightly different from the growth at pH 7.0, 5.5, 6.0 and 8.0, whereas the lowest growth was slightly different from the growth at pH 5.5 and 7.5.

The optimum growth of *Chlamydomonas noctigama* in this research was determined at pH 6.5 which is near about the pH condition of the habitat from which the species was isolated. Skjanes *et al.* (2008) also found optimum pH for the growth of *Chlamydomonas noctigama* collected from fresh water as 6.5.

The highest growth (19.019) and the lowest growth (9.951) as chlorophyll a per ml of Nostoc were found at pH 7.5 and pH 6.5, respectively. The highest and the lowest growth were statistically different from all other growth.

The optimum pH range for *Nostoc* sp. was reported by Rodriguez *et al.* (1986) as 6.4-8.2 which was almost similar to this research. Granhall (1975) reported optimum pH for the activity of *Nostoc punctiforme* as 7.6 which was also very close to the findings of this research. Lang *et al.* (2008) found optimum growth of *Nostoc* sp. at pH 7.0.

Temperature

The effect of different temperature on growth of *Chlamydomonas noctigama* and *Nostoc spongiaeforme* as O.D. (750 nm) was statistically different from each other.

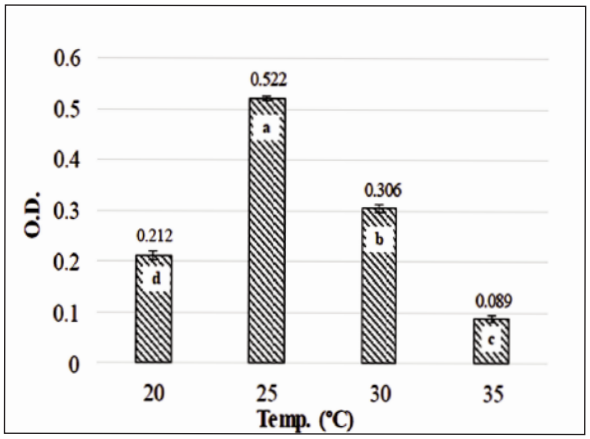


Fig. 6. Effect of temperature on the growth as O.D. of *Nostoc spongiaeforme*

The highest and lowest growth as O.D. (750 nm) was found at the temperature of 25°C and 35°C, respectively for both of the isolates.

From the result of the study the optimum temperature *Chlamydomonas noctigama* is 25°C which is similar to that of *Chlamydomonas reinhardtii* found by Akimoto *et al.* (1997). Harris (2009) reported that the usual laboratory species of *Chlamydomonas* grow well in the range of 20-25°C. Pocock *et al*. (2004) used 29°C as optimum temperature for the culture of *Chlamydomonas noctigama* in Bold's basal medium. The result varies might be due to the change in medium.

The growth increases with the increase in temperature up to its optimum and then declines with the increase in temperature. The optimum growth condition for the selected blue-green alga was achieved at temperature of 25°C which is similar to the finding of Li-Juan *et al.* (2011) on *Nostoc commune.* Spencer *et al.* (2011) found optimum growth of *Nostoc spongiaeforme* at 26°C on BG-11 medium.

Light intensity

The effect of different light intensities at 0 hr on the growth as O.D. (750 nm) of *Chlamydomonas noctigama* and *Nostoc spongiaeforme* was not statistically significant but the growth after 72 hr was statistically different from each other.

The highest growth (0.727) and lowest growth (0.621) were found at the light intensity of 110 and 30 $\mu Em^{-2}s^{-1.}$ for *Chlamydomonas noctigama* and of 70 and 30 $\mu Em^{-2}s^{-1.}$ for *Nostoc spongiaeforme*. The highest growth of *Chlamydomonas noctigama* had no statistical difference from the growth at the light intensity of 90 $\mu Em^{-2}s^{-1.}$.

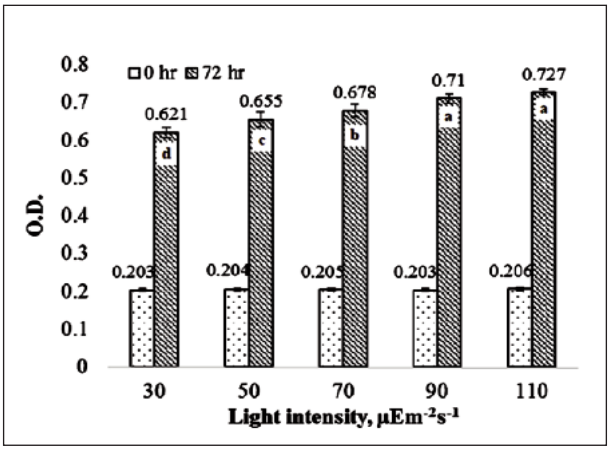


Fig. 7. Effect of light intensity on the growth as O.D. of *Chlamydomonas noctigama*

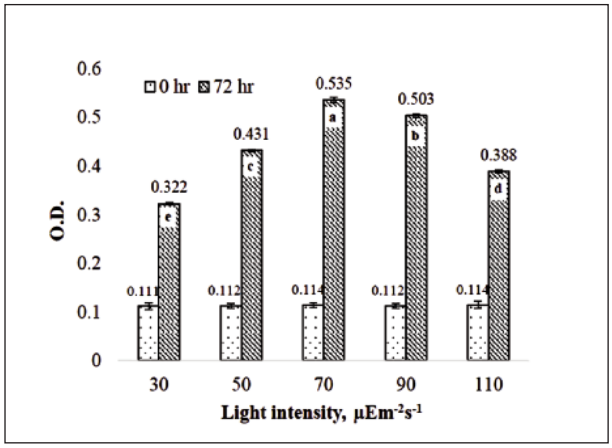


Fig. 8. Effect of light intensity on the growth as O.D. of *Nostoc spongiaeforme*

The result showed an increase in growth of *Chlamydomonas noctigama* with increasing light intensity. The optimal average light intensity for H_2 production in *Chlamydomonas reinhardti*i was found by Laurinavichene *et al.* (2004) as 30-40 $\mu Em^{-2}s^{-1.}$. For, *Nostoc spongiaeforme* the result showed an increase in growth with increasing light intensity up to 70 $\mu Em^{-2}s^{-1.}$ and then declines. So, optimum light intensity for the growth of this blue-green alga is 70 $\mu Em^{-2}s^{-1.}$. But Spencer *et al.* (2011) found that 227 $\mu Em^{-2}s^{-1.}$ of light was optimum for the growth of *Nostoc spongiaeforme* on BG-11 medium. The reason behind this might be the variation among the growth media. Besides, it is the limitation of the research that it was not feasible to provide light intensity more than 110 $\mu Em^{-2}s^{-1.}$ in the growth room.

Vitamin supplements

The effect of different vitamin on growth of *Chlamydomonas noctigama* and *Nostoc spongiaeforme* as O.D. (750 nm) was presented in Fig. 9 and 10, respectively.

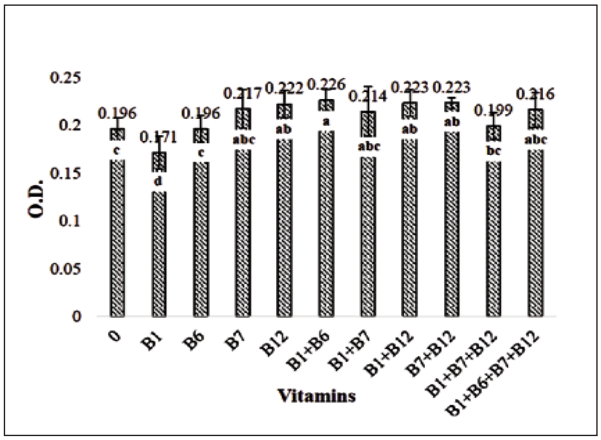


Fig. 9. Effect of vitamins on the growth as O.D. of *Chlamydomonas noctigama*

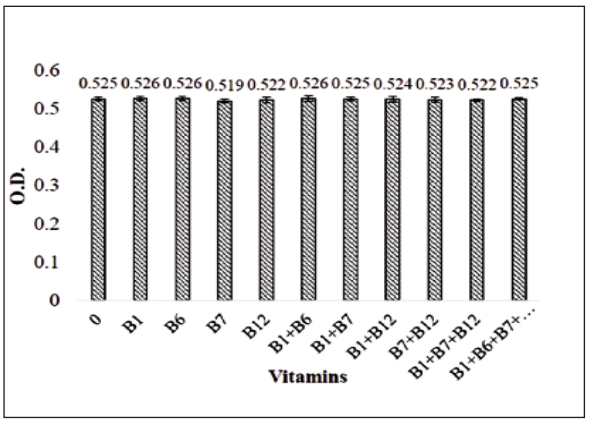


Fig. 10. Effect of vitamins on the growth as O.D. of *Nostoc spongiaeforme*

The result was statistically significant for *Chlamydomonas noctigama* and not statistically significant for *Nostoc spongiaeforme* at 5% level as the growth had no statistical difference to each other and also to the control.

The result showed that the highest growth (0.226) and the lowest growth (0.171) of *Chlamydomonas noctigama* were found at the vitamin B1+B6 and B1, respectively. The highest growth was slightly different from the growth at B7, B12, B1+B7, B1+B12, B7+B12 and B1+B6+B7+B12.

Croft *et al.* (2006) found that many algal species require exogenous cobalamin (vitamin B12), thiamine (vitamin B1) or biotin (vitamin B7) for growth. In this study, it had been found that the addition of vitamin B1+B6 in the media had a positive effect on the growth of *Chlamydomonas noctigama.* McVeigh and Brown (1954) found stimulating effect on the growth of *Chlamydomonas noctigama* after including vitamin B12 with the medium.

Kartz and Myers (1953) stated that numerous attempts to elicit a growth response from *Nostoc muscorum* by the addition of vitamins to a culture medium had proved fruitless. However, the addition of vitamins in the large scale commercial system might be proved to be advantageous.

Media concentration

The effect of different media conc. on growth of *Chlamydomonas noctigama* and *Nostoc spongiaeforme* as O.D. (750 nm) at 0 hr on the growth of both isolates was not statistically significant. But the result was statistically significant for both of them at 48 hr and 72 hr.

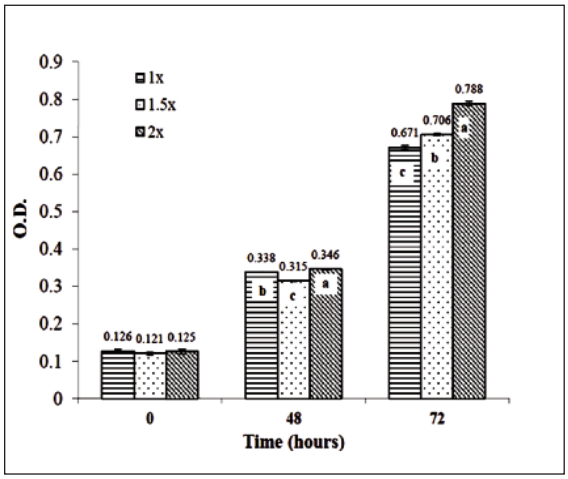


Fig. 11. Effect of nutrient concentration in medium on the growth as O.D. of *Chlamydomonas noctigama*

The highest growth (0.346) and the lowest growth (0.315) as O.D. (750 nm) of *Chlamydomonas noctigama* were found at 2x and 1.5x, respectively after 48 hr. whereas the highest growth (0.788) and the lowest growth (0.671) as O.D. were found at 2x and 1x, respectively after 72 hr. On the other hand, the highest growth (0.347) and the lowest growth (0.319) as O.D. (750 nm) of *Nostoc spongiaeforme* were found at 1x and 2x, respectively after 48 hr. The highest growth (0.536) and the lowest growth (0.515) as O.D. were found at 1x and 1.5x, respectively after 72 hr. Miller *et al.* (1999) found that the increased nutrient concentration in medium resulted in better growth and higher biomass of blue green algae.

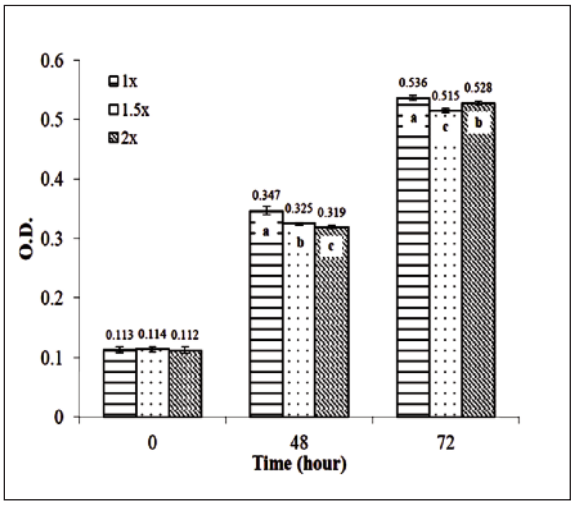


Fig. 12. Effect of nutrient concentration in medium on the growth as O.D. of *Nostoc spongiaeforme*

Air bubbling on growth

The effect of air bubbling on growth of *Chlamydomonas noctigama* and *Nostoc spongiaeforme* as O.D. (750 nm) was presented in Fig. 13.

The growth as O.D. of *Chlamydomonas noctigama* and *Nostoc spongiaeforme* with and without air bubbling were

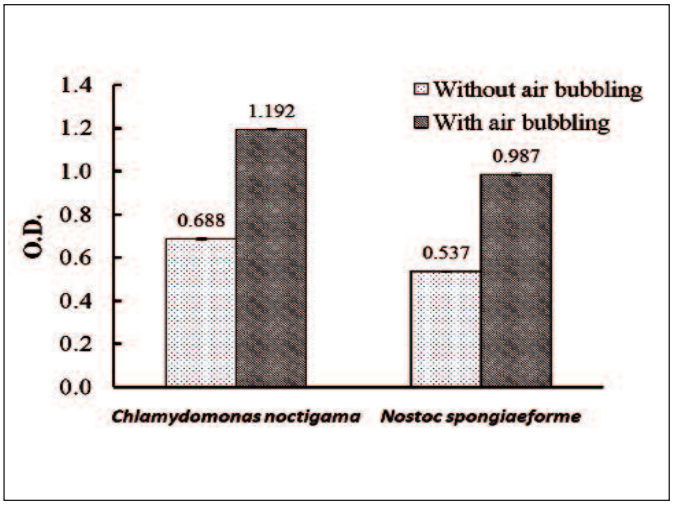


Fig. 13. Effect of air-bubbles supply on the growth as O.D. (at 750 nm) of *Chlamydomonas noctigama* and blue-green algae *Nostoc spongiaeforme.*

statistically different from each other at 5% level. An increase of 73% and 84% in the growth of the green alga and blue green alga, respectively was observed. It appears that both the isolates were responsive to air bubbling. It might be due to the utilization of CO_2 required for photosynthesis. Similar findings were reported by Evjen (2014) and Hassan *et al.* (2012) on *Chlamydomonas reinhardtii* and *Nostoc muscorum.*

Conclusion

The randomly isolated and selected algal strains in the present investigation i.e. *Chlamydomonas noctigama* and *Nostoc spongiaeforme* showed variable growth conditions and can be taken as baseline conditions. If growing in the optimum conditions, the algal strains can be used as a source of biomass for biofuel and other valuable products. Further research for large scale biomass production using the sets of growth conditions in raceway ponds and/or photo-bio reactor is required.

Acknowledgment

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