J. Asiat. Soc. Bangladesh, Sci. 42(2): 191-200, December 2016

OPTIMIZING CHLORELLA VULGARIS AND ANABAENA VARIABILIS GROWTH CONDITIONS FOR USE AS BIOFUEL FEEDSTOCK

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Abstract

Isolation and characterization of *Chlorella vulgaris* (green alga) and *Anabaena variabilis* (cyanobacterium) were made from natural and artificial water bodies of Dhaka University and Khulna, Bangladesh from March through December 2014 using modified Chu-10D medium to determine their potential as feedstock for biofuel production. Optimum growth measured as total chlorophyll and optical density under varying physical and chemical environments was determined. The optimum growth for *C. vulgaris* was obtained at pH 6.5 under light intensity of 110 µE m^{-2} s^{-1} and one and a half times the concentration of the Chu-10D. Compared to this, the optimum growth for *A. variabilis* was obtained at 7.0 pH, 90 µE m^{-2} s^{-1} light intensity and normal Chu 10D. Both organisms were grown at 25^{o} C temperature. Aeration of medium showed a significant positive growth for both the isolates. Supplementation of medium with vitamin B_1, B_6, B_7 and B_{12} would yield higher biomass of *C. vulgaris* as biofuel feedstock. Vitamins were not required for growing *A. variabilis.*

Key words: Microalgae, *Chlorella vulgaris*, *Anabaena variabilis*, Feedstock, Biofuel, Growth optimization

Introduction

Rising concern over depleting fossil fuel and greenhouse gas emissions has resulted in high level of interest in non-conventional fuel like biodiesel and bioethanol originating from bio-renewable sources including sugars, starches and ligno-cellulosic materials from solid wastes and plant biomass including algal biomass. Microalgae in particular have been reported to have several advantages which include high productivity, no competition with conventional agricultural land, utilization of waste water, brackish water and sea water, recycling of carbon dioxide, and compatibility with integrated production of fuels and co-products within bio-refineries such as agar, dye stuff, protein rich animal feed etc. (Sahoo *et al.* 2012 and Kumar *et al.* 2013).

Although the first-generation bioethanol production from food crops such as corn, grain, or sugar cane is well established and the industry is growing throughout the world, the use of these staple food crops as feedstock is not ideal because of the high price of raw

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materials, which account for almost 40–75% of total ethanol production cost (Jang *et al*. 2012). It has raised doubts about its possible impact on food supply and security, which is mainly reduced if its residues are used for bioethanol production (Song *et al*. 2013). In contrast, the second-generation bioethanol is derived from ligno-cellulosic feedstock. Currently, no commercial-scale cellulosic ethanol plants are in operation largely because of the high price of production, which is almost twice that of corn ethanol (Jones and Mayfield 2012). In view of the aforementioned issues, microalgae are gaining wide attention as an alternative renewable source of biomass for the production of biofuel, which is grouped under 'third-generation bioethanol' (Nigam and Singh 2011).

Certain species of microalgae have the ability to produce high levels of carbohydrates as reserve polymers instead of lipids. These species are ideal for the production of bioethanol as carbohydrates produce fermentative sugars. It has been estimated that approximately 46760-140290 L/ha ethanol can be produced from microalgae (Nguyen and Vu 2012). This yield is several orders of magnitude larger than the yields obtained from corn, soybean, etc. (Lombardi and Maldonado 2011). Green algae including *Spirogyra* and *Chlorococcum* were reported to accumulate high levels of polysaccharides both in their complex cell walls and as starch (Nigam and Singh 2011). This starch accumulation can be used in the production of bioethanol (Harun *et al.* 2010). Bioethanol is used in fuel mixtures such as E85 (a blended fuel of 85% ethanol and 15% gasoline) in Brazil and USA (Davis *et al*. 2000).

In Bangladesh, a large number of algal species were reported to occur in freshwater, brackish water and marine habitats (Web 1, Ahmed *et al*. 2008) which could be potential sources of biofuel feedstock, but the potential of algal biomass production for biofuel has not been properly addressed. Therefore, the present research was initiated to identify probable potential microalgae of Bangladesh for using as biofuel feedstock by optimizing their growth conditions.

Materials and Methods

Isolation of microalgae: Water samples with algal boom were taken in to plastic bottles as well as in glass vials containing sterilized modified Chu-10D medium (Aziz and Whitton 1987 adapted from Chu-10D of Sinclair and Whitton (1977) by a dropper from different fountains, ponds, ditches, etc. located at Dhaka University campus and Khulna for obtaining fast growing and frequently occurring microalgae. The liquid medium used in this study is not an absolute inorganic medium as two organic compounds, the EDTA as a chelating agent and HEPES as a buffer were used. Four vitamins i.e. B_1, B_6, B_7, B_{12} in six combinations i.e. B_1+B_6, B_1+B_7, B_1+B_{12}, B_7+B_{12}, B_1+B_7+B_{12} and B_1+B_6+B_7+B_{12} with a control in each case were used to find out their effects.

Unialgal culture was obtained by repeated subculturing in liquid and solid agar media using platinum wire loop or sterile Pasture pipette. In some cases, series of dilutions were

made in sterile medium using homogenized suspension of algae. Unialgal condition was confirmed by compound microscope.

Green algae and cyanobacteria of 8 to 30 days old cultures were used for microscopic study, described, photographed and identified following Ahmed *et al*. (2008), Starmach (1966) and Desikachary (1959) and Siddiqui *et al*. (2007).

Maintenance and subculture: The present experiments were conducted in the controlled growth room of National Professor KM Nurul Islam laboratory, Department of Botany, DU. Stock cultures were maintained in 30 ml liquid medium in the controlled growth room with an average temperature of 25^{o} C under continuous low light of *ca.* 40 µE m^{-2} s^{-1} provided by cool white fluorescent light from glass bottom. Subculture was made after about every three months. Stock cultures for experimentation were inoculated under a continuous average light flux of 71 µE m^{-2} s^{-1} and 4-7 days old culture was used as inoculum. Each treatment had four replicates and were randomized after every 24 hr.

Estimation of growth: Growth was estimated by measuring chlorophyll(s) and Optical density (O.D.). Chlorophyll *a* and *b* for *Chlorella vulgaris* were estimated following APHA (American Public Health Association 1985), only chl *a* for *Anabaena variabilis* following Marker *et al*. (1980) and O.D. in both cases by measuring absorbance at 750 nm wave length in spectrophotometer following Rodolfi *et al.* (2009).

pH was measured using Hanna pocket model and average light flux by Li-Cor, USA, using aerial probe. Temperature was measured by maximum-minimum wall thermometer.

Aeration: Aeration of the culture-flasks was done to optimize the effect of bubbling on algal growth (for CO_2 utilization) at a pressure of 0.0067 M Pa using an aquarium air pump with a pumping capacity of 8 L min^{-1} at 25 ± 1^{o} C temperature and at an average light flux of 71 µE m^{-2} s^{-1}.

The standard deviations were done to measure the sample variations, analysis of variance (ANOVA) of the data was computed to determine the F-value and test of significance was computed by Duncan's New Multiple Range Test (DMRT) in IBM SPSS statistics version 22.

Results and Discussion

Chlorella vulgaris (Class: Chlorophyceae, Order: Chlorococcales, Family: Chlorellaceae, Genus: *Chlorella*) and *Anabaena variabilis* (Class: Cyanobacteria, Order: Nostocales, Family: Nostocaceae, Genus: *Anabaena*) were isolated from freshwater bodies and characterized as follows:

***Chlorella vulgaris* Beyer (Figs. 1a-c)** (Ahmed *et al.* 2008)

Cell solitary or in small colony of indefinite shape; Individual cells spherical to broadly oval; Cell wall thin; Chloroplast massive cup-shaped, parietal with indistinct pyrenoid; Cell 6.5-7.5 µm long, 6.0-8.0 µm broad. Collected from TSC fountain, Dhaka University, planktonic

***Anabaena variabilis* Kütz. *ex* Born. *et* Flah (Figs. 1d-f)** (Siddiqui *et al.* 2007)

Thallus gelatinous; cells barrel-shaped, constricted at the cross walls**,** 4.5-6.0 µm broad, 5.4-6.5 µm long; end cells conical; heterocysts intercalary, oval, 7.5-12.0 µm broad, 7.8-10.8 µm long; akinete not differentiated in the present observation with 30 days old culture. Collected from a pond, Khulna University.

Optimization of growth conditions

Chlorella vulgaris grew better, measured as chlorophylls at pH 6.5 followed by 6.0. Growth at neutral and alkaline pH were significantly lower (Fig. 1). Mayo (1997)

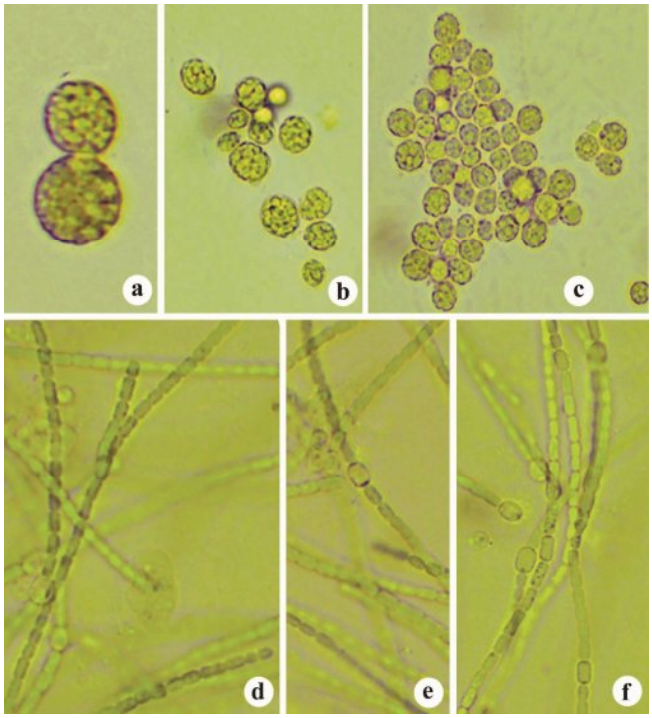

Plate 1. *Chlorella vulgaris*: a) Two associated cells enlarged showing nemerous starch granules around a pyrenoid; b) cells of various shape and size at lower magnification, smaller ones are released autospores; c) mature cells many having one pyrenoid in each cell and surrounding starch grains. d-f. *Anabaena variabilis*: d) young filaments as hormogonia, e) hormogonia and a mature filament with heterocyst, f) only mature filaments.

observed maximum growth more or less at 6.5 pH in *Chlorella* sp. In *C. vulgaris* a complete inactivation at acidic pH was found (Carberry and Brunner 1991). However, *Anabarna variabilis* had best growth at 7.0 pH (Fig. 3) which was similar to the findings of Yoon *et al.* (2008). In the same species Nagle *et al.* (2010) observed growth inhibition at <5.0 and >10.5 pH. The present organism also was severely affected at lower and much higher pH (Fig. 2).

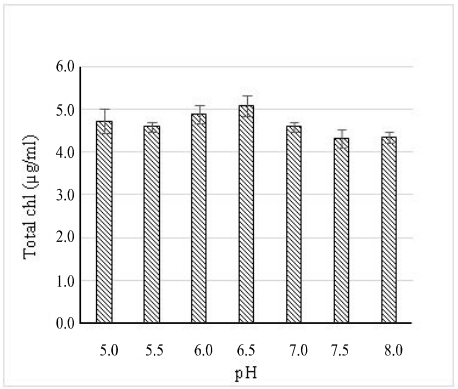

Fig. 1. Effect of pH on the growth as total chlorophyll (*chl a* and *b*) of *Chlorella vulgaris*. Significant at 5% level.

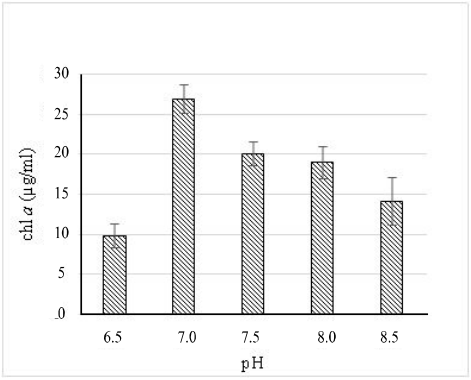

Fig. 2. Effect of pH on the growth as chlorophyll (*chl a*) of *Anabaena variabilis*. Significant at 5% level.

Chlorella vulgaris had best growth as optical density at 110 µE m^{-2} s^{-1} (Fig. 3). It is possible that continuous exposure of the cells under adequate light energy, in particular, during cell division process, *C. vulgaris* is able to grow faster. Similar observations were reported by Wijanarko *et al.* (2004) and Sharma *et al.* (2012). Best growth was in *Anabaena variabilis* observed at a light flux of 90 µE m^{-2} s^{-1} (Fig. 4).

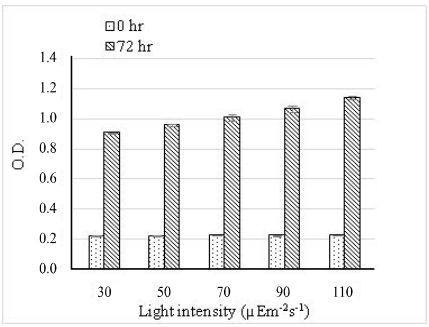

Fig. 3. Effect of light intensity on the growth of *Chlorella vulgaris*. Significant at 5% level.

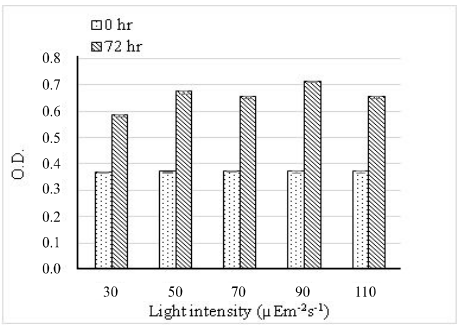

Fig. 4. Effect of light intensity on the growth of *Anabaena variabilis*. Significant at 5% level.

Temperature: Both *C. vulgaris* and *A. variabilis* responded similarly to temperature variations, highest growth as O.D. was found at 25° C and lowest at 35° C after 72 hr of incubation (Figs 6-7). Temperature ranging from 25 to 30° C was suggested to be favourable for the overall growth of *C. vulgaris* (Sharma *et al*. 2012). Specific growth rate of *C. pyrenoidosa*, increased uniformly with enhanced temperature, in the range 22° C to 30° C but dropped at higher temperature and cells were unable to grow at above 33° C (Ong *et al.* 2010). In *A. variabilis* and *A. nidulans*, Sato *et al.* (1979) obtained about double the cell growth at 25° C temperature.

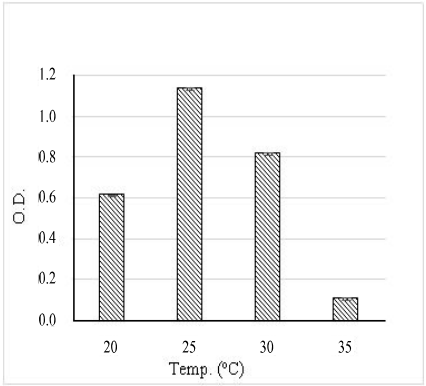

Fig. 5. Effect of temperature on the growth of *Chlorella vulgaris.* Significant at 5% level.

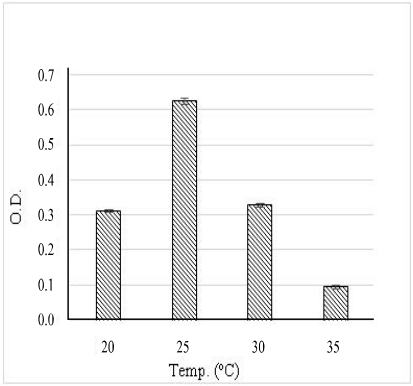

Fig. 6. Effect of temperature on the growth of *Anabaena variabilis.* Significant at 5% level.

Aeration: Effects of aeration for 72 hr on growth of *C. vulgaris* and *A. variabilis* are presented in Fig. 7. O.D. of the two algae increased by 81% and 90%, respectively due to bubbling for 72 hr. The positive effect was due to the utilization of CO_2 and continuous contact of organisms with the medium thereby helping nutrient absorption. These findings are similar to the works reported by Pirt and Pirt (1980) and Berberoğlu *et al.* (2008) on *C. vulgaris* and *A. variabilis*, respectively.

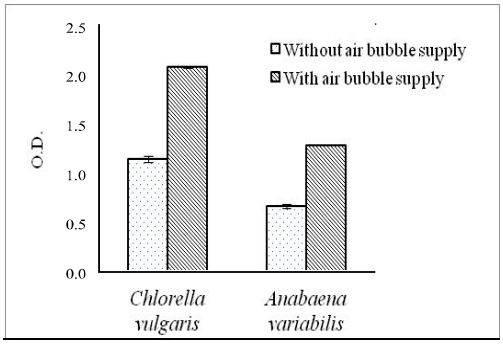

Fig. 7. Effects of air bubbling on the growth of two microalgae. Significant at 5% level.

Nutrient element concentration of Chu 10D: Chu 10D medium has relatively low concentration of elements compared to Bold's Basal Medium, in some cases less than half. Therefore, Chu 10D medium concentration was increased by one and one half and doubled. Increased nutrient status had significantly different effects on the growth as O.D. of both the organisms after 48 to 72 hr growth (Figs 8-9). Both the organisms were affected at double the strength of Chu 10D. Of the three concentrations *C. vulgaris* showed maximum growth at one and one half strength (Fig. 8) whereas *A. variabilis* showed maximum at normal Chu 10D strength (Fig. 9). The maximum in *A. variabilis* might be due to optimum concentration of nutrient elements in normal Chu 10D except nitrogen and the cyanobacterium supplemented it by fixing atmospheric N_2 (Stewart and Gallon 1980). At normal Chu 10D strength on the other hand *C. vulgaris* showed lowest growth and was most likely due to low nitrogen medium and inability of the organism to fix atmospheric N_2. However, Chia *et al.* (2013) showed that the growth obtained in *C. vulgaris* grown in the Chu-10D medium was the highest. Elser *et al.* (1990) suggested that N (also P) potentially limits algal growth where N-fixing cyanobacterium *Anabaena* has the ability to fix atmospheric N_2 when the water becomes N-depleted.

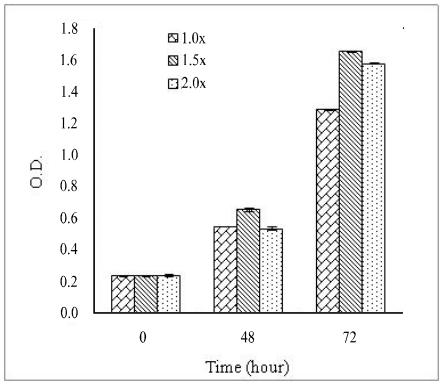

Fig. 8. Effect of nutrient concentration in medium on the growth of *chlorella vulgaris.*

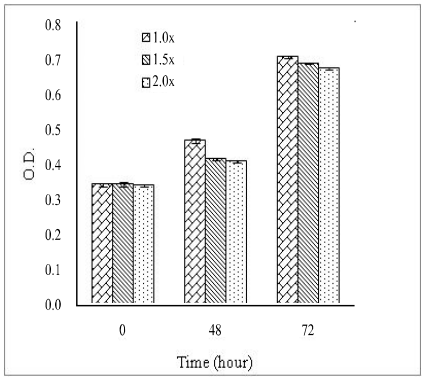

Fig. 9. Effect of nutrient concentration in medium on the growth of *A. variabilis.*

Vitamin supplement: Effect of vitamin B_6 alone or in combination with B_1, B_7 and B_{12} for *Chlorella vulgaris* is (Fig. 10). Therefore, supplementation of medium with vitamin B_1, B_6, B_7, B_{12} would yield higher biomass as feed stock for producing biofuel. In marine diatom *Chaetoceros calcitrans* vitamin B_6 addition also increased growth (Krichnavaruk *et al.* 2005). *Anabaena variabilis* does not require any vitamins and produced higher biomass after 72 hr growth than *Chlorella vulgaris* (Fig. 11).

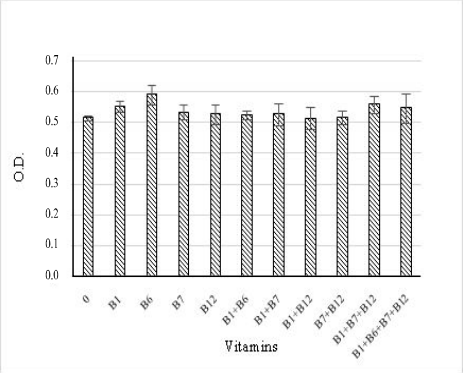

Fig. 10. Effect of vitamins on the growth of *Chlorella vulgaris*.
Significant at 5% level.

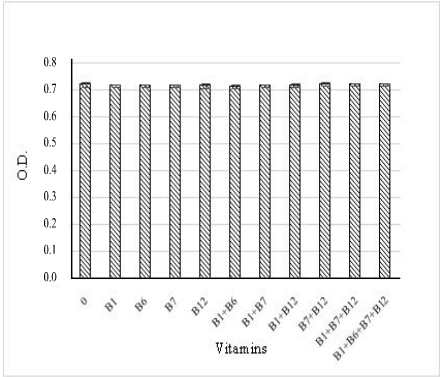

Fig. 11. Effect of vitamins on the growth of *Anabaena variabilis*.
Significant at 5% level.

Acknowledgments

Authors gratefully acknowledge the financial support from Centre for Advanced Studies and Research in Biological Sciences, University of Dhaka for carrying out the research and the Department of Botany, University of Dhaka for providing laboratory facilities.

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(Revised copy received on 15/8/2016)