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Effect of Varying Physicochemical Parameters on the Productivity and Phycobiliprotein Content of Indigenous Isolate *Geitlerinema sulphureum*

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Abstract: Cyanobacteria have long been studied to be potent candidates in biologically mediated carbon dioxide sequestration. Harvesting the grown culture for production of high value compounds such as the vibrantly coloured photosynthetic water soluble pigment phycocyanin could reduce the cost of sequestration. Indigenously isolated cyanobacterium *Geitlerinema sulphureum* is a proven candidate for CO_2 sequestration. An attempt to design a practical and efficient set of growth parameters to enhance the productivity and phycocyanin content of *Geitlerinema sulphureum* has been carried out. It is a known fact that varying physicochemical parameters such as light intensity, temperature, nutrient content such a nitrates and carbonates affect photosynthesis and thereby the growth of microalgae as well as the concentration of phycobiliproteins present in them. The productivity for the culture grown in optimized physicochemical conditions was 0.051 g L^{-1} day^{-1} while that of the culture grown in standard conditions was 0.025 g L^{-1} day^{-1}. Here, the culture which was provided with optimized conditions had a phycocyanin yield of 0.071 g L^{-1} of harvested culture while the phycocyanin yield of culture grown in standard media was 0.021 g L^{-1}. These results are more than promising in terms of *G. sulphureum* being an ideal candidate for commercial production of phycocyanin as an adjunct to a model organism for CO_2 sequestration.

Key words: Cyanobacteria, *Geitlerinema sulphureum*, phycocyanin yeild, phycobiliproteins, CO_2 sequestration

INTRODUCTION

In recent years microalgae have trumped other mediators of carbon dioxide sequestration for several reasons. Industrial flue gases contain traces of SO_x and NO_x which need to be removed while sequestering CO_2 which increases the cost of separation Microalgae can be fed with directly be fed with flue gas, without hindering their growth. Further, they have minimal requirements in terms of land usage; water quality etc. and the resulting biomass can be harvested and used for a variety of commercial products (Spolaore *et al*., 2006). This biomass consists of metabolites that can be used for high-value products including animal feed, nutritional supplements, algal biofuels, natural colourants etc. (Mostafa, 2012). Cyanobacteria, in particular are known to produce a wide variety of vibrant, water soluble photosynthetic pigments. These pigments could be used as natural colourants for food and cosmetic products as well as molecular fluorescent markers, depending on their purity grade (Chu, 2012).

In 2008, KET's VG Vaze College went into collaboration with a leading Indian cement company to carry out a preliminary study wherein *Chlorella vulgaris* SAG 211.12 was used as a model organism in a 2 L air lift photobioreactor for CO_2 sequestration experiments. In order to be deemed suitable for further experiments, the microalgal strain should have been be able to sustain temperatures beyond 30°C, as typically seen in Indian climatic conditions. *Chlorella vulgaris* SAG 211.12 did not survive at temperatures above 30°C. Hence, cyanobacterial species were isolated from hot springs, found in the Konkan region of West India (Adak and Deodhar, 2011). Three strains viz., *Limnothrix redekei*, *Planktolyngbya crassa* and *Geitlerinema sulphureum* could thrive in upto 28% of CO_2 without hampering biomass production. *G. sulphureum* was found to be a promising cyanobacterial species for CO_2 sequestration because it was found to tolerate high temperature typical of the tropical climatic conditions and also could sustain high concentration of CO_2 (Manjre and Deodhar, 2013).

Geitlerinema sulphureum has many positive characteristics. It has a high productivity (upto 50 mg L^{-1} day^{-1}) which is comparable to that of *Spirullina platensis* with a productivity of 50 mg L^{-1} day^{-1} as reported by Bhattacharya and Shivaprakash (2005). It is higher than the productivity of

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another strain of *Spirullina platensis* with a productivity 30.2±0.7 mg L^{-1} day^{-1} as reported by Colla *et al.* (2007). Another important factor is its ability to flourish under high temperature and light intensity (upto 35°C and 50 klux recorded in the summer), being an indigenous isolate. Further, it is an extreme alkalophile, making it easy to cultivate outdoors without risk of contamination. It can be easily harvested by sedimentation and it does not produce any mucilage, making it easy to use on a large scale. Its ability to grow in media containing high nitrate content also makes it an ideal candidate for production using waste waters high in nitrates.

Cyanobacteria possess water soluble photosynthetic pigments known as phycobiliproteins, allophycocyanin (APC), phycocyanin (CPC) and phycoerythrin (PE) which absorb maximally in the visible region of the spectrum at 650, 625 and 565 nm, respectively of which phycocyanin is mostly found to be predominant (Glazer and Fang, 1972). The growing awareness of the harmful effect of synthetic colourants has given rise to a demand for natural colours especially in foods and cosmetics (Sarada *et al.*, 1999). The prices of phycobiliproteins vary from US $ 3-25 mg^{-1} for food/cosmetic grade pigments but they can reach US$ 1500 mg^{-1} for highly purified molecular markers (with antibodies or other fluorescent molecules). In 1997, the value of these pigments in the commercial sector was estimated to be US$ 50 million worldwide (Spolaore *et al.*, 2006).

Geitlerinema sulphureum was also found to have comparable pigment production to many reported cyanobacteria. The investment in producing fine chemicals from the harvested microalgae can help in minimizing the cost that comes from carbon dioxide sequestration, thus serving a dual benefit of reducing the environmental impact as well as making profit out of it.

Gantar *et al.* (2012) reported a species of *Limnothrix* isolated from Crescent lake, Florida having a phyocyanin content of 18% as well as a biomass yield of 1.2 g L^{-1}. Shukia *et al.* (2008) reported a strain of Anabaena to have a phycocyanin percentage content of 7.9% while that of a strain isolated in Antarctica to have 14.6% PC. It is a known fact that varying physicochemical parameters such as light intensity, light regimen, temperature, nutrient content such a nitrates and carbonates affect photosynthesis and thereby the growth of microalgae as well as the concentration of phycobilproteins present in them.

An attempt to design a practical and efficient set of growth parameters to enhance the productivity and phycocyanin content in our isolated strain of *Geitlerinema sulphureum* has been carried out.

MATERIALS AND METHODS

Organism and culture condition: The freshwater cyanobacterium *Geitlerinema sulphureum* was isolated from a hot water spring located in Ganeshpuri (19.4867°N, 73.0258°E) situated in Thane district, Maharashtra, India (Manjre and Deodhar, 2013). This culture was grown in batch cultures in modified Zarrouk's medium. [Composition: $NaHCO_3$ 10 g, $NaNO_3$ 2.5 g, NaCl 1.0 g, K_2HPO_4 0.5 g, K_2SO_4 1.0 g, $MgSO_4$ 0.2 g, $CaCl_2.2H_20$ 0.04 g, $FeSO_4$ 0.01 g] at pH between 8-9 and temperature 27°C with light intensity of 1000 lux provided by cool-white fluorescent tubes with a Light: Dark cycle of 16 to 8 h. The cultures were placed in 250 mL volumetric conical glass flasks on a double decker orbital shaker for continuous aeration and agitation. All chemicals were obtained from Merck Ltd. of analytical grade quality.

Estimation of dry weight: Biomass was harvested from flask and separated from the surrounded media by centrifugation. Residual salts from the media were removed by washing the biomass pellet with distilled water. The resulting biomass was filtered through preweighed Whatman paper No. 1 and dried at 60°C for 24 h. The dried biomass was weighed and results were interpreted (Mohite and Wakte, 2011):

$$\text{Productivity (g L}^{-1}\text{ day)} = \frac{\text{Final dry biomass (g L}^{-1}\text{)-Initial dry biomass (g L}^{-1}\text{)}}{\text{No. of days of growth (Days)}}$$

Extraction of phycobiliproteins: The fresh cyanobacterial cells were harvested after 10 days of incubation under laboratory-controlled conditions (temperature, pH and light) by centrifugation at 5000 g for 20 min. The harvested cell mass was frozen at 0°C. One gram of frozen cell mass was suspended in 5 mL of sodium-phosphate buffer (0.01 M, pH 7.0), the suspended cell mass was disrupted by thawing at 4°C overnight. Repeated freezing and thawing was not necessary at the entire phycobiliprotein content was leeched in the first cycle itself and carrying out a second cycle of freeze-thawing gave rise to a supernatant devoid of phycobiliproteins. The cell lysate was subsequently centrifuged at 6000 g for 15 min at 8°C and a phycobiliprotein containing clear supernatant was collected.

Estimation of phycobiliproteins: The pigments were diluted (2 mL distilled water and 0.4 mL extract) and the absorbances of phycobiliprotein containing supernatant were measured on a Genesys Scan UV-Vis, NIR spectrophotometer by Thermo Scientific at wavelengths 620, 652 and 562 nm for calculating the concentrations of

C-PC, APC and PE, respectively (taking into consideration the dilution factor), using the following equations (Bennet and Bogorad, 1973):

$$\text{C-PC mg mL}^{-1} = [A_{620}\text{-}0.474(A_{652})]/5.34$$

$$\text{APC mg mL}^{-1} = [A_{652}\text{-}0.208\ (A_{620})]/5.09$$

$$\text{PE mg mL}^{-1} = \{A_{562}\text{-}2.41\ [PC]\text{-}0.849[APC]\}/9.62$$

where, A620 is the absorption maxima for C-Phycocyanin, A652 is the absorption maxima for Allophycocyanin, A562 is the absorption maxima for Phycoerythrin.

Spectroscopic measurement: All UV-Vis absorption spectra of C-PC samples were recorded on a CARY 500 Scan UV-Vis, NIR spectrophotometer with a 1 cm path length. In the visible region, C-PC and APC show maximum absorption at 620 and 652 nm, respectively. The purity was evaluated according to the absorbance ratios (A_{620}/A_{280}) (Boussiba and Richmond, 1979).

Experimental set-up

Effect of inoculum size: To study the effect of inoculum size on growth of *Geitlerinema sulphureum,* 100 mL of media was inoculated with varying concentration of *Geitlerinema sulphureum* 0.1, 0.2 and 0.3 g L^{-1}, respectively. The growth was monitored on day 0, 2, 4, 6, 8 and day 10. Flasks were harvested every alternate day in order to determine the dry weight of the biomass (in g L^{-1}). Observations were made in triplicates and the average productivity of the culture at each concentration was calculated. The growth curve was plotted.

Effect of light intensity: Media with a total volume of 100 mL was inoculated with a fixed density of culture in triplicates in two sets. One set each was subjected to varying light intensities viz., 700, 1000 and 1400 lux for a period of ten days. The growth was recorded in terms of dry biomass and productivity was calculated from the initial and final dry weight of the culture. Percentage of C-Phycocyanin was measured per mg dry biomass.

Effect of temperature: Media with a total volume of 100 mL was inoculated with a fixed density of culture in triplicates in three sets. One set each was subjected to varying temperatures viz., 20, 25 and 30°C for a period of ten days. The growth was recorded in terms of dry biomass and productivity was calculated from the initial and final dry weight of the culture. Percentage of C-Phycocyanin was measured per mg dry biomass.

Effect of nitrate concentration: Media with a total volume of 100 mL was inoculated with a fixed density of culture in triplicates in four sets. One set each was subjected to varying nitrate concentrations with sodium nitrate being the nitrate source. The concentrations of sodium nitrate chosen were 1.5, 2.5, 3.5 and 4.5 g L^{-1}. The cultures grown at 2.5 g L^{-1} where used as the control since the media contains 2.5 g L^{-1} of sodium nitrate as a standard concentration. The growth was recorded in terms of dry biomass and productivity was calculated from the initial and final dry weight of the culture. Percentage of C-Phycocyanin was measured per mg dry biomass.

Effect of different concentrations of sodium hydrogen carbonate: Media with a total volume of 100 mL was inoculated with a fixed density of culture in triplicates in six sets. One set each was subjected to varying bicarbonate concentrations with sodium hydrogen carbonate being the carbonate source. The concentrations of sodium bicarbonate chosen were 0, 2, 4, 6, 8 and 10 g L^{-1}. The cultures grown at 10 g L^{-1} where used as the control since the media contains 10 g L^{-1} of sodium bicarbonate as a standard concentration. The growth was recorded in terms of dry biomass and productivity was calculated from the initial and final dry weight of the culture. Percentage of C-Phycocyanin was measured per mg dry biomass.

Effect of different concentrations of sodium carbonate: Media with a total volume of 100 mL was inoculated with a fixed density of culture in triplicates in five sets. One set each was subjected to varying carbonate concentrations with sodium hydrogen carbonate being the carbonate source. The concentrations of sodium carbonate chosen were 3.12, 6.24, 9.3 and 12.5 g L^{-1} of sodium carbonate. The cultures grown at 10 g L^{-1} where used as the control since the media contains 10 g L^{-1} of sodium nitrate as a standard concentration. The growth was recorded in terms of dry biomass and productivity was calculated from the initial and final dry weight of the culture. Percentage of C-Phycocyanin (in mg) was measured per mg dry biomass.

Effect of varying growth period: To study the effect of varying growth period size on growth of *Geitlerinema sulphureum*, 100 mL of media was inoculated with a fixed density. The growth was monitored on day 0, 5, 10, 15 and

20. Flasks were harvested on these days in triplicates in order to determine the dry weight of the biomass (in g L^{-1}). Observations were made in triplicates and the productivity of the culture at each concentration was calculated. The growth curve was plotted. The growth was recorded in terms of dry biomass and productivity was calculated from the initial and final dry weight of the culture. Percentage of C-Phycocyanin was measured per mg dry biomass.

Effect of optimization of all parameters: Media with a total volume of 100 mL was inoculated with a fixed density of culture in triplicates in two sets. One set had media modified with 3.5 g of $NaNO_3$ and 6.35 g L^{-1} of Na_2CO_3 for a period of fifteen days with 1400 lux light intensity at 30°C. The second set was grown in the standard modified Zarrouk's media for a period of fifteen days (so as to have a comparable productivity) with 1000 lux light intensity at 27°C. Their growth in terms of productivity as well as percentage C-Phycocyanin content was calculated.

RESULTS AND DISCUSSION

Effect of inoculum size of *Geitlerinema sulphureum*: Flasks inoculated with 0.1 g L^{-1} (in dry weight) inoculum showed a steady rise throughout the growth period upto the 10th day as seen in Table 1. In media inoculated with 0.2 g L^{-1} (in dry weight) inoculum and 0.3 g L^{-1} (in dry weight) inoculum, the log phase was observed up to the 6th day and after which stationary phase was achieved. With all three inoculum sizes, there is no significant difference between the production of biomass at the end of the 10th day. For 0.1 g L^{-1} inoculum size, the biomass increase is approximately 6 fold and becomes 0.61 g L^{-1} whereas, for 0.3 g L^{-1} inoculum size, the increase in biomass is only 1.83 fold and the resulting biomass is 0.648 g L^{-1}. Hence, 0.1 g L^{-1} inoculum size was selected as the optimal inoculum size for further studies. It however, maybe be observed, that the productivity may vary with age of the inoculum used, due to which simultaneous inoculation of experimental sets is necessary so as to draw an unbiased observation of the effect of variation made in every parameter. In 2008 Lopez-Elias *et al*. (2008) carried out a similar study where the effect of varying inoculum size on the growth of microalga *Chaetoceros muelleri* in mass outdoor cultures was analysed. They found that a cellular density of 0.4×10^6 cells mL^{-1} reached the optimal density required before harvested in the shortest amount of time as compared to higher inoculum densities. They postulated that, higher inoculum densities could lower growth rate of the cultures due to the phenomenon of 'autoshading' by the same organisms in the culture itself, resulting in poor light penetration, thereby lowering the growth rate of the culture.

Effect of light intensity on C-Phycocyanin production of *Geitlerinema sulphureum*: Light intensity and light regime significantly affect the photosynthetic rate of an organism which in turn influences its growth (Pandey *et al*., 2010). Table 2 illustrates the differences in productivity under varying light intensities, with productivity at lower light intensity (700 lux) being 0.017 g L^{-1} day^{-1}, while that at high light intensity (1400 lux) was 0.029 g L^{-1} day^{-1}. The productivity of the culture with intermediate light intensity was found to be 0.025 g L^{-1} day^{-1}. This could be explained by the fact that the photosynthetic rate increases with an increase in incident light intensity. Pandey *et al.* (2010) assessed the effect of varying light intensity on *Spirullina platensis* where the culture was subjected to varying light intensities between 3000 to 5000 lux. Highest biomass (in terms of dry weight) was observed at 5000 lux with 1700 mg L^{-1}. At the lowest light intensity used (3000 lux), the biomass achieved was 1200 mg L^{-1}. Similar observations were made by Mohite and Wakte (2011) in 2011 when they were assessing the physical factors influencing the growth and C-Phycocyanin production in *Arthrospira platensis*. The culture was subjected to various light intensities between 2500 to 10,000 flux. The inoculated flasks were kept under growth conditions for 12 days. The isolate showed the optimal growth at light intensity of 2500 flux with a productivity of 0.031 g L^{-1} day^{-1}. At the highest light intensity i.e., 10,000 lux, the productivity significantly decreased

Table 1: Indicates the increase in dry weight (in g L^{-1}) of grown biomass over a period of ten days

Dry weight (g L^{-1})	Day 0 (g L^{-1})	Day 2 (g L^{-1})	Day 4 (g L^{-1})	Day 6 (g L^{-1})	Day 8 (g L^{-1})	Day 10 (g L^{-1})	Overall productivity (g L^{-1} day^{-1})
0.1	0.120	0.163	0.268	0.445	0.558	0.610	0.0490
0.2	0.212	0.277	0.396	0.545	0.597	0.517	0.0305
0.3	353.000	0.440	0.548	0.656	0.653	0.648	0.0295

Table 2: Indicates the effect of varying light intensity on productivity and PBP content of *G. sulphureum*

Light intensity (lux)	Productivity (g L^{-1} day^{-1})	CPC content (%)	APC content (%)	PE content (%)	A620:A280 ratio
700	0.017	8.94	2.28	0.37	1.15
1000	0.025	5.62	0.35	0.32	1.08
1400	0.029	4.45	0.28	0.27	0.94

Table 3: Indicates the effect of varying temperature on productivity and PBP content of *G. sulphureum*

		PBP content (% mg dry wt.$^{-1}$)		
Temperature (°C)	Productivity (g L^{-1} day^{-1})	CPC	APC	PE
20	0.007	5.730	1.370	0.244
25	0.015	5.270	0.435	0.278
30	0.025	2.880	0.405	0.150

to 0.020 g L^{-1} day^{-1}. It could be concluded that beyond a threshold light intensity, that higher light decreases maximizes the productivity of the given culture.

But an increase in light intensity brought about a decrease in in phycocyanin content from 8.93% at 700 lux to 4.45% at 1400 lux (Table 2). At intermediate light intensity 1000 lux, the phycocyanin percentage was found to be 5.62% A decrease was also seen in allophycocyanin content from 2.27% in the culture grown at 700 lux to 0.28% in the culture grown at 1400 lux. It was observed that the relative concentration of allophycocyanin increases at lower light intensity, this is because allophycocyanin is a more efficient harvestor of light as compared to C-phycocyanin (Lemasson *et al*., 1973). Hence, its concentration increases when there is a dearth of light so as to maximize the efficiency of the photosynthetic process. Change in light intensity did not cause much change in phycoerythrin content. It decreased from 0.37% at 700 lux to 0.27% at 1400 lux. High light intensity not only alters the percentage phycocyanin content but also alters the purity ratio of C-Phycocyanin to other proteins. At 700 lux, the purity ratio was 1.15 whereas, it decreased to 0.94 at 1400 lux imparting a greenish tinge to the crude PBP extract which can be subsequently removed by ammonium sulphate fractionation. Velea *et al.* (2011) studied the effect of varying light intensity on growth and PBP production in *Porphyridium purpureum.* They reported a drastic decrease in the phycobiliprotein concentration of red alga *Porphyridium purpureum.* At light intensity 120 μE m^{-2} sec^{-1}, the phycoerythrin was 10.23% whereas, at 240 μE m^{-2} sec^{-1}, it reduced to 1.85%. The percentage of phycocyanin and allophycocyanin also decreased. At similar observations were made by Mohite and Wakte (2011) and Zucchi and Necchi (2001).

Effect of temperature on *Geitlerinema sulphureum*: Table 3 shows that as the temperature of the growth environment increased, the biomass significantly increased. At 20°C, a very negligible productivity of 0.007 g L^{-1} day^{-1} was seen. At 25°C, a productivity was 0.015 g L^{-1} day^{-1}. This increase was two folds than that at 20°C. The productivity at 30°C was 0.0259 g L^{-1} day^{-1}, nearly 3.7 times that at 20°C. This is because the culture was isolated from hot water springs. These results are encouraging, since it indicates that the culture could be used in the outdoors and hence, production could be scaled up. This was in coherence to the findings of Soundarapandian and Vasanthi (2008). They tested the effect of temperature on biomass of five strains of *Arthrospira platensis.* viz., 15, 20, 25, 30, 35, 40°C. The highest biomass was recorded in between 30 and 40°C for all cultures, the highest biomass being 0.017 g mL^{-1} for strain CS-1 at 35°C, indicating that the strain was better adapted to higher temperatures.

As the temperature increased, the phycocyanin content decreased. At 20°C it was 5.73% at 20°C and slightly decreased to 5.27% at 25°C (Table 3). But at 30°C, the phycocyanin content substantially decreased to 2.88%. A decline in the APC content is observed when the temperature is increased from 1.37% at 20°C to 0.435% at 25°C. However, this lower concentration is maintained even when the temperature rises to 30°C at 0.405%. Phycoerythrin present in trace amounts did not show a drastic change as the temperature is increased. At 20°C, it is found to be 0.244% and decreases to 0.15% at 30°C. This could be because, an increase in photosynthetic rate, favours the production of chlorophyll over accessory pigments. Similar effect of temperature on the concentration of C-PC in *Arthrospira platensis* was observed by Mohite and Wakte (2011). The pigment concentration is found to be maximal (29.38 mg mL^{-1}) at 28°C and the pigment concentration decreased at 30°C to 20-25 mg mL^{-1}, indicative that an increase in temperature caused a decrease in phycocyanin content, similar to our findings.

Effect of nitrate concentration on C-Phycocyanin production of *Geitlerinema sulphureum*: In *Geitlerinema sulphureum,* it was observed that at low nitrate concentrations i.e., 1.5 g L^{-1}, the productivity was very negligible as seen in Table 4. Hence, further studies on the concentration of C-PC were not made. At 2.5 g L^{-1} of sodium nitrate, a productivity of 0.0154 g L^{-1} day^{-1} was observed. With an increase in nitrate, the biomass concentration as well as the productivity increased. At 3.5 mg mL^{-1}, a productivity of 0.023 g L^{-1} day^{-1} was observed. At the highest concentration of 4.5 g L^{-1}, the increase was not as significant as compared to that at 3.5 g L^{-1}, the biomass was productivity was 0.025 g L^{-1} day^{-1}. Hence, 3.5 g L^{-1} of sodium nitrate may be utilized for optimized biomass production. Nitrogen is

Table 4: Indicates the effect of varying nitrate concentration on productivity and PBP content of *G. sulphureum*

Concentration of $NaNO_3$ (g L^{-1})	Productivity (g L^{-1} day^{-1})	Percentage CPC content	Percentage APC content	Percentage PE content
1.5	0.006	Productivity was low, hence, concentration was not evaluated		
2.5	0.015	5.27	0.43	0.27
3.5	0.023	8.81	1.93	0.96
4.5	0.024	9.90		

Table 5: Indicates the effect of varying bicarbonate concentration on productivity and PBP content of *G. sulphureum*

Concentration of $NaHCO_3$ (g L^{-1})	Productivity (g L^{-1} day^{-1})	Percentage CPC content	Percentage APC content	Percentage PE content
0	No growth observed			
2.0				
4.0	0.0030	5.54	1.48	0.230
6.0	0.0040	5.03	1.16	0.097
8.0	0.0100	5.67	0.84	0.350
10.0	0.0252	5.76	1.37	0.430

part of the building blocks of every living cell. Its presence, in the surrounding media, has a positive effect on the microalga's growth. Similarly, Nigam *et al*. (2011), found that increasing nitrate concentration increased biomass production of green alga *Chlorella pyrenoidosa*. The original nitrogen source concentration in the medium was 0.2 g L^{-1} KNO_3 in Fogg's medium. The experiment was performed in 0, 1/4, 1/2 and double of the original nitrogen source concentration. No growth was observed, in absence of a nitrogen source. Maximum biomass concentration of 0.315 g L^{-1} was achieved with double the concentration of nitrate (0.4 g L^{-1} KNO_3). Dry matter of 0.296, 0.246 and 0.127 g L^{-1} was found when the culture was supplemented with 0.2 g L^{-1} KNO_3 (control), 0.1 g L KNO_3(1/2 of control) and 0.05 g L^{-1} KNO_3 (1/4th of control), respectively.

Table 4 indicates the C-PC percentage at a standard nitrate concentration is 5.27%. At 3.5 g L^{-1} of sodium nitrate, the C-PC percentage drastically increases to 8.8%. At 4.5 g L^{-1} of $NaNO_3$, the percentage of C-PC is 9.96% which is not significantly different from that found when the concentration is 3.5 g L^{-1}. An approximately five fold increase is seen in the percentage content of both allophycocyanin and phycoerythrin in the cultures grown with 3.5 and 4.5 g L^{-1} of sodium nitrate. The standard concentration of nitrate (2.5 g L^{-1}) shows a concentration of 0.43 and 0.27% of allophycocyanin and phycoerythrin. This increased to 1.99 and 0.89%, respectively at a concentration of 4.5 g L^{-1}. Since the main product of interest is C-PC, it would be ideal to utilize 3.5g L^{-1} of sodium nitrate in terms of optimizing the phycocyanin content.

Similar to our work, Urek and Tarhan (2012) used *Spirulina maxima* SAG 84.79 to study the effect of nitrates on phycocyanin production. In growth conditions of *S. maxima*, 10 mM (limited) and 50 mM (supplemented) $NaNO_3$ was used as a nitrogen source. The highest phycocyanin content was determined in nitrate supplemented conditions on day 13, at 190.68±9.3 mg g^{-1}. Phycoyanin and other pigments increased with 1.7 and 1.2 times in *Spirulina maxima* when grown in higher (upto 50 mM) concentratations of nitrate in comparison to be grown in nitrate depleted and control conditions. Abd El-Baky (2003) reported that *Spirulina platensis* at high nitrogen level gave highest percentage of total phycocyanin 9.94% and R-PC 5.75%. Pereira *et al*. (2012) also concluded there was a direct relationship in between the phycocyanin content with the concentration of nitrogen sources (ammonium ions, nitrates, nitrites etc.) in *Gracilaria domingensis*.

Effect of carbonate concentration of *Geitlerinema sulphureum*: Modified Zarrouk's (1966) medium contains a very high quantity (10 g L^{-1}) of sodium hydrogen carbonate. An experiment was carried out with a commercial point of view to observe whether lowering the carbonate concentration had any effect on the productivity as well as phycobiliprotein content of the culture.

Table 5 suggests the standard concentration of 10 g L^{-1} of sodium hydrogen carbonate is the minimum required for growth of the culture as the productivity of the culture at 8 g L^{-1} was 0.01 g $L^{-1}day^{-1}$ but it rose to 0.025 g L^{-1} day^{-1} at 10 g L^{-1}. It is therefore necessary to supplement the medium with a minimum of 10 g L^{-1} of $NaHCO_3$ for suitable growth to occur. This maybe because the preferential uptake of carbonate ions over bicarbonate ions by the cell. Velea *et al*. (2011) studied two variables; light and sodium bicarbonate on growth and phycoerythrin production of *Porphyridium purpureum*. They supplemented ASW medium with additional amount of $NaHCO_3$ (1, 2 and 3 mg mL^{-1}) as in comparison to the standard concentration (0.51 g L^{-1}). The highest exponential growth was observed at 2 g L^{-1} $NaHCO_3$ when the cultures were exposed to 120 µE m^{-2} sec^{-1} of light. The biomass produced in standard ASW medium was 3.5 g L^{-1}. When ASW was supplemented with 2 g of $NaHCO_3$ at the same light intensity produced a biomass of 4 g L^{-1}.

Table 6: Indicates the effect of varying concentration of sodium carbonate on productivity and PBP content of *G. sulphureum*

Concentration of sodium carbonate (g L^{-1})	Productivity	Percentage CPC content	Percentage APC content	Percentage PE content
3.12 Na_2CO_3	0.035	8.75	1.010	0.189
6.24 Na_2CO_3	0.045	8.53	0.450	0.314
9.3 Na_2CO_3	0.049	6.74	0.252	0.102
12.5 Na_2CO_3	0.056	1.88	0.050	0.140
10 $NaHCO_3$	0.026	5.34	0.952	1.070

The PBP content of the culture did not show any considerable variation with increase in concentration of sodium carbonate as indicated in Table 5. At 4 g L^{-1} of $NaHCO_3$ supplemented medium which is the minimum required to sustain *G. sulphureum*, the C-PC concentration was found to be 5.54%. As the concentration was increased to 6 and 8 g L^{-1} of $NaHCO_3$ supplemented medium, the C-PC content was found to be 5.060 and 5.67%, respectively. At the standard concentration of the media (10 g L^{-1}), the C-PC content was found to be 5.76%. Allophycocyanin and phycoerythrin concentration did not show any significant change either.

In coherence to our findings, at low light intensity, 120 μE m^{-2} sec^{-1}, Velea *et al.* (2011) found that the standard ASW medium showed a phycoerythrin content of 10.23% whereas at same light intensity, ASW medium supplemented with 2 g L^{-1} $NaHCO_3$ produced 12.7% of phycoerythrin.

Effect of varying concentration of sodium carbonate of *Geitlerinema sulphureum*: Table 6 demonstrates that the increasing concentration of sodium carbonate does seem to have a positive effect on the growth and productivity of *G. sulphureum*. Four concentrations of sodium carbonate were chosen for this experiment, the highest being equimolar to that of the standard $NaHCO_3$ concentration. It was seen that even the lowermost concentration of sodium carbonate showed higher productivity than the standard concentration of sodium bicarbonate. At 3.12 g L^{-1} Na_2CO_3 concentration the productivity was found to be 0.035 g L^{-1} day^{-1}. When the concentration was increased to 6.24 g L^{-1} Na_2CO_3 concentration and 9.3 g L^{-1} Na_2CO_3 concentration, the increase in productivity was 0.045 g L^{-1} day^{-1} and 0.049 g L^{-1} day^{-1} respectively. The culture grown in equimolar concentration of Na_2CO_3 showed the highest productivity i.e., 0.056 g L^{-1} day^{-1} as compared to the culture grown in standard medium containing 10 g L^{-1} of $NaHCO_3$ i.e., 0.026 g L^{-1} day. Similarly, Soundarapandian and Vasanthi (2008) studied the effect of different carbonate sources on growth of five different isolated of *Spirullina platensis*. They used five different carbonate sources, $(NH_4)_2CO_3$, $CaCO_3$, $NaHCO_3$ and Na_2CO_3. In all five cultures, biomass production was significantly higher when $NaHCO_3$ was replaced with Na_2CO_3. For CS1 isolate, when $NaHCO_3$ was used, the biomass produced was 5.78 mg mL^{-1} (dry weight) at the end of ten days. But when it was replaced with Na_2CO_3, the biomass production rose to 7.51 mg mL^{-1}. However, the authors did not study the effect of varying carbonate source on the PBP content.

Increasing concentration of sodium carbonate has a negative effect on the percentage of phycocyanin as observed in Table 6. At the lower most concentration of sodium carbonate (3.12 g L^{-1}), the phycocyanin content is found to be 8.75% CPC, 1.01% APC and 0.189% PE. At 6.24 g L^{-1} Na_2CO_3 concentration, the phycocyanin is somewhat similar with 8.53% CPC, 0.45% APC and 0.314% PE. At 9.3 g L^{-1} Na_2CO_3 concentration, the phycocyanin content decreases to 6.74% CPC, 0.252% APC and 0.102% PE. The most drastic decrease is found with equimolar concentration of Na_2CO_3, where the phycobiliprotein concentration decreases to 1.88% CPC, 0.05% APC and 0.14% PE. The culture grown in standard media (with 10 g L^{-1} sodium hydrogen carbonate) had a phycobiliprotein concentration of 5.34% PC, 0.952% APC and 1.07% PE. Hence, 3.12 g L^{-1} of Na_2CO_3 can be used as a replacement for $NaHCO_3$ to increase the biomass production as well as phycocyanin content. Recently, Deshmukh and Puranik (2012) statistically evaluated the impact of nutritional components on phycocyanin production in *Synechocystis* sp. When the media contained 0.036 g L^{-1} $CaCl_2$ and 0.06 g L^{-1} of Na_2CO_3, the PC content was 13.8 mg L^{-1}. However, when the media was supplemented with 0.04 g L^{-1} $CaCl_2$ and 0.04 g L^{-1} of Na_2CO_3, the PC content was increased to 16.7 mg L^{-1}.

Effect of varying growth period of *Geitlerinema sulphureum*: The productivity of *G. sulphureum* differs greatly depending upon the phase of growth as indicated in Table 7. During the first five days of growth, the productivity is 0.011 g L^{-1} day^{-1}. Further between day 0 and 10, the productivity increases to 0.023 g L^{-1} day^{-1}. The productivity between day 0 and 15 is maximal at 0.029 g $L^{-1}day^{-1}$ and drop to 0.014 g L^{-1} day^{-1} between day 15 and 20. Hence, extending the growth period to 15 days would maximize the yield of the culture for a given inoculum concentration of 10% along with other optimised physiochemical parameters. Soundarapandian and Vasanthi (2008) studied the effect of altering various physicochemical factors on five different strains of *S. platensis*. It was observed that when standard conditions where applied, all strains showed maximal growth on day 30.

Table 7: Indicates the effect of varying time period of growth on productivity and PBP content of *G. sulphureum*

Days	Productivity (g L^{-1} day^{-1})	PC (%)	APC (%)	PE (%)
0-5	0.011	7.15	0.740	0.540
0-10	0.023	7.11	0.780	0.320
0-15	0.029	11.28	4.041	0.506
0-20	0.014	9.80	2.090	0.312

Table 8: Indicates the effect of optimized physicochemical parameters on productivity and PBP content of *G. sulphureum*

Parameter	Productivity (g L^{-1} day^{-1})	PC (%)	APC (%)	PE (%)	PC yield (g L^{-1})
Test	0.051	9.30	0.18	0.53	0.071
Std	0.025	5.43	0.41	0.27	0.021

The phycocyanin content varies depending upon the phase of growth since it is a nitrogen storage compound within the cell. At day 5 and 10 the percentage is around 7% and increases to 11.28% at day 15. At day 20, it However, dips to 9.8%. Hence, harvesting on day 15 would be favourable in terms of biomass production as well as PC content. Soundarapandian and Vasanthi (2008) also studied the change in phycocyanin content per unit dry weight with respect to growth period. The increase in PC content too was directly proportional with lengthening the growth period. The PC content was found to be highest at day 30 as compared to day 10 and 20 in every parameter studied including carbonate source, pH, temperature and light wavelength.

Effect of optimizing all growth parameters on *Geitlerinema sulphureum*: Media was supplemented with 3.5 g of $NaNO_3$ and 6.24 g L^{-1} of Na_2CO_3 for a period of fifteen days with 1000 lux light intensity at 30°C. The second set was grown in the standard modified Zarrouk's media for a period of ten days with 1000 lux light intensity at 27°C. Their growth in terms of productivity as well as percentage C-Phycocyanin content was calculated and results were as seen in Table 8.

Applying optimised parameters for growth to test their combined effect on the growth of *G. sulphureum* had very promising results. The productivity for the culture grown in optimized physicochemical conditions was 0.051 g L^{-1} day^{-1} while that of the culture grown in standard conditons was 0.025 g L^{-1} day^{-1}.

The effect of applying optimized physicochemical parameters had an overall positive effect on the phycocyanin content, from the original 5-6 to 9.3%. The phycocyanin yield which is a combined reflection of the PC content as well as the final biomass concentration showed that the culture which was provided with optimized conditions had a phycocyanin yield of 0.071 g L^{-1} of harvested culture while the phycocyanin of culture grown in standard media was 0.021 g L^{-1}. These results are more than promising in terms of *G. sulphureum* being a candidate for commercial production of phycocyanin as an adjunct to a model organism for CO_2 sequestration.

CONCLUSION

G. sulphureum, a proven shortlisted candidate for CO_2 sequestration, was found to have a phycocyanin concentration of about 5-6%. However, the productivity of the strain along with the phycocyanin yield could be manipulated by optimizing physicochemical factors such as temperature, light, growth period, nitrate concentration as well as carbonate source and concentration. The application of these optimized parameters resulted in a significant increase in overall volumetric productivity 0.05 from 0.025g L^{-1} day^{-1} and PC percentage of 9.3 and 5.43%, respectively. But the overall PC yield of 0.071 g L^{-1} the culture grown with optimized parameters was greater than the standard yield of 0.021 g L^{-1}. Efforts are underway to apply these parameters for biomass and pigment production in a lab scale tubular photobioreactor using *G. sulphureum.*

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