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RESEARCH ARTICLE

Optimization of physico-chemical parameters for the production of phycobilin protein blue pigment, phycocyanin from the cyanobacterial strain *Pseudanabaena limnetica* (Lemmermann) Komarek

Ashwini Tribhuvan, Manjushri Deodhar & Ajit Kengar*

Department of Botany, Kelkar Education Trust's V. G. Vaze College of Arts, Science and Commerce (Autonomous), Mithagar Road, Mulund (E), Mumbai-400081, India

*Email: ajitkengar@vazecollege.net

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Abstract

Pseudanabaena limnetica (Lemmermann) Komarek, the cyanophycean microalga, like other members of Cyanophycea, is an excellent source of pigments such as phycocyanin, proteins, carotenoids and polysaccharides. These strains also form a large proportion of algal biomass. The *P. limnetica* strain can grow in the extreme environmental conditions and it grows well in SW-BG 11 medium under laboratory conditions. In the present investigation, this cyanobacteria strain was isolated from the salt pans of Mulund Mumbai areas and it was cultivated in the lab under controlled conditions of light and temperature with optimum parameters of nitrate and carbonate concentrations. The culture was cultivated in the 60L photo bioreactor systems with the (65,000-85,000 lux) at 45°C in the SW-BG11 medium. The optimization experiments were carried out at the indoor and outdoor conditions. The nitrate and carbonate concentrations were optimized for obtaining maximum amount of algal biomass along with the blue-green phycocyanin pigment. The phycocyanin pigment was lyophilized for its further incorporation into the food and cosmetics products. The spectroscopic calculations of phycocyanin, allophycocyanin and phycoerythrin was done at 620, 650 and 562 nm using the Bennett and Bogorad equation. From the results obtained, it was concluded that 0.1 g/L and 1.5 g/L of the carbonate and nitrate concentrations, respectively, were the ideal concentrations for the further experiments for the cost effective production of *P. limnetica* in the SW-BG 11 medium. The outdoor conditions were found to be favorable for obtaining the maximum biomass and phycocyanin pigment production which would - make it more cost effective, commercially.

Keywords

phycocyanin pigment, *Pseudanabaena limnetica*, physico-chemical optimization, cyanobacteria strain, photo bioreactor systems

Introduction

Culturing of the microalgae to obtain secondary metabolites in the cost-effective manner is considered to be a bio-refinery concept. Algae are considered as the sources of various secondary metabolites such as polysaccharides, phycobiliprotein pigments, carotenoids and proteins. The 'bio-refinery concept' which has been recently developed has emerged as a boon to the algal biomass production. The production of a large amount of

the algal biomass would further help the curbing of carbon dioxide and also in the process of bioremediation.

Cyanophycean species are considered to be containing various light harvesting pigments. The phycobiliproteins are classified into various classes of pigments such as the phycocyanin, allophycocyanin and phycoerythrin. These pigments are reported to possess several anti-oxidant, anti-inflammatory and anti-cancer properties (1, 2). Several companies such as Parry Nutraceuticals and Blue-tech have exploited the commercial strain *Spirulina* for obtaining the phycocyanin pigment on a large scale. The phycocyanin pigment has potent anti-inflammatory and immunomodulatory properties shown by the commercial cyanophycean strain *Spirulina platensis* (*Arthrospira platensis*) (3). The phycocyanin pigment protein was also shown to possess anti-cancer effect on malignant tumors (4).

The main source of phycocyanin on a commercial scale are *A. platensis*, which generally contains around 7% phycocyanin (dry weight basis), however values of up to 18% have also been reported (5). The biomass productivity of *A. platensis* have been reported up to 0.32 g/L and 1.59 g/L for open raceway ponds and novel tubular reactors, respectively (6,7). Even though *A. platensis* is widely cultivated, limitations still exist under elevated temperatures and light intensities (8). This is especially a concern for production as in tropical climatic conditions, the temperatures and light intensities can reach extreme levels. High light intensities and temperatures do not only have a negative effect on biomass productivity, but can also have a negative effect on the phycocyanin content of the strain (9). Recently researchers have isolated a thermotolerant marine *Leptolynbya* spp. from Qatar and assessed its performance in desert conditions with temperature up to 45 ° C and light intensities up to 1800u /m^2/s (10). The biomass produced was 45% higher than *A. platensis* and it was a potential candidate for phycocyanin production in desert conditions (11). *P. limnetica* isolated form salt pans of Mumbai suburban region was cultured and was put in 1000L flat panel photobioreactor maintained in outdoor conditions (temperature up to 45°C and light intensity up to (65,000-85,000 lux). The biomass produced in batch mode was 1.57 g/L and in semi-continuous operations for 3 months produced 3 g/L of biomass. The objective of the present study was to test whether variations in the amount of CO_3 and NO_3in modified BG11 medium used by earlier workers (11) can optimize the production of phycocyanin in *P. limnetica.* The study was aimed to obtain maximum production of phycocyanin pigment with optimized physicochemical parameters.

Materials and Methods

Culture conditions

The modified sea water BG-11 medium was used for the cultivation of the strain. The chemicals used were from Loba Chemie Pvt Ltd. (Mumbai, India).

Photobioreactor and culture conditions

Optimization experiments conducted for obtaining the Phycocyanin pigment:

The culture of cyanobacteria strain *Pseudanabaena limnetica* (Lemmermann) Komarek was scaled up to 1000L photobioreactor systems and was maintained with light conditions of 185-222 umol $m^{-2}sec^{-1}$ intensity for 12 hours/day. The experiment was carried out in the 60 L of the photobioreactor systems containing the SW-BG11 medium and cultures were maintained in the indoor and outdoor conditions (Lab and Terrace). The experiment was carried out for 20 days in total. The biomass and the pigment produced were interpreted after an interval of every 4 days. The biomass and pigment production were carried out in outdoor conditions and indoor conditions. The outdoor conditions were of higher light intensity (65,000-85,000 lux) and higher temperature (35°C-45°C). The biomass and pigment production obtained were recorded.

Optimization of the nitrate concentration:

In the SW-BG11 medium, the sodium nitrate $NaNO_3$ is the main source of nitrate to the culture of *P. limnetica*. The usual concentration of the nitrate in the SW-BG11 is 1.5 g/L. Various concentrations of sodium nitrate ($NaNO_3$) such as 0.5 g/L, 1.0g/L, 1.5 g/L and 2.0 g/L were used to study the effect of nitrate concentrations on the biomass and the pigment production. The experiment was carried out in the 60L photobioreactor systems in indoor and outdoor conditions for 20 days. The amount of the total biomass produced as well as the pigment production were interpreted at an interval of every 4 days.

Optimization of the carbonate concentration

In the SW-BG11 medium, the sodium carbonate Na_2CO_3 is the main source of carbonate for the culture of *P. limnetica*. Different concentrations of sodium carbonate such as 0.01 g/L, 0.02 g/L, 0.1 g/L and 0.2 g/L were used to study the effect of the various carbonate concentrations on the biomass and phycocyanin pigment production. The experiment was carried out for 20 days. The amount of the total biomass produced as well as the phycocyanin pigment produced was interpreted at interval of every 4 days (12).

Spectroscopic determination of the phycocyanin pigment production from the cyanobacterial strain P. limnetica calculations of the total phycocyanin pigment

The UV-VIS absorption spectroscopy of the phycocyanin pigment extracted from the *Pseudanabaena limnetica* cyanobacterial strain was carried out at 620nm. The pigment concentration was recorded using the Thermofisher Scientific Genesys UV-Vis Spectrophotometer with the path length of 1cm. The values of the CPC (Phycocyanin pigment) were calculated using the total percentage of phycocyanin production (12).

The Phycocyanin pigment concentration was calculated at 620nm and 562nm respectively using the below equation (12):

$$CPC\ (gms) = OD_{620}nm - 0.474(OD_{652}nm)/5.34$$

Results and Discussion

In order to make the process of CO_2 sequestration more cost effective, *Pseudanabaena limnetica* strain was grown in the outdoor conditions and for enhancing the CO_2 absorption, it was cultured using the Na_2CO_3 enriched SW-

BG11 medium. The objective of this experiment was to check the effect of these culture conditions on the phycocyanin pigment production and to correlate it with the biomass production. So, initially the indoor and outdoor conditions on biomass as well as phycocyanin pigment production were studied. Then the effect of Na_2CO_3 on biomass as well as on the CPC pigment production were studied. Accordingly, the effect of nitrate source in the medium was also optimized.

Effect of indoor and outdoor conditions on biomass and phycocyanin production

The experiment was carried out in two different environmental conditions (indoor and outdoor conditions). For the indoor cultivation, the experiment was carried out in the 60L capacity air lift photobioreactors systems which was provided with the artificial light source (6 white tube lights). For the outdoor conditions, the photobioreactors were kept on the terrace and were exposed to the natural sunlight (65,000- 85,000 lux) and temperature conditions (35°C-38°C).

Effect of the various indoor and outdoor conditions on the biomass productivity from P. limnetica

When the algal cultures were subjected to indoor conditions and the total biomass production was noted after the interval of 4 days. On day 0, the total biomass obtained was 0.38 g/L which did increase to 0.47 g/L on the day 4. There was an exponential growth of the biomass seen between day 8^{t} and day 12 with the total biomass production of 0.55 g/L and 0.68 g/L. The culture was in its log phase even on day 16 with the biomass of 0.74 g/L. Further on day 20, the culture did enter its stationary phase with the biomass production of 0.63 g/L.

When the same experiment was conducted in the outdoor conditions, the biomass production obtained on the day 0 was found to be 0.37 g/L. From the day 4 to the day 16, the culture was seen to be in the log phase with a substantial increase in the biomass. The total biomass production obtained was 0.74 g/L, 1.09 g/L, 1.33 g/L on the days 8, 12 and 16, respectively. After the day 16, the culture did enter its stationary phase with little reduction in the biomass concentration, 1.27 g/L on the day 20. As shown in Table 1 and Fig 1, at the end of day 16, the biomass produced is almost doubled, when compared to indoor conditions

On days 12, 16 and 20, the biomass produced in outdoor conditions were significantly greater than the amount of biomass produced in outdoor conditions at $P<0.01$, when tested with one-way Anova with post hoc-Tukey test.

Effect of indoor and outdoor conditions on phycocyanin pigment production (mg/ml)

When the culture was subjected to the indoor conditions, the total phycocyanin production found on the day 4 was 0.02 mg/ml. The phycocyanin content did show a negligible increase on the day 4 with 0.08 mg/ml. Post the day 4, there was an increase in the concentration of the phycocyanin content up to the day 16 which was found to be 0.15 mg/ml. On day 8 and day 12^{h} , there was a slight increase in the phycocyanin content in the culture to 0.08 mg/ml and 0.11 mg/ml. Later, on day 20, it was seen that the culture did enter its stationary phase with concentration of the phycocyanin pigment showing to be 0.04 mg/ml (Fig 2).

In outdoor conditions, the phycocyanin concentration obtained on day 0 was found to be 0.03 mg/ml. On day 4, the phycocyanin concentration was found to be 0.09 mg/ml which did show a slight increase of 0.15 mg/ml on day 8. The concentration of phycocyanin pigment was seen to increase on day 12 and day 16 to 0.25 mg/ml and 0.28 mg/ml, respectively. On day 20, there was a decline in the concentration of phycocyanin pigment concentration when cultures entered the stationary phase (Fig 2).

In both outdoor and indoor conditions, the phycocyanin content started increasing as the cultures entered into the log phase, the highest amount of phycocyanin was found to be in the late log phase and as stationary phase was achieved there was a decline in phycocyanin content (Fig 2). On Days 12, 16 and 20, both biomass and Phycocyanin produced in outdoor conditions were significantly greater than the amount of biomass produced in outdoor conditions at $P<0.01$ when tested with one-way Anova with post hoc Tukey test. Similar observations were made in *Limnothrix* sp. (13). In lag phase phycocyanin content was 6 mg/ml, in mid log phase it was increased to 8 mg/ml which was again increased to 14 mg/ml in late log phase and at stationary phase it again decreased to 8 mg/ml.

The outdoor conditions brought about a substantial increase in both biomass and phycocyanin content compared to the indoor conditions. At late log phase, on day 16 in outdoor conditions, the biomass produced was 0.051 g/L and phycocyanin produced was 0.071 mg/ml which was almost two times greater than phycocyanin produced by indoor cultures which lead to increased productivity of phycocyanin. From the present observations it can be inferred that the outdoor conditions were more favorable for the consistent growth of the biomass as well as the total phycocyanin pigment production in the SW-BG11 medium.

Growth and phycocyanin content in Antarctic and tropical cyanophytes, *Anabaena, Nostoc* and *Phormidium* species, in batch culture and indoor and outdoor mass cultivation units were already investigated (14). The indoor culture unit consisted of 20L glass culture unit provided with a magnetic stirrer. The outdoor culture unit was tubular made up of polypropylene 5M in length and 1.25cm in diameter. It was connected to a culture tank which was maintained at 20° C. All Antarctic species showed significant increase in biomass as well as phycocyanin content when grown in outdoor conditions. In Antarctic *Phormidium* species, the indoor biomass productivity was 0.49 g/L and it was 0.58 g/L in outdoor conditions. Phycocyanin produced in indoor conditions was 198.2 ug/mg dry biomass whereas in outdoor unit, phycocyanin was 249.6 ug/mg of dry biomass.

Table 1: Effect of indoor and outdoor conditions on biomass and phycocyanin pigment concentration

S.No	No. of Days	Biomass concentration g/L (Indoor)	Phycocyanin production Indoor (mg/ml)	Biomass concentration g/ L (Outdoor)	Phycocyanin production (Outdoor) mg/ml
1.	Day 0	0.38 g/L	0.02 mg/ml	0.37 g/L	0.03 mg/ml
2.	Day 4	0.47 g/L	0.03 mg/ml	0.45 g/L	0.09 mg/ml
3.	Day 8	0.55 g/L	0.08 mg/ml	0.74 g/L	0.15 mg/ml
4.	Day 12	0.68 g/L	0.11 mg/ml	1.09 g/L	0.25 mg/ml
5.	Day 16	0.74 g/L	0.15 mg/ml	1.33 g/L	0.28 mg/ml
6.	Day 20	0.63 g/L	0.04 mg/ml	1.27 g/L	0.10 mg/ml

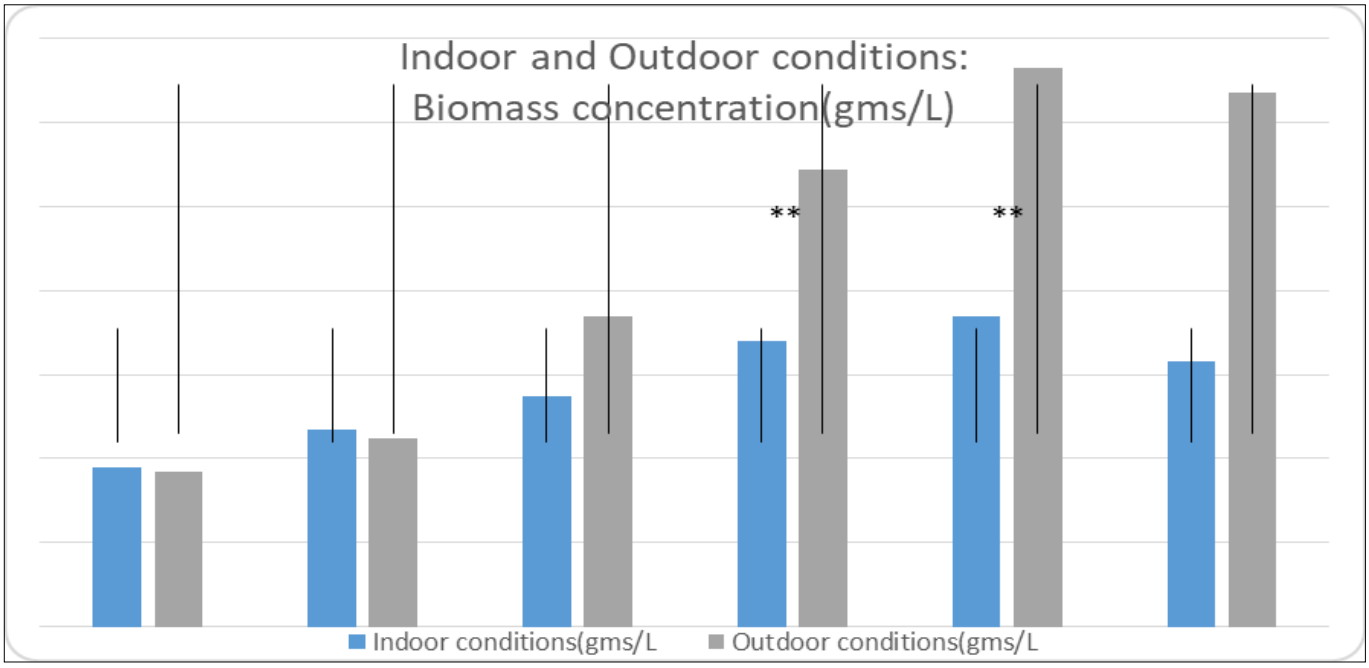

Fig 1: Effect of indoor and outdoor conditions on biomass production (g/L).

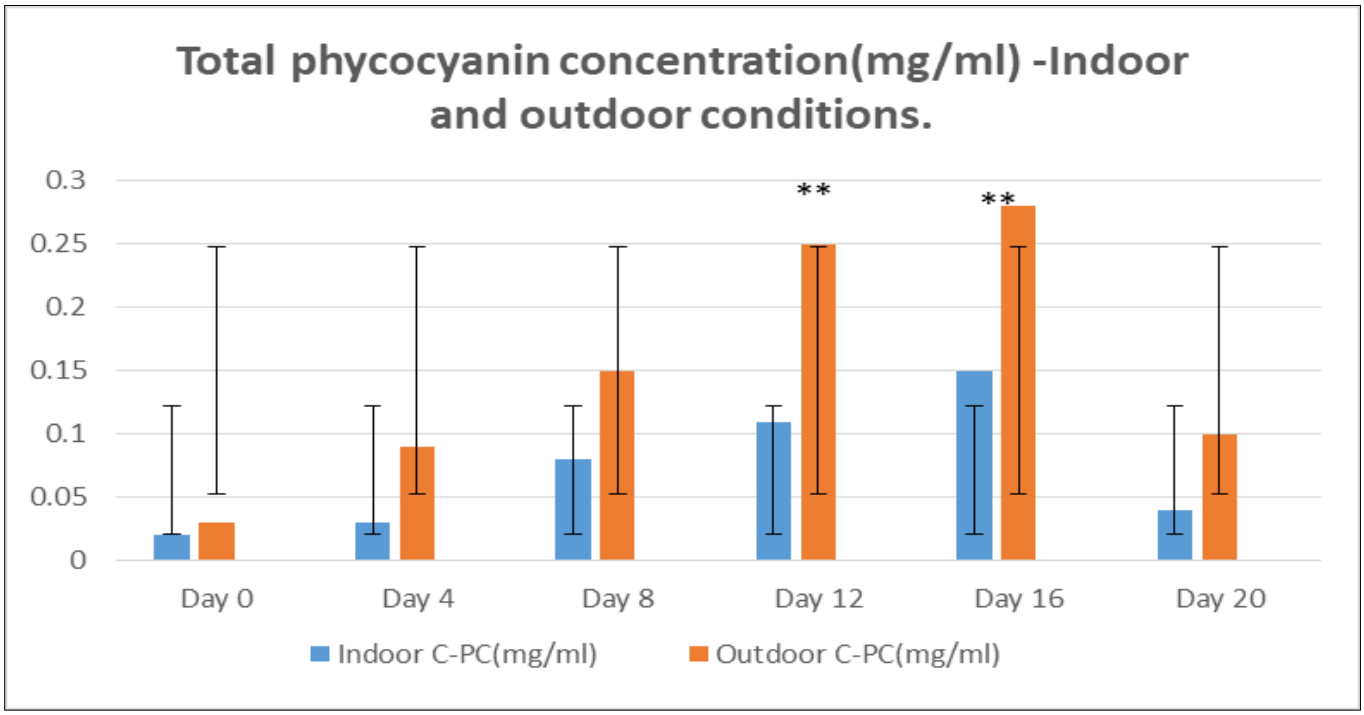

Fig 2: Effect of Indoor and Outdoor condition on the total Phycocyanin Phycocyanin production.

Production

Carbonate is considered as the essential nutrient for the growth of the algal cells. It is one of the nutrients that is required for the photosynthesis and hence for the algal cell growth and reproduction. In the SW-BG11 medium, sodium carbonate (Na_2CO_3) is used for the optimization of the

carbonate concentration for the biomass and phycocyanin pigment production. The different concentrations of the sodium carbonate used were 0.01 g/L, 0.02 g/L, 0.1 g/L and 0.2 g/L.

The objective was to optimize the levels of carbonates to obtain maximum production of phycocyanin pigment as well as biomass when *P. limnetica* was cultivated in outdoor conditions in the SW-BG11 medium.

Effect of various carbonate concentrations on the biomass productivity P. limnetica

On addition of the 0.01 g/L of the carbonate concentration, the biomass produced on day 3 was found to be 0.4 g/L which did increase to 0.59 g/L on the day 6. Further, from day 6 to day 12, the culture did enter the log phase (Fig 2) which did show a biomass concentration of 0.739 g/L on the day 9 and 0.836 g/L on day 12, respectively. Further, on day 15, the culture did show a decline in the growth achieving a stationary phase showing a biomass production of 0.703 g/L on day 15 (Table 2 and Fig 3). When 0.02 g/L of the carbonate concentration was added to the SW-BG11 medium, the biomass obtained on day 3 was found to be 0.331 g/L which did show an increase to 0.455 g/L on the day 6. From day 6, to day 12, the culture did show a log phase with a good biomass growth of 0.619 g/L and 0.825 g/L on the day 9 and day 12, respectively. After day 12, the biomass did show a very less increase in biomass which showed that the culture had achieved its stationary phase with a biomass production of 0.830 g/L the on day 15.

On addition of 0.1 g/L of the carbonate further, the biomass produced on day 3 was found to be 0.39 g/L. On day 6, the biomass produced was 0.574 g/L. From day 6 onwards, the culture entered into the log phase, till day 12 showing a biomass production of 0.768 g/L on the day 9 and 0.878 g/L on the day 12. Even on the day 15, the biomass production was found to be 0.995 g/L which did show that the culture did not enter the stationary phase even on the 15th day. When 0.2 g/L of the carbonate was supplied to the SW-BG11 medium, the culture did enter a lag phase on day 3 which showed a biomass production of 0.574 g/L. After day 6 , the culture entered a log phase up till day 15 showing that biomass production was fairly good till the day 15. The biomass produced on day 6 was 0.76 g/L and 0.808 on the day 9. The culture showed an exponential growth of biomass on day 12 of 0.936 g/L. The culture did not achieve a stationary phase even on day 15 of the biomass produced.

The increase from day 0 to day 3 was found to be in the magnitude of 0.1866 which is higher than p>0.05 and so p-value is insignificant at p>0.05. The value from day 3 day 6 did decrease with p value less than 0.05 and so the p value shows a high significance at p<0.01. Again on day 6 up to the day 9, p value was found to decrease with a highly significant value at p<0.01. From the day 9-day 12, the p-value was decreasing at p<0.05, and the Tukey p-value was highly significant at p<0.01.

The value for day 0 to day 3 does show an increase in the value which is p>0.05 and the Tukey tests p-value is highly insignificant with the p-value of 0.105. Further, at day 6 the P-value is lesser than 0.05 and Tukey test value is highly significant with p<0.01and p-value of 0.001. At the day 9 the p-value was found to decrease at p<0.05 and a very higher significant value at p<0.01 with a p-value of 0.00100.

At the concentration 0.1 g/L on the day 3, the p-value was found to be p<0.05 and the Tukey test value p<0.01 which is highly significant. At day 6,the p-value is p>0.05 and the Tukey test value is highly significant at p<0.01 with a p value of 0.001. Further on the day 9, the p-value was 0.421 which was found to be higher than 0.05, p>0.05 and Tukey test value is insignificant at p-value 0.421. After 12 days, the p-value was found to be higher than 0.05 at p>0.05 whereas the p-value was insignificant at p>0.01.

At the day 3, the p-value was found to be lower than p<0.05 with p-value at 0.0002 whereas the p<0.01 which is highly significant at day 3. At the day 9, the p-value is lesser than 0.05 which indicates that Tukey test p-value is highly significant at p<0.01. At the day 12, the p-value is lower than 0.05 which is 0.0003 whereas the Tukey test is highly significant at p<0.01.

Amongst all the carbonate concentrations, good growth of biomass was seen at 0.1 g/L and 0.2 g/L. But the optimum and exponential growth of the culture was seen with a carbonate concentration of 0.2 g/L which was most conducive for the good biomass production and hence it was shortlisted for the optimum growth of the cyanobacterial culture. The effect of various physico-chemical parameters on the biomass productivity and phycocyanin pigment production of the *Geitlerinema sulphureum* cya-

Table 2: Effect of the Various Carbonate Concentrations on the Total Biomass Production

Concentration of carbonate (g/L)	0.01 g/L	0.02 g/L	0.1 g/L	0.2 g/L
No. of days	Total biomass(g/L)	Total biomass (g/L)	Total biomass g/L	Total biomass g/L
0 day	0.369 g/L	0.396 g/L	0.365 g/L	0.315 g/L
3 days	0.40 g/L	0.331 g/L	0.384 g/L	0.574 g/L
6 days	0.587 g/L	0.455 g/L	0.574 g/L	0.760 g/L
9 day	0.739 g/L	0.619 g/L	0.768 g/L	0.808 g/L
12 day	0.836 g/L	0.825 g/L	0.878 g/L	0.936 g/L
15 day	0.703 g/L	0.830 g/L	0.995 g/L	1.150 g/L

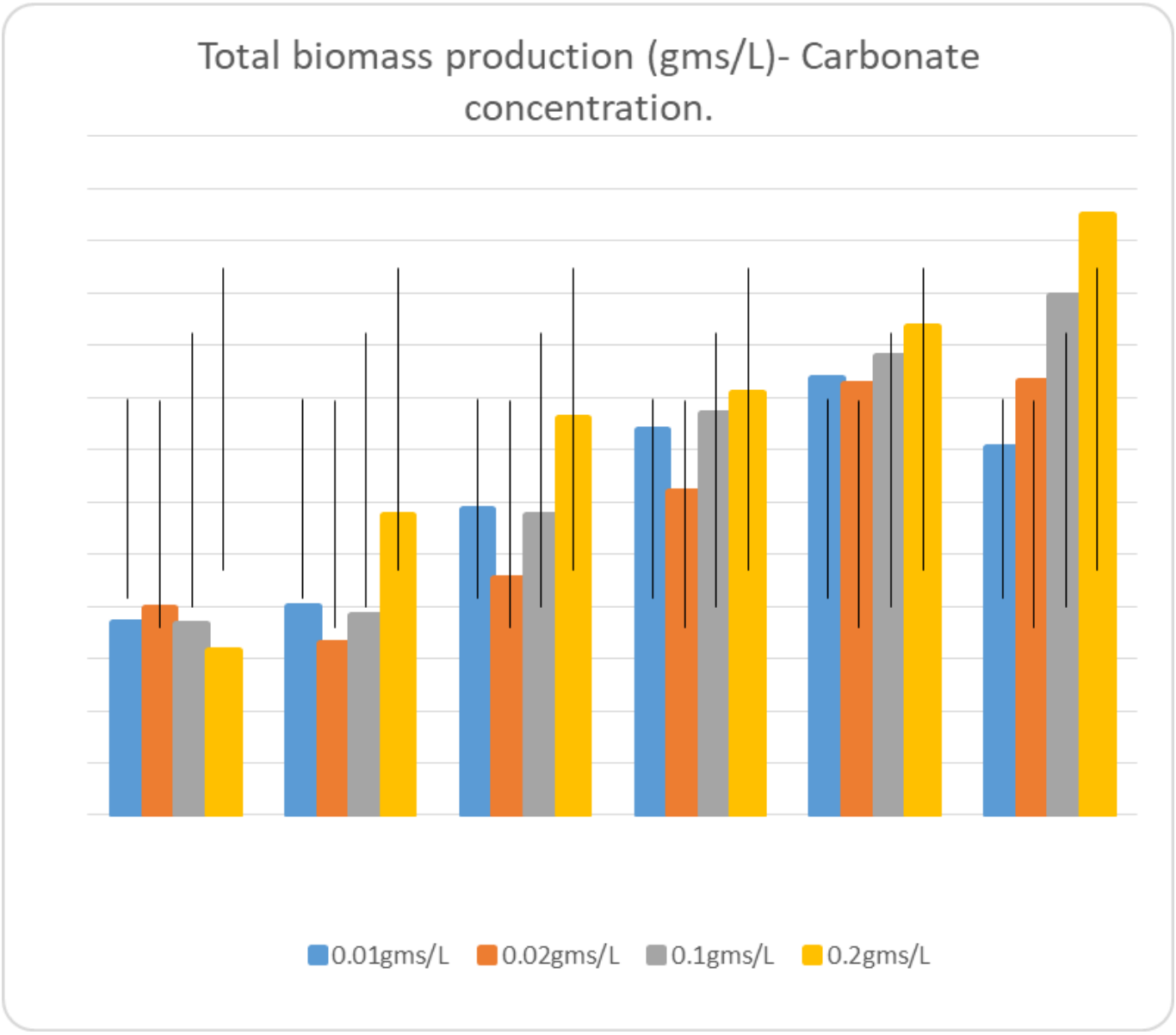

Fig 3: Effect of carbonate concentration on the biomass production of *P. limnetica.*

nobacterial strain was also carried out through similar experiments (15). Effect of various carbonate concentrations was checked on the same strain. From the results it was observed that at the higher concentrations of carbonate concentrations, the production of the pigment was found to be higher as well as the productivity of biomass was found to be higher.

When 0.01 g/L of the carbonate concentration was added to the SW-BG11 medium, the phycocyanin content obtained on day 3 was found to be 0.045 mg/ml. There was a negligible increase in the phycocyanin content on the day 6 which was 0.041 mg/ml. There was a rise in the concentration of the phycocyanin content up to the day 15, after which the culture was seen to attain the stationary phase. After day 6, the culture has entered its log phase since day 9 to day 15 with the CPC concentration to be 0.073 mg/ml and 0.099 mg/ml on day 9 and day 12^{-} day, respectively. On addition of the 0.02 g/L of the carbonate concentration the phycocyanin concentration obtained on day 3 was found to be 0.045 mg/ml which increased to 0.077 mg/ml and 0.073 mg/ml on the day 6 and day $9^{,}$ respectively. From day 12 and the day 15 , there was a decline in the concentration of the phycocyanin pigment which did show that the culture had entered its stationary phase. The culture entered its logarithmic phase since day 3. When the carbonate concentration of 0.1 g/L was added to the SW-BG11 medium, the phycocyanin concentration was obtained on day 3 was found to be 0.025 mg/ml. It showed a rise in the concentration of the phycocyanin production up to day 12. Further, there was a decline in the concentration of CPC on day 15 and day 18 which did prove that the culture has entered the stationary phase. The rise in the phycocyanin production was seen since day 6 when the concentration of phycocyanin was seen to be 0.048 mg/ml and 0.114 mg/ml on day 12. On addition of 0.2 g/L of the carbonate concentration in the SW-BG11 medium, the phycocyanin concentration on the day 3^{-} was found to be 0.021 mg/ml. It did show a slight increase of 0.045 mg/ml on the day 6^{-} to day 12 which indicates that the culture has entered the logarithmic phase. The concentration of phycocyanin on day 12 was found to be 0.184 mg/ml. On day 15, there was a decline seen in the concentration of pigment phycocyanin to 0.052 mg/ml which is an indication of attainment of the stationary phase. Thus, in present experiment, increase in CO_3 content brought

about substantial increase in phycocyanin produced. On day 12, at 0.01 g/L of $Na2CO_3$ the Phycocyanin produced was 0.99 mg/ml but when the cultures were supplemented with 0.2 g/L of $Na2CO_3$ it increased to 0.184 mg/ml.

As concentration of carbonate increased from 0.01 to 0.02 g/L, the increase in phycocyanin content was not significant in initial days up to day 6. On day 9, at all carbonate concentrations, increase in carbonate concentration brought about significant increase in amount of phycocyanin produced. On day 15, as carbonate concentration increased from 0.1 to 0.2 g/L, though there was little increase in phycocyanin produced which was not significant at $P<0.01$ when tested with One-way Anova with post hoc Tukey test.

But as far as biomass production is concerned, at every carbonate concentration applied, increase in carbonate concentration brought about significant increase in the biomass and the increase was significant right from day 3 to day 15 ($p<0.01$) when tested with One-way Anova with post hoc-Tukey test (Fig 4 and Table 3).

However, some researchers have made observations contradictory to this (16). They studied the effect of carbon content, salinity and pH on *S. platensis* for the CPC, APC and PE accumulation in the medium. In standard used, the medium contained 18.0 g/L of $NaHCO_3$. The *S. platensis* was grown in 100% C-deprived medium which contained no $NaHCO_3$, 75% C-deficient medium with 4.5 g/L of $NaHCO_3$ and 50% C-deprived medium containing 9 g/L of $NaHCO_3$. In all C-deprived media CPC, APC and PE production was enhanced. Highest CPC was produced in 100% C-deprived condition and highest APC and PE was found in 50% deprived condition. Some researchers studied the effect of the carbonate source on the phycocyanin pigment production in the *Synecocystis* spp also (17). It was seen that the effect of lower concentration of carbonate, the phycocyanin content was 13.8 m g/L which on further decrease in concentration of carbonate, was found to be 0.04 g/L and the CPC concentration increased to 16.7m g/L.

Another group of researchers made similar observations while standardizing physicochemical parameters for production of phycocyanin from *Geitlerinema sulphurium* (15). To make the process cost effective, $NaHCO_3$ in Zarrouk's medium was replaced by Na_2CO_3. The amount of Na_2CO_3 in medium was varied (12.5 g/L, 9.3 g/L,6.24 g/L and 3.12 g/L.) 10 g/L $NaHCO_3$ was used as control. At the end of 15 days, the biomass productivity in control medium was 0.026 g/L/D and % CPC was 5.34%. When $NaHCO_3$ was replaced by 12.5 g/L of Na_2CO_3, the biomass productivity was doubly increased to 0.056 g/L/D, but Phycocyanin % was substantially reduced to 1.88%. When the amount of Na_2CO_3 was reduced by half (6.24 g/L), there was little decline in productivity (0.045 g/L/D), but % of phycocyanin produced was increased to 8.53%. In present communication, increase in phycocyanin production was observed, when Na_2CO_3 concentration in SWBG11 medium was increased form 0.02 g/L in control to 0.2 g/L. The reason for this is outdoor cultivation conditions as per another research which studied the effect of increased CO_3 in indoor and outdoor culture conditions, in the same algal strain *P. limnetica,* in the same 60L photobioreactor. In indoor conditions, in control, SWBG11 medium with 0.02 g/L of Na_2CO_3, the stationary phase was achieved on day 15 and the highest biomass produced on this day was 1.00 g/L. But, in indoor conditions, when medium was supplemented with 0.2 g/L of Na_2CO_3, the stationary phase was achieved very early on day 9, and on this day, the biomass

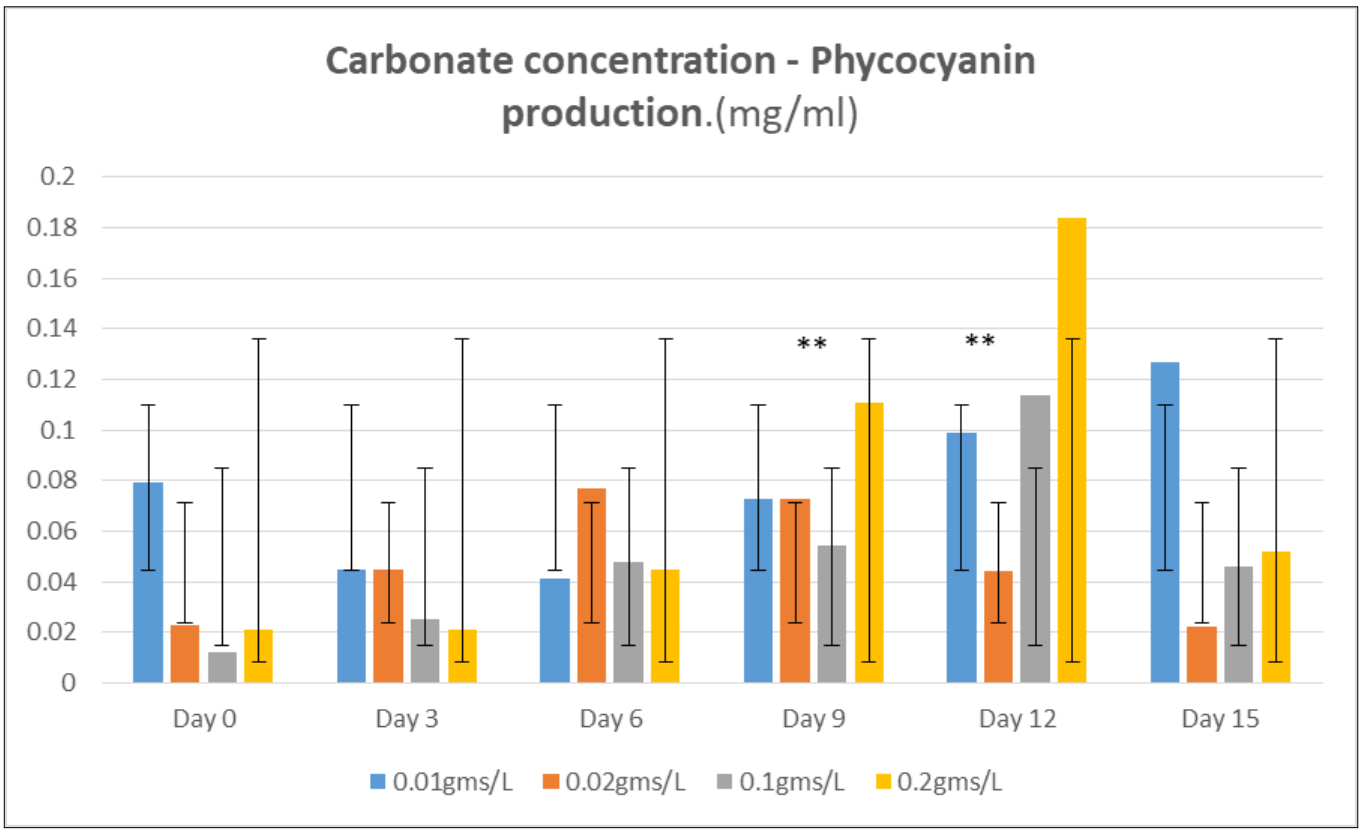

Fig 4: Effect of the carbonate concentration on the phycocyanin pigment production.

Table 3: Effect of various carbonate concentrations on the total Phycocyanin pigment production

Carbonate concentration	0.01 g/L	0.02 g/L	0.1 g/L	0.2 g/L
No. of days:-	Total Phycocyanin production (mg/ml)	Total Phycocyanin production (mg/ml)	Total Phycocyanin production (mg/ml)	Total Phycocyanin production (mg/ml)
0 day	0.079 mg/ml	0.023 mg/ml	0.012 mg/ml	0.021 mg/ml
3 days	0.045 mg/ml	0.045 mg/ml	0.025 mg/ml	0.021 mg/ml
6 days	0.041 mg/ml	0.077 mg/ml	0.048 mg/ml	0.045 mg/ml
9 day	0.073 mg/ml	0.073 mg/ml	0.054 mg/ml	0.111 mg/ml
12 day	0.099 mg/ml	0.044 mg/ml	0.114 mg/ml	0.184 mg/ml
15 day	0.127 mg/ml	0.022 mg/ml	0.046 mg/ml	0.052 mg/ml

produced was 0.98 g/L which was increased to 1.15 g/L on day 15.

Contrary to this, in outdoor conditions at 0.02 g/L of Na_2CO_3, the stationary phase was not achieved even on day 18 and biomass produced on this day was much less 0.88 g/L. But when 0.2 g/L Na_2CO_3 was used in medium, there was substantial increase in biomass, which was 1.37 g/L on day 18. As light intensity increases, requirement of CO_3 and N increases and that leads to higher biomass. The nitrate requirement of *P. limnetica* when cultured indoor and outdoor conditions was studied earlier (18). The $NaNO_3$ concentration used in BG11 medium was 1.5 g/L. SW-BG11 medium was supplemented with 0.3, 0.7 and 1.5 g/L of $NaNO_3$ to optimize growth of *P. limnetica*. In indoor conditions, the optimum growth of 1.41 g/L was obtained when supplemented with 0.7 g/L of $NaNO_3$. The higher concentration 1.5 g/L inhibited growth of *P. limnetica* in indoor conditions. In outdoor conditions, the highest growth was obtained at 1.5 g/L. In present communication, our interest was to study effect of various $NaNO_3$ concentrations on phycocyanin production.

Effect of nitrate concentration of the phycocyanin pigment

For the further optimization of the experiment, the nitrate content in the SW-BG11 medium, the carbonate content in the SW-BG11 medium was kept constant of 0.2 g/L concentrations. The various concentrations of nitrate used for optimization were 0.5 g/L, 1g/L, 1.5 g/L and 2 g/L. In certain earlier experiments performed, in the outdoor experiments, as the concentration of the nitrate increased, the biomass also increased with a concentration of 1.5 g/L. In the current experiment, the effect of various concentrations of nitrate was studied on the phycocyanin pigment production (Table 4). The experiment was carried out for 15 days. At the internal of every 4 days, the biomass and phycocyanin content production were analyzed. The results were interpreted in Table 4 and 5.

Effect of various nitrate concentrations on the biomass productivity of P. limnetica

When 0.5 g/L of the nitrate concentration was added to the SW-BG11 medium, the biomass produced on the day 3 was found to be 0.4 g/L which did show a rise in biomass production, on day 6 of 0.596 g/L. Further, from day 6 to day 12, the culture entered the log phase showing a biomass production of 0.861 g/L on day 9 and 1.0 g/L on day 12. On day 15, there was a decline in the growth of biomass which did show a growth of 0.869 g/L. It did show that the culture did achieve a stationary phase on day 15 after which the culture did not show growth of the biomass (Fig 5).

Table 4: Effect of various nitrate concentration on the total biomass production

Nitrate concentration:(g/L)	0.5g/L	1.0g/L	1.5g/L	2g/L
No. of days	Total biomass g/L	Total biomass g/L	Total biomass g/L	Total biomass g/L
Day 0	0.387 g/L	0.356 g/L	0.385 g/L	0.367 g/L
Day 3	0.4 g/L	0.339 g/L	0.450 g/L	0.480 g/L
Day 6	0.596 g/L	0.606 g/L	0.953 g/L	0.919 g/L
Day 9	0.861 g/L	0.999 g/L	0.989 g/L	1.153 g/L
Day 12	1.0 g/L	1.161 g/L	1.208 g/L	1.253 g/L
Day 15	0.869 g/L	0.757 g/L	1.596 g/L	1.667 g/L

After the addition of 1g/L of the nitrate concentration, the biomass produced on day 3 was 0.339 g/L. From day 6, the culture did enter the logarithmic phase up till the day 12 with a biomass production of 01 g/L on day 9^h and 1.161 g/L on day 12. On day 15, the growth of biomass did show a decline in the biomass of 0.757 g/L which did show that culture as achieved the stationary phase (Fig 4).

On addition of 1.5 g/L of nitrate concentration, the culture entered a lag phase on day 3 with a biomass production of 0.45 g/L. On day 6, the biomass produced was found to be 0.96 g/L and the culture entered the logarithmic phase till day 15. The biomass production seen on day 9 and day 12 was 0.989 g/L and 1.2 g/L, respectively. Even up to day 15, the culture did not attain the stationary phase and did show a biomass production of 1.596 g/L (Fig

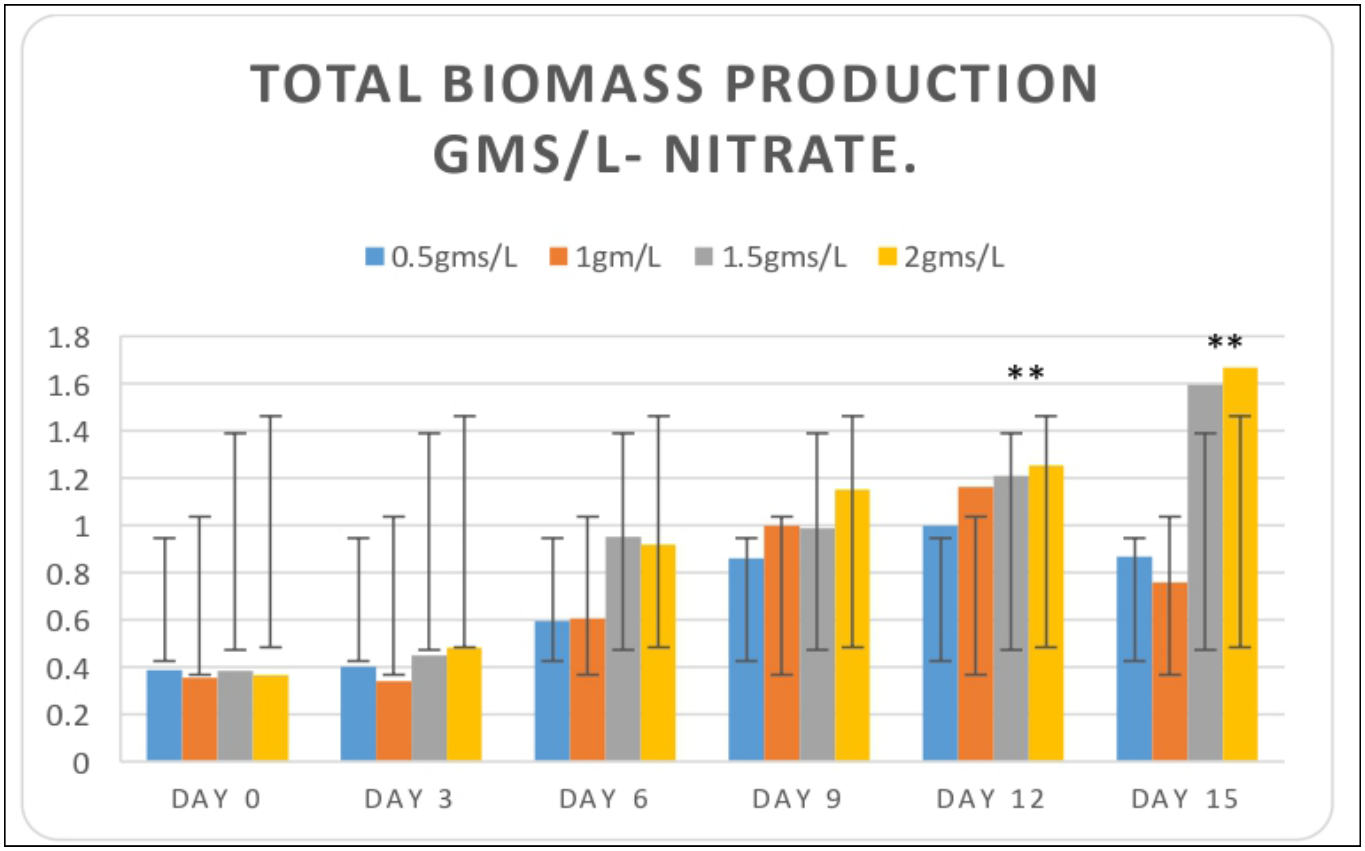

Fig 5: Effect of nitrate concentrations on the biomass production.

5).

Further, on addition of 2 g/L of nitrate concentration, the biomass produced on day 3 was 0.48 g/L. There was an exponential growth of biomass from day 6 to the day 15. The biomass produced from day 6 was found to be 0.919 g/L and 1.153 g/L on the day 9. Even on day 12, and on day 15, very good biomass production was seen of 1.253 g/L on day 12 and 1.667 g/L on day 15. The culture did not achieve the stationary phase even up to the day 15 of the experiment (Fig 5).

As mentioned in the Table 5 and Fig 6 above, when 0.5 g/L nitrate concentration was added into the SW-BG11 medium, the concentration of phycocyanin pigment obtained on day 3 was found to be 0.031 mg/ml which did show a negligible increase on the day 6 to 0.042 mg/ml. On day 9, day the concentration of phycocyanin pigment was 0.549 mg/ml. From day 9 , day the pigment concentration of phycocyanin was found to enhance much to 0.947 mg/ml which showed that the culture had entered its logarithmic phase, even up to day 15 when the growth was seen to be 0.163 mg/ml. Further, on day 18, the culture was found to enter its stationary phase with the phycocyanin pigment concentration of 0.130 mg/ml. On addition of 1g/L pf the nitrate concentration, the pigment concentration obtained on day 3 was found to be 0.020 mg/ml which did show a slight increase to 0.036 mg/ml on day 6. There was an enhancement in the concentration of phycocyanin pigment from day 9 onwards with 0.047 mg/ml which showed that culture was in its log phase. On the day 12, the pigment concentration was found to be 0.125 mg/ml which did show a decline in concentration of the pigment to 0.090 mg/ml and 0.093 mg/ml on the day 15 and day 18 which showed that the culture had entered its death phase. When 1.5 g/L of the nitrate content was added into the SW-BG11 medium, the concentration of the phycocyanin pigment obtained was 0.031 mg/ml on day 3. Initially there was a slight decline in the pigment content on day 6 which was 0.028 mg/ml. From day 9 onwards the culture had its logarithmic phase when concentration of pigment was 0.043 mg/ml and 0.069 mg/ml on day 12. Even up to day 15, there was a good increase in the phycocyanin pigment concentration which was 0.195 mg/ml further which a decline was seen on day 18 towards 0.176 mg/ml which showed that the culture had entered it's death phase later on. When 2 g/L of the nitrate concentration was added in the medium, the total pigment production obtained on day 3 was 0.044 mg/ml which showed a decline in phycocyanin content on day 6 to 0.041 mg/ml. From day 9 onwards, there was a good growth of the phycocyanin pigment concentration from 0.056 mg/ml to 0.103 mg/ml on day 12. Even up to day 15 and day 18 there was an increase seen in total phycocyanin pigment concentration which was 0.17 mg/ml and 0.108 mg/ml which showed that even up to day 18, the culture was in its logarithmic phase (Fig 6).

Though initially up to day 9, the increase in nitrate concentration did not significantly contributed to phycocyanin production on day 12 or day 15, the lower nitrate concentrations like 0.5 g/L or 1g/L or 1.5 g/L were effective in phycocyanin production. But further, the increase in nitrate concentration do not contribute significantly to the increase in phycocyanin. The increase in nitrate concentrations also brought about significant increase in biomass, particularly on day 12 and 15 (Table 5).

Table 5: Effect of various nitrate concentrations on the phycocyanin pigment production.

Nitrate concentration: g/L	**0.5 g/L**	**1.0 g/L**	**1.5 gms /L**	**2.0 g/L**
No. of Days:	**Phycocyanin production mg/ml**	**Phycocyanin production mg/ml**	**Phycocyanin Production mg/ml**	**Phycocyanin Production mg/ml**
DAY 3	0.031 mg/ml	0.020 mg/ml	0.031 mg/ml	0.044 mg/ml
DAY 6	0.042 mg/ml	0.036 mg/ml	0.028 mg/ml	0.041 mg/ml
DAY 9	0.549 mg/ml	0.047 mg/ml	0.043 mg/ml	0.056 mg/ml
DAY 12	0.947 mg/ml	0.125 mg/ml	0.069 mg/ml	0.103 mg/ml
DAY 15	0.163 mg/ml	0.090 mg/ml	0.195 mg/ml	0.17 mg/ml

Similar observations were made by other researchers also who studied the effect of light conditions and nitrate conditions on the biomass and phycobiliproteins production from *Arthrospira platensis* culture (19). It was found that under higher irradiance conditions (111.67 umol photon/$m^{-2}S^{-1}$) and low nitrate conditions of 1.5 g/L, maximum amount of the phycobiliproteins were produced along with maximum biomass production. Out of all the concentrations, the phycocyanin pigment production was higher at 1.0 g/L and 1.5 g/L concentration of the nitrate concentration. But the optimum growth of the pigment was seen at 1.5 g/L of the nitrate concentration. In the present study, it was seen that the biomass as well the phycocyanin pigment concentration was found to increase even in 1.5 g/L of the nitrate component concentration. Hence, 1.5 g/L concentration was shortlisted as a significant concentration for maximum production of biomass as well as for the extraction of phycocyanin pigment.

When all culture conditions were standardized in 60L photo bioreactor in batch mode, the biomass produced was 1.56 g/L and the highest phycocyanin produced was 0.195 mg/ml. Hence in batch culture, *P. limnetica* produced 125 mg/gm of phycocyanin. *Leptolyngbya* in desert climatic conditions of Qatar where the temperature reaches to 49°C and light intensity goes beyond 2200 umol/m2/s, the biomass productivity was reported to be 1.09 g/L/day and phycocyanin content was 72.12 mg/gm. (10**).** There are reports of *A. platensis* producing PC upto184mg/g. However, optimum light intensity for phycocyanin production is very low, 80umol/m2/s and shows a sharp decline of 53% and 78% when the light intensity increased up to 300umol and 1800 u mold/m^2/S. For tropical climatic conditions in India, *P. limnetica* also is proved to be the most suitable strain. In outdoor climatic conditions of temperature up to 40°C and light intensity of 65,000-85,000 lux, biomass production of 1.56 g/L was ob-

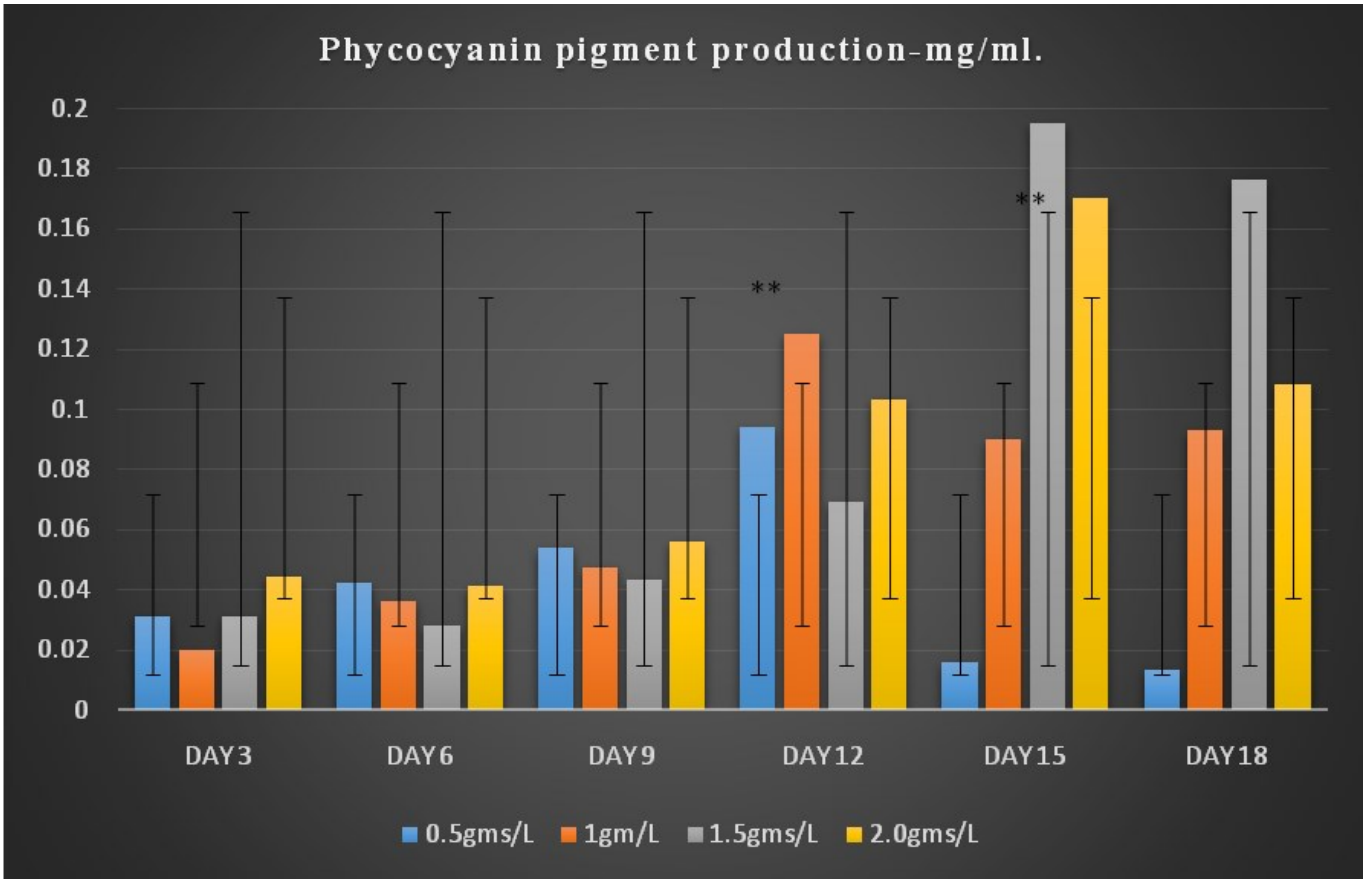

Fig 6: Effect of Various Nitrate concentrations on the phycocyanin pigment production.

served in batch mode and the phycocyanin content was 0.195 mgs/ml.

Phycocyanin productivity of P. limnetica

In our earlier reports, when all culture conditions were standardized in 60L photobioreactor systems in the batch mode, the biomass produced was 1.56 g/L and when the reactor is operated in semi-continuous mode, about 2.5 g/L of biomass was produced every day.

The amount of phycocyanin produced was 14.15 mg/g when extracted by freeze-thawing method. Hence in batch mode, the amount of phycocyanin produced was 22.07 mg. And in the semi-continuous mode, phycocyanin produced is anticipated to be 35.375 mg/L/day.

The efficient extraction of phycocyanin from the biomass is essential to accurately determine the phycocyanin content and the productivity potential. A number of different methods have been published, however they all differ considerably, mainly in terms of cellular disruption method, type of extraction buffer, biomass-buffer ratio and extraction time. Moreover, the optimal extraction method can differ from strain to strain. The extraction buffer used and cell disruption method employed can have profound effect on phycocyanin content and it's purity. Sonication in phosphate buffer showed the highest extraction yields, with phycocyanin contents of 169.89 ± 3.63 and 111.03 ± 5.41 mg PC·gX −1, after 96 and 48 hours of incubation, for *Leptolyngbya* sp. Whereas when freeze-thaw method was employed for *Leptolyngbya* sp., the phycocyanin content was only 2.73 ± 0.10 mg PC·gX −1(10**).**

Studies and research works are still going on, in order to standardize the efficient extraction procedure for *P. limnetica* and incorporating phycocyanin into viable food products.

Conclusion

The objective of the present study was the optimization of the parameters such as temperature, carbonate content and nitrate content for maximum production of biomass as well as for the exo-polysaccharide production from the cyanobacterial strain *P. limnetica.* Amongst the 2 different temperature conditions, maximum biomass and phycocyanin production was seen in the outdoor conditions. The most favourable condition was observed on day 16 of the experiment, on which the biomass production was seen to be 0.051 g/L and phycocyanin production was 0.071 mg/ml. This was much higher when compared to indoor conditions (please heck this statement. Is this, "when compared to indoor conditions". For the carbon optimization experiment, it was carried out for 15-20 days using 4 different concentrations, namely 0.01 g/L, 0.02 g/L, 0.1 g/L and 0.2 g/L for the biomass as well as phycocyanin production. From the results it was observed that 0.2 g/L was the favourable concentration for the maximum biomass production along with a good percentage of phycocyanin production at the day 12^{-} of the experiment which was found to be 1.150 g/L and 0.184 mg/ml respectively. For the nitrate concentration optimization, 4 different concentration were used namely 0.5 g/L, 1g/L, 1.5 g/L and 2 g/L. Out of which, the optimum concentration which was shortlisted for the maximum production of the biomass as well as the good phycocyanin production of nitrate was 1.5 g and 0.2 mg/ml of carbonate concentration. The percent yield of phycocyanin pigment obtained was 2-3% along with standardization of the phycocyanin pigment extraction protocol. Hence, it is proved that the cultivation of the *P. limnetica* strain at the lab scale level is cost effective and easy to maintain.

From the above results, it can be inferred that these optimum concentrations were most favorable for the maximum phycocyanin and biomass production during the scaling up process which can be used for the further experiments in the SW-BG11 medium for its further application in the food and cosmetics by cost effective production of the strain *P. limnetica.* The phycocyanin pigment has a good nutritional value and it adds to the aesthetic appeal of the food and cosmetic product into which it is incorporated.

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Authors contributions

MD has been the pioneer in shaping the experiments. AT has been instrumental in completing the table work and prepared manuscript. AK reviewed, edited and did formatting of the manuscript. The final manuscript was read and approved by all authors.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None.

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