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Effect of pond depth and lining plastic color on growth and nitrogen fixing capacity of the cyanobacteria, *Anabaena* sp. E3

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Cyanobacteria are a cheap source of nitrogen and quite suitable for farmers of developing countries. Although, they live in a diverse range of environments, different environmental variables influence their nitrogen fixing ability. Thus, this study evaluated the effect of pond depth and lining plastic colors on nitrogen fixing capacity of *Anabaena* species strain E3. Factorial combinations of four pond lining plastic colors and two depths were laid out in a complete randomized design with three replications. The ANOVA results revealed that the 20 cm depth pond had a higher mean growth rate (0.063 OD day^{-1}), dissolved oxygen (17.63 mg L^{-1}), dry biomass (0.58 g L^{-1}) and total nitrogen (27.77 mg L^{-1}) than the 40 cm depth. The highest mean growth rate (0.089 OD day^{-1}), dissolved oxygen (19.07 mg L^{-1}), dry biomass (0.66 g L^{-1}) and total nitrogen (41.60 mg L^{-1}) were registered in the treatment with a transparent lining plastic color. Moreover, it was noted that, the test strain under the treatment combinations of 20 cm depth and transparent plastic lining expressed the highest mean optical density (1.227), heterocyst frequency (2.92%) and pH (10.28). Therefore, for mass-production of E3-strain-based biofertilizer, the strain should be grown in ponds of 20 cm depth with transparent plastic.

Key words: Cyanobacteria, dry biomass, growth rate, shallow pond, total nitrogen.

INTRODUCTION

Cyanobacteria (formerly classified as “blue-green algae”) are among the largest, most diverse and widely distributed group of prokaryotes. They are highly adaptable, and some species exhibit wide ecological tolerance and gliding mobility. They can be found in almost any environment, including extreme ones (e.g. benthos, cold and hot deserts, and Antarctic dry valleys) (Cohen and Gurevitz, 2006; Stal, 2007). Although, all existing cyanobacteria have the ability to perform oxygenic photosynthesis, they use H_2O as an electron donor, and some are able to grow as anaerobic photo-autotrophs using H_2S as an alternative electron donor

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(Cohen et al., 1986). This represents a unique additional capability of anoxygenic photosynthesis in these organisms. Some species produce toxins that affect animals and humans. People may be exposed to cyanobacterial toxins by drinking or bathing in contaminated water. The most frequent health effects are caused by drinking water containing the toxins (cyanobacteria), or by ingestion during recreational water contact (Ian, 1996). Cyanobacteria are equipped with nitrogenase enzymes, thus they can fix atmospheric nitrogen (N_2) into a biologically accessible form and thereby play a key role in the nitrogen cycle of the biosphere (Bothe et al., 2010; Zehr, 2011). Consequently, they are used as biofertilizer to maintain and improve soil fertility (Ahmed, 2001).

One way to improve soil fertility is the use of inorganic fertilizers. Adugna and Hiruy (1988) reported that the majority of Ethiopian soils gave a large yield response to applied nitrogen. Nevertheless, the use of this input among smallholder farmers is currently very low in the country. High fertilizer costs, marketing problems and poor infrastructure are some of the major reasons for low use of fertilizers (Schneider and Anderson, 2010). Moreover, synthetic N fertilizers have lower agronomic use efficiency due to losses of applied N through volatilization, leaching and denitrification (Havlin et al., 2010). Excess use of chemical fertilizers may result in multi-nutrient deficiencies and nutrient imbalance in soil. Furthermore, it also generates several environmental problems including acidification of water (Choudhury and Kennedy, 2005). The Ethiopian Ministry of Agriculture has identified 19 soil types throughout the country. The big proportion of the country's landmass is covered by lithosols, nitosols, cambisols and regosols in order of their importance (MoA, 2000). The country's soils, similar to other agricultural soils of the tropics, are generally low in nitrogen and phosphorus (Desta, 1982; Pulschen, 1987).

Therefore, the use of alternative options for soil fertility replenishment is indispensable to maintain soil fertility and productivity. Cyanobacteria offer an economically attractive and ecologically sound alternative to chemical fertilizers for realizing the ultimate goal of increased productivity (Mishra and Pabbi, 2004; Rajasulochana and Krishnamoorthy, 2014; Shweta and Kritika, 2015).

Phylogenetic analysis on endogenous Cyanobacteria *Anabaena* sp. E strains was performed in Colorado State University, USA, and *Anabaena torulosa* A525, *Anabaena oscillarioides* BECID22, *Anabaena sphaerica* RPAN 38 and *A. sphaerica* UTEX B 1616 were found to be close phylogenetic neighbors of *Anabaena* sp. E3 with greater than 97% similarity as summarized in Table 1. The phylogenetic tree for *Anabaena* sp. E3 and other similar endogenous cyanobacterial strains is also presented in Figure 1.

Although, the cyanobacteria live in a diverse range of environments, a number of environmental variables influence their photosynthetic and nitrogen fixing ability (Behl, 2013). Prior to this, a research was conducted (in 2012 at the same geographical location) to evaluate the effect of cyanobacterial based biofertilizer on maize yield, indicating that strains grown in ponds with two colors produced different cyanobacterial biomasses. This observation ignited an idea that pond plastic color may also influence the growth of *Anabaena* sp. E3. In general, studies on an organism's atmospheric nitrogen fixation capacity have to be done in laboratory settings prior to mass-production and formulation as biofertilizer. Therefore, the objectives of this study were (1) to evaluate the effect of pond depth and lining plastic color on nitrogen fixing capacity of cyanobacteria *Anabaena* sp. E3 and (2) to develop recommendations for pond depth and lining plastic color that optimize growth and development of cyanobacteria *Anabaena* sp. E3.

Table 1. Estimate of evolutionary divergence between *Anabaena* sp. E3 and reference cyanobacterial species registered in GenBank.

Strains	Similarity with E3 (%)
Anabaena torulosa A525	97.05
Anabaena oscillarioides BECID22	97.36
Anabaena sphaerica RPAN 38	97.34
Anabaena sphaerica UTEX B 1616	97.34

MATERIALS AND METHODS

Description of the study area

The laboratory phase of the experiment was carried out in the soil microbiology laboratory at Hawassa University, College of Agriculture and the glasshouse phase at Hawassa University main campus, Hawassa, Ethiopia, from March 2014 to October 2014.

Source of cyanobacterial strain

Heterocytous cyanobacteria, *Anabaena* sp. E3 was used. The strain was previously isolated from a pigeon pea field in Ziway, Ethiopia by Colorado State University, USA and was obtained from soil microbiology laboratory of Hawassa University College of Agriculture. In an earlier study, in comparison with strains E2, E5 and E9, the test strain in this study was proven to be the strain with the highest nitrogen fixing capacity under different environmental conditions (Girma et al., unpublished data).

Experimental details

Strain activation and inoculum preparation

Allen-Arnon medium was prepared (Allen and Arnon, 1955) using tap water. The only source of N in the Allen-Arnon medium is the ammonium metavanadate (NH_4+VO_3). For strain activation, *Anabaena* sp. E3 was transferred from a preserved sample to Allen-Arnon media in a 1:10 ratio and incubated for two weeks as

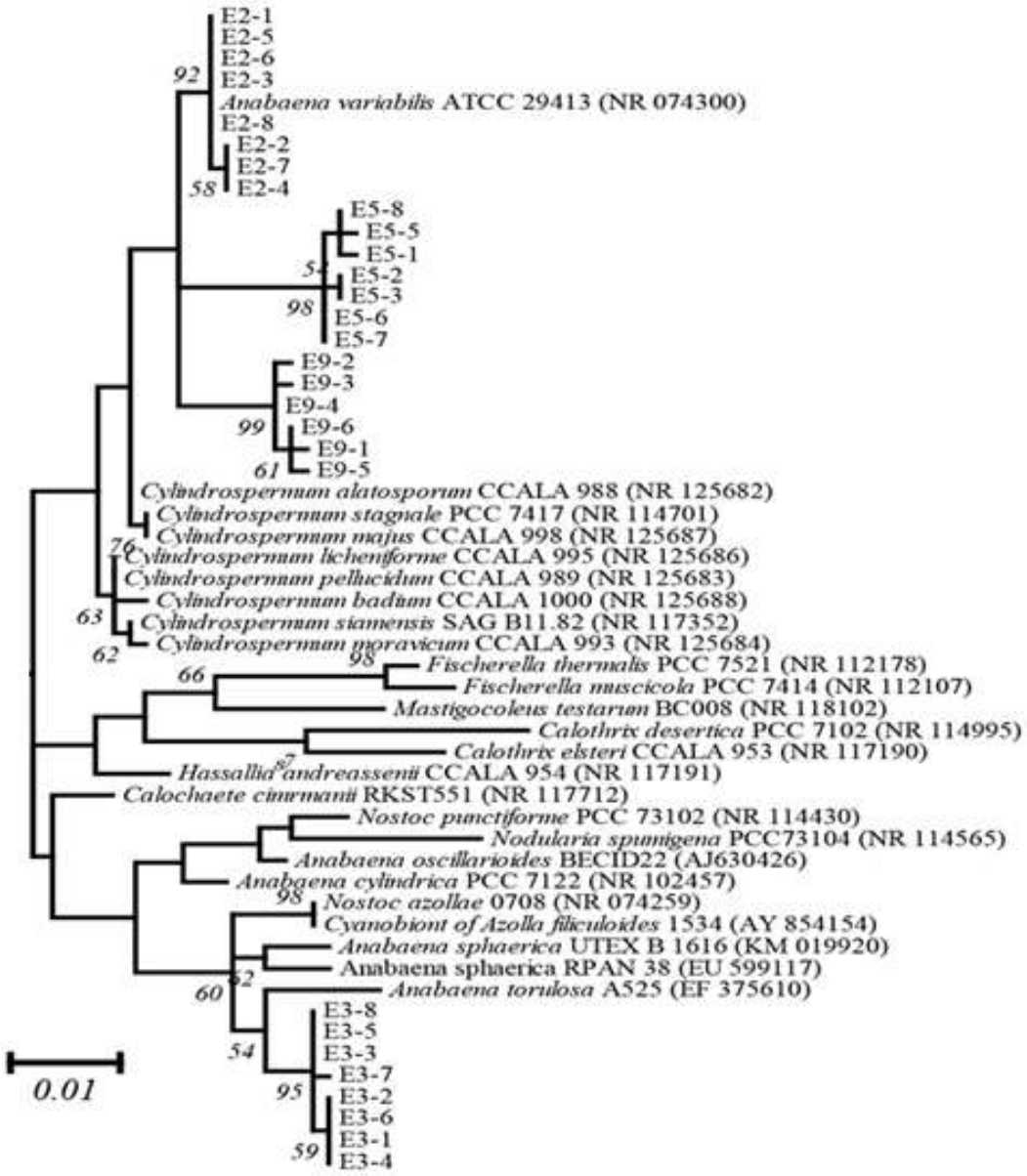

Figure 1. Phylogenetic tree based on 16S rRNA gene sequences, showing the relationships among the different initial Ethiopian cyanobacterial strains and reference cyanobacterial species registered in GenBank. Accession numbers are given in parentheses. Trees were constructed by the maximum likelihood method using MEGA version 5 (Tamura et al., 2011). Bootstrap values over 50% are shown at each node. Bars, % estimated substitution expected number of changes per site.

shown in Figure 2. Then, the activated culture was transferred to fresh media made of double distilled water and grown for two weeks.

For inoculant culture preparation, the strain was inoculated in growing media in a 1:50 culture dilution. The culture was maintained under uniformly controlled conditions of continuous light of 2500 lux (Briand et al., 2004) during the day time (12 h) using cool fluorescent tubes at 27-30°C (Heino et al., 2009). Air was supplied to the growing culture in the box by means of a compressor for six hours day time only. The cultures were allowed to grow for two weeks before the formulation of secondary metabolites (Amal et al., 2010). The inoculant was described microscopically at the beginning and weekly throughout this phase of the experiment and hence, normal and healthy appearance of the inoculum culture was verified.

Pond design

A variety of cultivation systems for microalgae was developed. The only one used on a large scale and a commercial basis is the shallow, open raceway. These ponds are usually not more than 30 cm deep, and the water with nutrients and microalgae is circulated with a paddle wheel (Janssen, 2002). A factorial combination of two

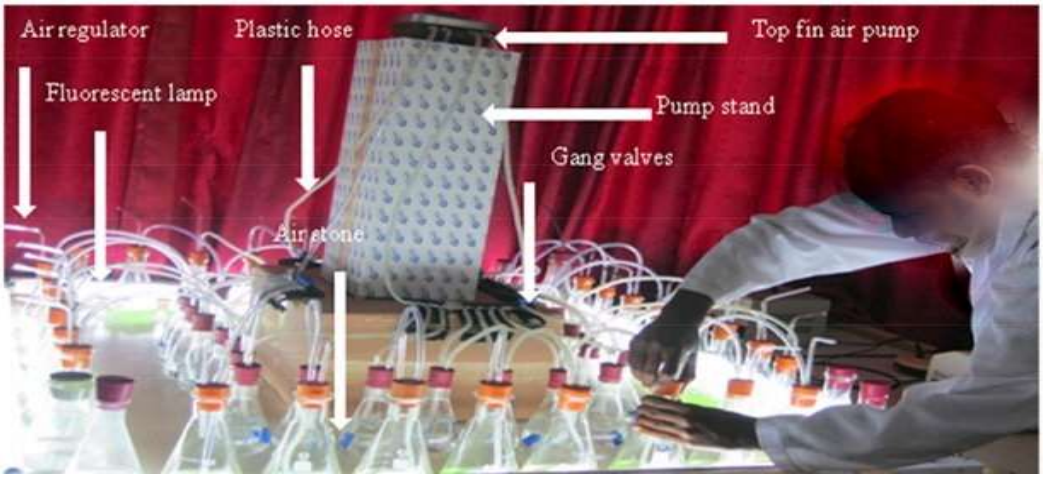

Figure 2. Cyanobacteria *Anabaena* species strain E3 activation for inoculum preparation.

Figure 3. Aeration by modified rotating paddle wheel.

depths (40 and 20 cm) and four pond lining colors (transparent, black, blue and red), altogether eight treatments were used. To control the effect of extraneous variables and assume any significant difference in the outcome variables is fairly due to the pond depth and lining plastic color, the treatments were laid out in a complete randomized design with three replications. In total, 24 experimental units were constructed. Plastic sheets purchased from the local market were used for pond lining after the ponds were constructed from wood in the glasshouse. The size of the two sets of twelve ponds were 50 (width) x 100 (length) x 20 cm (depth) and 50 (width) x 100 (length) x 40 cm (depth). Air was supplied to the growing culture in the ponds by means of a modified paddle wheel (rotating wheel) as shown in Figure 3. The wing length of the paddle wheel in ponds with a 20 cm depth was 10 cm whereas in 40 cm depth ponds it was 20 cm. As a result, the entire culture was set in motion to optimize gas exchange with the atmosphere in a similar manner in all the ponds.

Strain E3 was cultured in the Allen-Arnon media as described above, and this was used as the inoculant culture. The ratio of inoculant to media was 1:15. Four pond lining plastic colors (transparent, black, blue and red) and two depths (20 and 40 cm) with three replicates were assigned to the experimental units. The air temperature range in the glasshouse was 27-38°C, and the light incidence range was 5745.6 to 7711.2 lux with 12 h light/dark cycle for two weeks.

Data collection and analysis

Cyanobacterial growth attributes

Optical density (OD): Optical density was determined at 655 nm at the end of the experiment using JENWAY model 6300 spectrophotometer (Briand et al., 2004).

Growth rate (GR): The growth of cyanobacterial culture was calculated with the optical density result from the following equation (Tang et al., 1997; Gokason et al., 2007): $GR= (X_2-X_1) /(T_2-T_1)$,

Table 2. Interaction effects of pond depth and lining plastic color on optical density of E3 at the end of the two weeks growth period.

Treatment	Optical Density (655nm)				
Pond depth	**Trans**	**Blue**	**Red**	**Black**	**Depth mean**
20 cm	1.227[a]	0.955[c]	0.815[d]	0.614[e]	0.90
40 cm	1.083[b]	0.927[c]	0.804[d]	0.595[e]	0.85
Lining plastic color mean	1.15	0.94	0.81	0.60	
LSD (5%) = 0.08					
CV (%) = 4.74					

Means across all rows and columns followed by the same letter are not statistically different at $p < 0.05$.

where X_1 is the OD at the beginning of a time interval T_1, and X_2 is the optical density at the end of time interval T_2.

Heterocyte frequency (HF): Heterocyte frequency was determined by counting the number of heterocytes (nitrogen fixing cells) in a given filament under microscopic observation and expressed as % frequency (Singh et al., 2011).

Dry biomass (DB): The dry biomass of cyanobacteria was measured by taking 100 ml of culture from each pond at the end of the growth period and filtering it through Whatman No. 42 filter paper, then oven drying at 75°C for 24hrs. Finally, the measured g $100mL^{-1}$ value was converted to g L^{-1}.

Dissolved oxygen (DO): Dissolved oxygen in the culture was measured by Thermo Scientific ORION 5 STAR at the end of the growth period.

pH: The pH of cultures was measured every day during midday time throughout the growth period of the culture by Thermo Scientific ORION 5 STAR.

Total nitrogen (TN): After two weeks of culturing, total nitrogen of each culture was determined using Kjeldahl procedure as described by Nelson and Sommers (1980) and APHA (1999).

Statistical analysis

The triplicate sets of data for various parameters were subjected to analysis of variance (ANOVA) in accordance with the experimental design (completely randomized design) using Statistical Analysis System (SAS, 2003) to quantify and evaluate the source of variation. The means were compared using the least significant difference (LSD) test at the 5% significance level. Simple correlation analysis was conducted between cyanobacterial strain nitrogen fixing capacity and growth attributes.

RESULTS AND DISCUSSION

Chemical properties of the water source

The mean pH of tap water was 7.04, which was within the acceptable range for cyanobacteria growth (Roger and Reynaud, 1979). In addition to this, the amount of total nitrogen in the tap water was found to be 0.30 mg L^{-1}.

Growth performance of strain E3

Optical density

The main effects of pond depth ($p<0.01$), lining plastic color ($p<0.001$) and their interactions ($p<0.05$) were significant on final culture optical density at the end of the experiment. The maximum mean optical density (1.227) was recorded in the 20 cm deep transparent plastic lined ponds whereas minimum OD (0.595) was recorded in 40 cm deep black plastic lined ponds (Table 2). The depth effect was only significant in the ponds with transparent plastic lining; for the blue, red and black plastics, depth did not significantly affect OD.

In cyanobacterial culture, light is the source of energy that drives photosynthesis for assimilation of inorganic carbon which results in increased synthesis of carbohydrates, and thereby increased growth and optical density (Briand et al., 2004). Therefore, the difference in optical density with respect to the interaction effect of the two factors could be attributed to the highest illumination due to shallow depth (20 cm) and the higher light intensity reflected back to the culture as a result of transparency of the lining plastic color. This was consistent with other findings like that of Bernhard et al. (1966). A study conducted by Fernandez et al. (1998) revealed that a transparent glass tank reflected more light back to the culture and thus recorded highest optical density values. Similarly, Park et al. (2011) noticed that an increase in light intensity resulted in higher photosynthetic activity and higher optical density. Since all photoautotrophic organisms, that is cyanobacteria, photosynthetic eubacteria, algae and higher plants, are able to convert light energy into chemical energy by means of photosynthesis, undoubtedly light becomes the most limiting factor in a number of ways. But the light requirement varies from organism to organism. A laboratory scale study by Behl (2013) indicated that higher light intensity resulted in higher optical density, growth rate and dry biomass for cyanobacteria *Synechococcus* BG0011. Different light intensities such as 3000, 4000, 5000 and 6000 lux light were directly

Table 3. Growth parameters of strain E3 at the different pond depth and lining plastic colors. Growth rate (GR), dissolved oxygen (DO), dry biomass (DB) and total nitrogen (TN).

Treatment	GR (OD day^{-1})	DO (mg L^{-1})	DB (g L^{-1})	TN (mg L^{-1})
Pond lining plastic color				
Transparent	0.089^{a}	19.07^{a}	0.66^{a}	41.60^{a}
Blue	0.058^{b}	17.67^{b}	0.53^{b}	25.56^{b}
Red	0.049^{c}	16.90^{b}	0.46^{c}	20.85^{c}
Black	0.039^{d}	15.19^{c}	0.37^{d}	17.22^{d}
LSD (5%)	0.003	0.84	0.02	1.32
Pond depth				
20 cm	0.063^{a}	17.63^{a}	0.584^{a}	27.77^{a}
40 cm	0.054^{b}	16.79^{b}	0.428^{b}	24.85^{b}
LSD (5%)	0.0021	0.59	0.014	0.93
CV (%)	4.15	3.99	3.30	4.09

Means across all columns followed by the same letter are not statistically different at $p < 0.05$.

proportional to light intensity with cyanobacterium *Arthrospira maxima* growth rate, optical density and dry biomass (Pandey et al., 2011). Furthermore, cyanobacterial culture maintained under higher illumination produced chlorophyll concentrations of 25 mg L^{-1} which was slightly higher than the low-light counterparts that produced 20 mg L^{-1} thus, resulting in increased optical density (Wen et al., 2005). The rapid rate of increase in culture absorbance at A_{655} obtained in ponds with highest illumination was largely due to an increase in the volume of cells, their granular content and division rate.

Growth rate

The difference in growth rate of the E3 strain was observed to be highly significant ($p < 0.001$) among the pond liners with different plastic colors. The highest (0.089 OD day^{-1}) and the lowest (0.039 OD day^{-1}) growth rates were observed in ponds lined with transparent and black plastic, respectively (Table 3).

The difference in growth rate could be related to the difference in light intensity saturation that was being reflected by the culture (Bernhard et al., 1966). A study conducted by Alan and David (1990) revealed that cyanobacterial culture grown under high light intensity up to the saturation point induces a higher growth rate than the low light intensity. Similarly, a study conducted by Oliveira et al. (2014) revealed a progressive change in growth response curve and biomass for cyanobacteria (*Anabaena* PCC7120 and *Anabaena variabilis*) related to an increase in light intensity. The same research team indicated that biomass yield of *Nostoc* species F105 was found to be a function of light intensity.

Growth rate of the culture strain was found to be significantly ($p < 0.001$) influenced by the pond depth. However, the interaction of depth and lining color was not significant. Higher growth rate value (0.063 OD day^{-1}) was obtained in ponds with 20 cm depth, whereas a lower value (0.054 OD day^{-1}) was obtained in ponds with 40 cm depth (Table 3).

The lower growth rate of *Anabaena* sp. E3 in 40 cm depth ponds could be due to the decrease in photon flux density of the incoming light. Sunlight heated the surface and was absorbed by the culture; in a relative sense, in 40 cm deep ponds, the photon flux density and light intensity probably decreased with increasing depth as compared to the 20 cm deep ponds. Thus, the lower growth rate was recorded in 40 cm deep ponds. This phenomenon was visualized by Janssen (2002) for ponds with 30 cm depth; the photon flux density was 1000 µmol m^{-2}s^{-1} but decreased with increasing depth. A potential toxin producing cyanobacterial strain of *Cylindrospermopsis raciborskii* (coiled and straight) showed faster growth rate when exposed to higher light intensity (Oliveira et al., 2011). Similarly, the growth rate of cyanobacterial strain PCC 7936 was greatly influenced by higher light intensity as observed by Otero and Vincenzini (2003).

Heterocyte frequency

The main effects of pond lining plastic color, depth and their interaction were highly significant ($p < 0.001$) on the mean heterocyte frequency of the strain grown under different treatment combinations. The maximum (2.92%) and minimum (1.17%) mean heterocyst frequencies were observed in shallow (20 cm) transparent and deep (40 cm) black plastic lined ponds, respectively (Table 4).

The maximum heterocyte frequency in 20 cm transparent plastic lined ponds could be in response to the increased light intensity which resulted in an increased

Table 4. Interaction effect of pond depth and lining plastic color on heterocyte frequency of E3.

Treatment	Heterocyte frequency (%)				
Pond depth	**Trans**	**Blue**	**Red**	**Black**	**Depth mean**
20 cm	2.92[a]	2.21[b]	1.92[c]	1.64[d]	2.17
40 cm	2.15[b]	1.72[d]	1.64[d]	1.17[e]	1.67
Lining plastic color mean	2.535	1.965	1.78	1.405	
LSD (5%) = 0.116					
CV (%) = 3.672					

Means across all rows and columns followed by the same letter are not statistically different at $p < 0.05$.

population of heterocyte along the filament. Similarly, Pisciotta et al. (2010) noted the presence of double heterocytes resulting from an increase in the symmetry of cell division due to incubation of cyanobacteria in a pond having relatively higher illumination. A research report by Adams and Carr (1981) indicated that incubation of *Anabaena cylindrica* at high light intensity resulted in an increase in the frequency of pairs of adjacent double heterocytes (DHC).

In addition, heterocyte formation increased with increased cyanobacteria growth in response to optimum intensity of light (Sven et al., 2010). In the same way, heterocyte frequency of cyanobacteria strain PCC 7936 increased significantly with higher light intensity (Otero and Vincenzini, 2003).

Dissolved oxygen

The difference in mean dissolved oxygen level measured during the growth period of strain E3 was found to be highly significant ($p < 0.001$) among the ponds lined with different plastic colors. The highest (19.07 mg L^{-1}) and the lowest (15.19 mg L^{-1}) dissolved oxygen concentration were observed in ponds lined with transparent and black plastic color, respectively (Table 3).

Pond lining plastic color could reflect a relatively higher light intensity and could produce a higher photosynthetic rate of oxygen evolution. Correspondingly, Baosheng and Kunshan (2002) reported that an increase in cyanobacterial photosynthesis could result in increased dissolved oxygen concentration in the suspension. Pond water day time DO levels could increase to more than two fold saturation with intense photosynthetic activity (Garcia et al., 2000).

The amount of mean dissolved oxygen under the two depths was found to be significantly ($p < 0.01$) influenced by pond depth. However, the interaction of depth and lining color was not significant. Higher (17.63 mg L^{-1}) and lower (16.79 mg L^{-1}) dissolved oxygen of the culture strain was recorded in ponds with 20 and 40 cm depth, respectively (Table 3).

The higher dissolved oxygen level of *Anabaena* sp. E3 in ponds with 20 cm depth could be due to the higher illumination in shallow depth that comparatively enhanced photosynthetic rate and hence oxygen evolution. Similarly, Huisman et al. (2004) noted that increased light intensity resulted in higher photosynthetic activity and consequently, this resulted in enhanced oxygen production. Furthermore, Kranz et al. (2010) suggested that the effect of optimum light could increase optical density and growth rate of a cyanobacterial culture by promoting photosynthetic activity which in turn resulted in increased oxygen level.

pH of the culture

The difference in mean pH of the culture measured during the growth period was found to be significant ($p < 0.05$) among the different treatment combinations. The maximum mean pH (10.28) was recorded in 20 cm deep transparent plastic lined ponds whereas the minimum (8.88) was recorded in 40 cm deep black plastic lined ponds (Figure 4).

Prior to the inoculation of the test strain in the different treatment units, the pH of the cyanobacterial growing culture was 7.97. Nevertheless, pH increased in the whole treatment units during the growth period.

Higher illumination due to the shallow pond depth (20 cm) and transparency of the lining color resulted in higher photosynthetic activity which in turn caused an increased consumption of CO_2 and HCO_3^-. This phenomenon leads to the accumulation of OH^- ions in the medium (Park and Craggs, 2010). Photosynthetic rates can be considerable within millimeter-thin layers of cyanobacteria at the air–water interface because of high light intensities and efficient gas exchange with the atmosphere in shallow ponds (20 cm) which were lined with transparent plastic; accordingly, high CO_2 uptake resulted in a relatively higher pH value (Briand et al., 2004). Moreover, Gao et al. (2012) indicated that an increase in cyanobacterial biomass caused an elevation in pH (9-10.5) for weeks in the shallow and tidal-fresh regions of the Sassafras

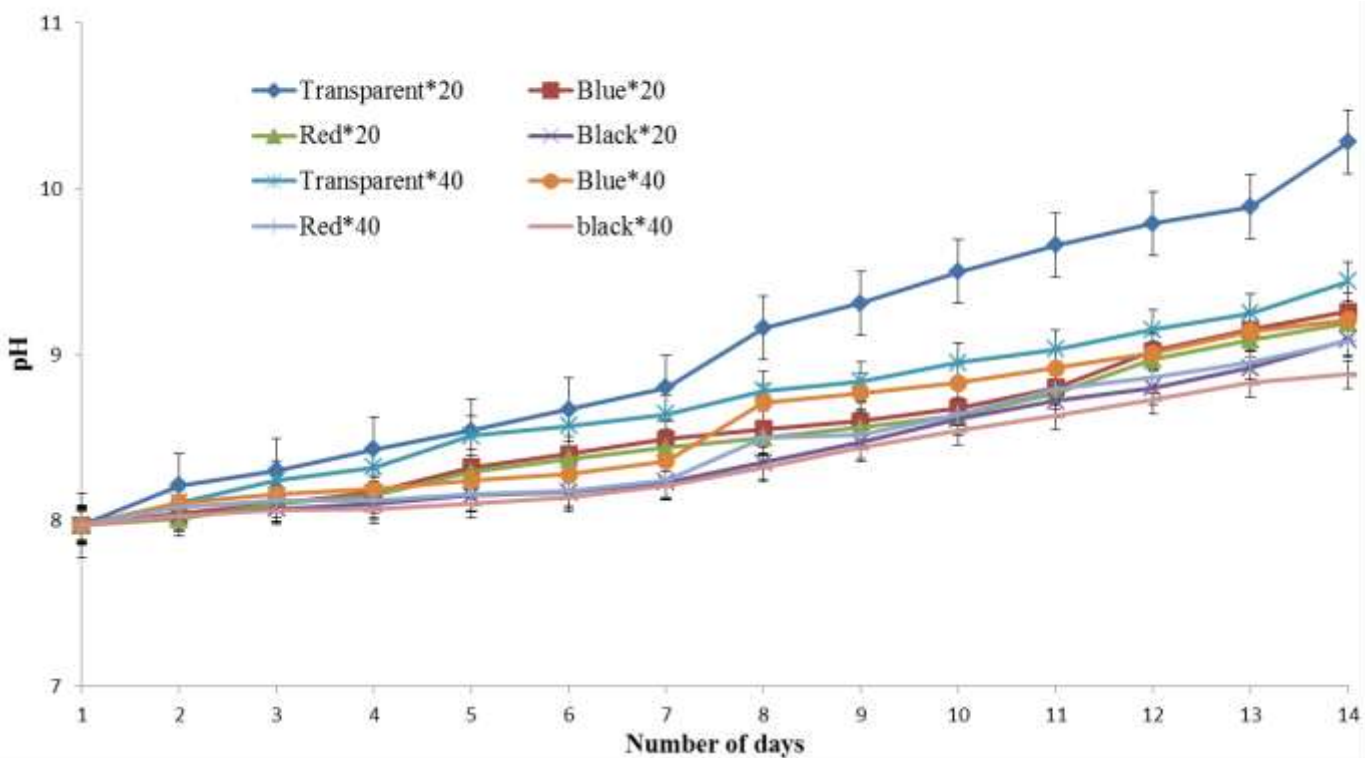

Figure 4. The pH of growing culture in different treatment units and it was measured during the midday time. Error bars indicate the 95% confidence intervals based on the Students T distribution. The numbers 20 and 40 after the respective lining plastic colors indicates depth of the ponds in cm.

River, a tributary of the Chesapeake Bay (USA).

Dry biomass

The difference in dry biomass was observed to be highly significant ($p < 0.001$) among the ponds lined with different plastic colors. The highest (0.66 g L^{-1}) and lowest (0.37 g L^{-1}) dry biomass were recorded in transparent and black plastic color lined ponds, respectively (Table 3).

This difference in dry biomass among the ponds lined with different plastic colors could be a reflection of the difference in light intensity reflected by the culture that had direct impact on photosynthetic rate and thus the dry biomass (Tredici, 1999). The influence of depth on dry biomass was highly significant ($p < 0.001$). However, the interaction of depth and lining color was not significant. Higher (0.58 g L^{-1}) and lower (0.43 g L^{-1}) dry biomass of cyanobacteria *Anabaena* sp. E3 was recorded in ponds with 20 and 40 cm depth, respectively (Table 3).

The difference in dry biomass under the two depths could be due to variation in the efficiency of light utilization, which can be expressed as the biomass yield of light energy in grams of dry weight of cyanobacteria *Anabaena* sp. E3 per amount of light energy absorbed. The poor volumetric dry biomass productivity of the culture strain in 40 cm deep ponds could be related to the relatively long light path which decreased photosynthetic efficiency. Thus, lower biomass was observed (Janssen, 2002). In addition, the findings of this research strongly agreed with Sven et al. (2010) that optimum light intensity had a pronounced effect on the growth and dry biomass accumulation of cyanobacterial culture. In addition, the report of Sven et al. (2010) again found that the biomass of cyanobacterial culture did not increase even by two fold when grown at a low light intensity of 2000 lux. However, higher biomass of 1.2366 g L^{-1} was acquired when grown under light intensity of 6000 lux.

Total nitrogen content

The difference in total nitrogen was observed to be highly significant ($p < 0.001$) among the ponds lined with different plastic colors. The highest (41.60 mg L^{-1}) and lowest (17.22 mg L^{-1}) total nitrogen concentrations were recorded in ponds lined with transparent and black plastics, respectively (Table 3).

The significant difference in total nitrogen content of *Anabaena* sp. E3 in ponds with different lining color could be attributed to relatively higher nitrogenase enzyme activity of the cells resulting from the difference in light

intensity (Levitan et al., 2010). Similarly, the effect of optimum light intensity can stimulate photosynthesis and nitrogen fixation of cyanobacteria (Kranz et al., 2010). The same research study reported that gross photosynthesis increased with light intensity, which in turn, increased total nitrogen, carbon content and growth rate of cyanobacterium, *Trichodesmium* IMS101. Moreover, high light intensity increases cellular iron uptake which could eventually be responsible for higher nitrogen fixation (Milligan and Harrison, 2001).

Total nitrogen content of the culture was found to be significantly ($p < 0.001$) influenced by pond depth. However, the interaction of pond depth and lining color was not significant. Higher nitrogen concentrations were recorded in ponds with 20 cm (27.77 mg L^{-1}) depth as compared to the 40 cm depth (24.85 mgL^{-1}) (Table 3).

The significant difference in total nitrogen content under the two depths could be attributed to variation in photon flux density which further created variation in illumination and intensity of light (Janssen, 2002). Similarly, it was observed that an increase in light intensity up to the saturation point could accelerate photosynthesis and thus enhance both growth and N_2 fixation of cyanobacteria (Alan and David, 1990). The total nitrogen content was positively and significantly ($p \leq 0.001$) correlated with optical density ($r = 0.92$), growth rate ($r = 0.98$), heterocytes frequency ($r = 0.93$), dissolved oxygen ($r = 0.84$) and dry biomass ($r = 0.85$). These correlations indicate that TN increased due the increase in these parameters.

Conclusion

Biological nitrogen fixation through microbial processes ensures great quantitative impact on the nitrogen cycle and has tremendous potential for the contribution of fixed nitrogen in the soil. Cyanobacterial based biofertilizer mass production conditions can greatly influence distribution and concentrations of photosynthetic and UV-screening pigments which, in turn, can critically alter light attenuation, photosynthetic rates and nitrogenase activity. Therefore, from the study findings, it could thus be concluded that the test strain in this study should be grown in ponds of 20 cm depth lined with transparent plastic for large-scale production. However, the issue related to cyanotoxins cannot be overlooked, thus further research aiming at exploring the safety of this strain should be taken into consideration for large-scale production and dissemination of biofertilizer derived from this strain.

Conflict of interest

The authors declare that there is no conflict of interest with regards to this study.

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