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Optimization of C-phycocyanin production by *Limnothrix* sp. 37-2-1

Vijay Patel[3], David E. Berthold[1], Sivanesan Dhandayuthapani[2], Appu Rathinavelu[2], and Miroslav Gantar[1]

[1]Department of Biological Sciences, Florida International University, Miami, FL 33199, USA
[2]Rumbaugh Goodwin Institute for Cancer Research, Nova Southeastern University, Plantation,
[3]School of Life Sciences, North Maharashtra University, Jalgaon, Maharashtra 425001, INDIA

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Abstract

C-phycocyanin (C-PC) is a blue colored photosynthetic accessory pigment in cyanobacteria. We have previously shown that C-PC purified from our cyanobacterial isolate *Limnothrix* sp. 37-2-1, has cytotoxic activity against prostate and breast cancer cell lines. Furthermore, we have shown that when used in combination with currently used anticancer drugs, C-PC enhances the potency of those drugs and therefore potentially allows for a lower dosage. The medically significant characteristics of C-PC have geared the scientific community and industry towards finding a productive strain of C-PC as well as determining the optimal conditions for C-PC production. In this work, environmental growth conditions such as temperature, light intensity and quality, and medium chemical composition for maximum biomass and C-PC yield were investigated. In addition, *Limnothrix* sp. was cultivated in selenium (Se)-supplemented medium in order to obtain Se-containing C-PC. The cyanobacterium was grown in a standard BG-11 medium that was used as a control. Results showed that the highest growth yield does not necessarily provide the highest C-PC accumulation. By supplementing the medium with Se, it was possible to increase Se-content in C-PC 144 times. In conclusion, by manipulating the growth conditions of *Limnothrix* sp. 37-2-1 it was possible to improve the biomass yield as well as the quantity and quality of C-PC.

Introduction

C-PC is a blue photosynthetic accessory pigment found in cyanobacteria and some genera of eukaryotic algae. In cyanobacteria, the pigment is found organized together with other phycobiliproteins into globular structures called phycobilisomes. The pigment aggregates are attached to thylakoids that are involved in light harvesting and transferring of energy toward photosystem II reaction centers (MacColl, 1998). C-PC is composed of a protein portion containing two subunits (α, β) and a chromophore group – phycocyanobilin (Glazer, 1994). The reported molecular mass of an intact C-PC molecule varies and ranges between 50kDa (Gantar et al. 2012) to 215 kDa (Soni et al., 2006) depending on the organism from which it is derived.

In addition to the physiological role played in cyanobacteria, CP-C is involved in appealing to the food, nutraceutical, and biotechnology industries. In the neutraceutical industry C-PC is considered to be a profitable commodity owing to its antioxidative activity. There are numerous reports on radical scavenging activity of this blue pigment have (Romay et al., 1998; Benedetti et al., 2004; Gantar et al., 2012). The antioxidative activity of C-PC is primarily attributed to phycocyanobilin (Lissi et al. 2000) even though antioxidative activity has been also reported for the apo-protein that does not contain this chromophore group (Ge et al. 2006). Being natural and non-toxic, C-PC is used in Japan as a colorant in food and cosmetic industry (Prasanna et al., 2007). Due to its binding affinity to DNA, C-PC has been suggested as an alternative to carcinogenic chemical DNA fluorescent dye (Paswan et al., 2015). C-PC and other phycobiliproteins are highly fluorescent with high molar extinction coefficient and when conjugated to immunoglobulins can be developed into fluorescent probes to be used for various diagnostic applications (Eriksen, 2008).

The most important application of C-PC involves its potential administration as a therapeutic agent. It has been reported that C-PC induces apoptosis in carcinogenic cell lines (Reddy et al. 2003; Bobbili et al. 2003). This pigment is also shown to reduce antigen specific IgE antibody (Nemoto-Kawamura, 2004) and therefore being able to suppress allergic inflammation reactions in different animal models (Remirez et al., 2002). Nagaoka et al., (2005) reported on hypocholesterolemic effect of *Spirulina platensis* concentrate which was actually attributed to C-PC and its effect on inhibition of both jejunal cholesterol absorption and ileal bile acid reabsorption.

Recently we have reported on anticancer activity of C-PC that was isolated from the cyanobacterium *Limnothrix* sp. 37-2-1 (Gantar et al. 2012b). By combining C-PC with
topotecan, a widely used anticancer drug, the use of only 10% of a typical dose of this drug killed the cancer cells at higher rate than when topotecan was used alone at full dose. Traditionally C-PC is isolated from *Spirulina*. Since our novel isolate *Limnothrix* sp. 37-2-1 is a more productive source of C-PC than *Spirulina* (Gantar, et al. 2012a), we have conducted this research to optimize the culture conditions for C-PC production.

Materials and Methods

Organism and culture conditions

The filamentous cyanobacterium *Limnothrix* sp. 37-2-1, part of the microalga culture collection at the Florida International University, used in this study was isolated from the freshwater habitat in Florida, USA. The taxonomic identity of the isolate was based on morphological characteristics (Anagnostidis and Komarek, 1988) and confirmed by 16S rRNA sequencing and BLAST searches (Gantar et al., 2012). The cyanobacterium was maintained in BG-11 medium (Rippka et al., 1979) under cool white light (20 µmol photons $m^{-2}s^{-1}$), at 24° C. The pH of the medium was adjusted to 7.2.

Growth and phycocyanin content determination

Limnothrix sp. 37-2-1 was grown in 4-liter flasks containing 3 liters of BG-11 medium (Ripka et al, 1979), and aerated with sterile air. The growth rate was determined by measuring the weight of dry biomass. During the course of the experiment, ten ml samples were taken every other day and filtered through pre-weighed filters (1.2 µm GF-C Whatman glass microfiber filters). The filters were dried at 60°C to a constant weight. Simultaneously, another 10 ml of the culture was centrifuged and the pellet was frozen and later used for C-PC determination. In order to determine C-PC content, the pellets of triplicate samples were freeze-thawed three times in order to lyse the cells. To the cell lysate, 2 ml of distilled water was added and centrifuged. C-PC content was determined spectrophotometrically according to the method of Bennet and Bogorad (1973).

Effect of different concentrations of nitrate and phosphate

Limnothrix sp. 37-2-1 was cultivated in medium with five different concentrations of $NaNO_3$ (14, 16, 17.6, 20 and 22 mM), where the concentration of 17.6 mM is the original one in BG-11 medium and that served as a control. In a separate experiment, BG-11 medium was supplemented with different concentrations of K_2HPO_4 (0.1, 0.2, 0.25, 0.3 and 0.4 mM) using 0.25mM as the control. The flasks were inoculated with a log phase culture to an optical density (600nm) of 0.03, and incubated as described above. Triplicate samples for biomass and C-PC determinations were taken every second day for 17 days.

Effect of different light intensity

A set of 250 ml Erlenmeyer flasks containing 150 ml BG-11 media, were inoculated with a log phase culture providing an initial OD (600 nm) of 0.03. The cultures were incubated under standard conditions with the exception of exposed light intensities: 100, 75, 50, 25, 10 and 5 µmol $m^{-2}s^{-1}$. Each light level was inoculated in triplicates. Samples for biomass and C-PC determinations were taken every second day for 17 days. The flasks were illuminated from the bottom, while the rest of the flask was wrapped with aluminum foil. The light intensity was measured at the bottom surface of the flask. The presented data are results obtained as means of triplicate samples.

Effect of different light quality

In order to assess the effect of light quality on phycocyanin production, a log phase culture was inoculated into a 2-liter photobioreactor containing BG-11 medium. The photobioreactor was supplemented with a fully adjustable wavelength (Red-Green-Blue) LED light platform (UTEX, Austin, USA). The photobioreactor was aerated with sterile air and

temperature maintained at 24°C. The culture was initially grown under white light for 13 days, and then the light was switched to red, blue or green in separate experiments. The cultures were then cultivated for another 6 days. The triplicate samples for biomass and phycocyanin determination were taken on 19th day.

Effect of selenium

The effect of selenium on the growth and C-PC production in *Limnothrix* sp. 37-2-1 was assessed by using the modified method of stepwise supplementation of the medium with Se (Chen et al. 2006). A set of 4-liter Erlenmeyer flasks with 3 liters of BG-11 medium was inoculated to provide a uniform cell density of 0.03 (O.D. at 600nm) and incubated under cool white light (30 µmol photons $m^{-2}s^{-1}$) at 25°C with aeration. Selenium was added stepwise in each flask in the form of sodium selenite (Na_2SeO_3) (Sigma, St. Louis, MO, USA) to obtain the final concentrations of 200, 250, and 300 mgL^{-1}. Triplicate samples of cells were harvested after 13 days by centrifugation at 5000 rpm (Beckman GPR Centrifuge, Model: SER9D037, USA) for 25 min at 20° C. The biomass was washed with sterile distilled water to remove any excess selenium, centrifuged again and freezer-dried.

Selenium content was determined both in control C-PC and in that obtained from Se-enriched cultures. The concentration of selenium was determined by using inductively coupled plasma mass spectrometry (ICP-MS, ELAN DRCe, PerkinElmer SCIEX). Dry C-PC was dissolved in 2% (v/v) nitric acid (trace metal grade, Fisher Scientific) to facilitate the liberation of selenium from the matrix, and then run on ICP-MS. An external calibration curve was run prior to sample analysis and used to quantify the concentrations of selenium in the samples using PC-based data acquisition and analysis software (ELAN version 3.4, PerkinElmer SCIEX). An internal standard, yttrium, was added to all calibration standards and samples at a concentration of 0.020 mg/L to correct instrumental drift during analysis.

Radical scavenging activity of C-phycocyanin

Radical scavenging activity of purified C-PC from control and Se-enriched biomass of *Limnothrix* sp. 37-2-1 was analyzed by using the method described by Ahire et al. (2013).

A 0.8 ml of C-PC and 1 ml of freshly prepared α,α-diphenyl-β-picrylhydrazyl (DPPH) solution (0.2 mM in methanol) were mixed and allowed to react for 30 min in dark at room temperature. Similarly, control sample contained pure methanol only. The percentage of scavenging activity was determined spectrophotometrically.

Statistical analysis

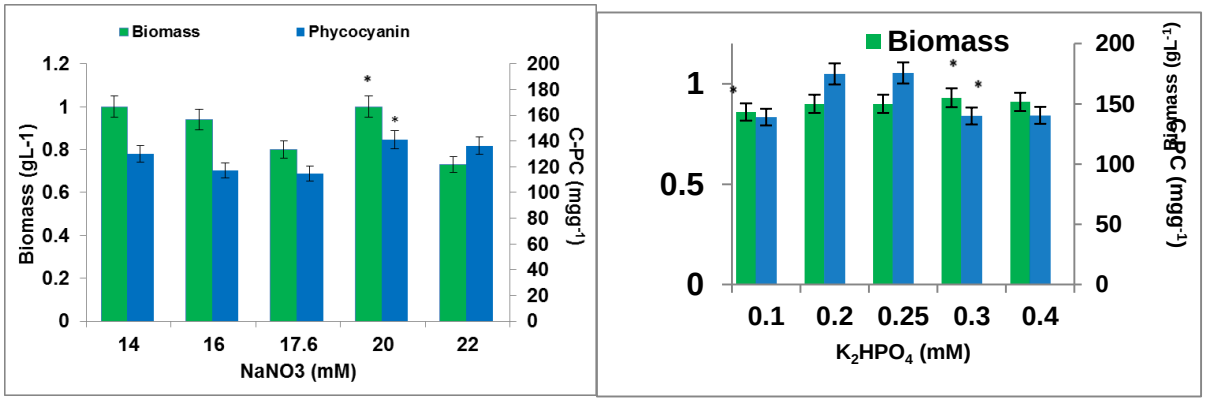
Different growth conditions used in this work were compared by analysis of variance (ANOVA). Tukey's test was used at a significance level of 5%.

Results

Medium composition

The effect of different concentrations of nitrate and phosphate on the biomass yield and C-PC production is presented in Fig. 1. Both, the highest biomass and C-PC yield were achieved at the concentration of 20mM of $NaNO_3$, which is higher than that in the original BG-11 medium. At the concentration of 22mM of $NaNO_3$, the growth was suppressed even though C-PC production was comparable to that obtained at the concentration of 20mM. The most favorable concentration of phosphate in terms of C-PC yield was that of the original BG-11 medium (0.25 mM).

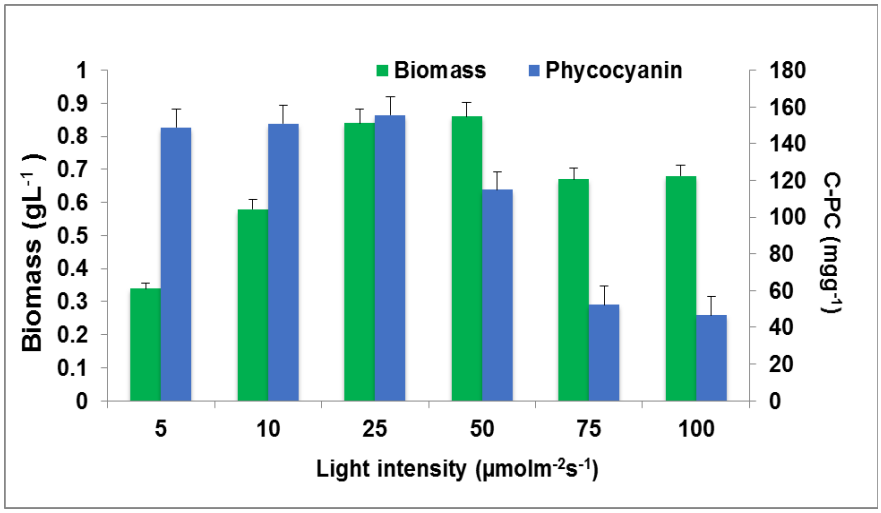
Fig. 1 Effect of different concentrations of nitrate (a) and phosphate (b) in BG11 medium on biomass and C-phycocyanin production after 17 days of cultivation. *Statistically significant difference as compared to control (17.6 and 0.25 mM $NaNO_3$ and K_2HPO_4 respectively, P- value is <0.05)



Light intensity

The highest biomass yield was achieved at light intensities of 25 and 50 µmol photons $m^{-2}s^{-1}$ (Fig. 2). Light intensities higher and lower than these resulted in lower biomass yield. C-PC production was the highest at 25 µmol photons $m^{-2}s^{-1}$. In most of the cases the highest level of C-PC was reached between 13th and 16th day of cultivation declining on the last day of experiment (data not shown). The only exception that C-PC content continued to increase throughout the experiment was at the light intensity of 25 □mol photons $m^{-2}s^{-1}$.

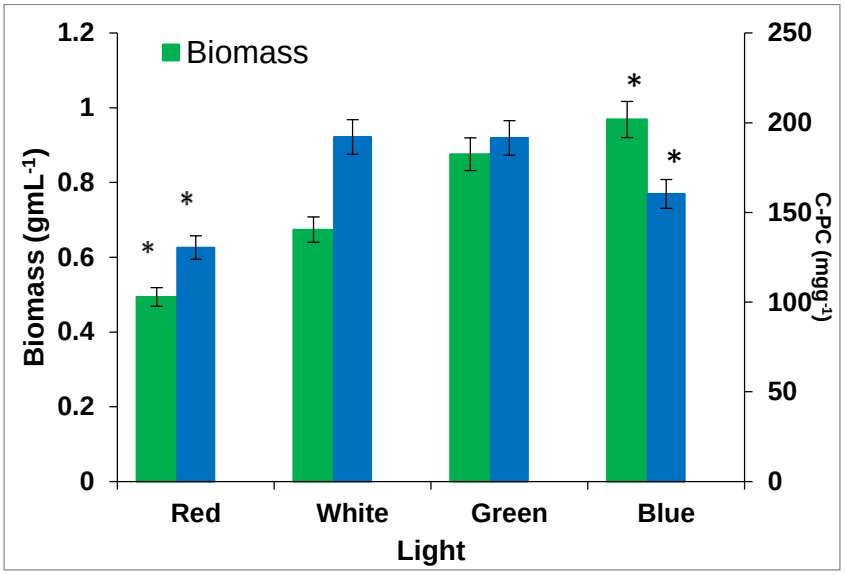
Fig. 2 Effect of light intensity on biomass and C-PC yield.



Light quality

The highest biomass yield was achieved under the blue light, while the highest content of C-PC was recorded with the white light (Fig. 3). The strongest inhibition of both growth and C-PC production was recorded with the red light.

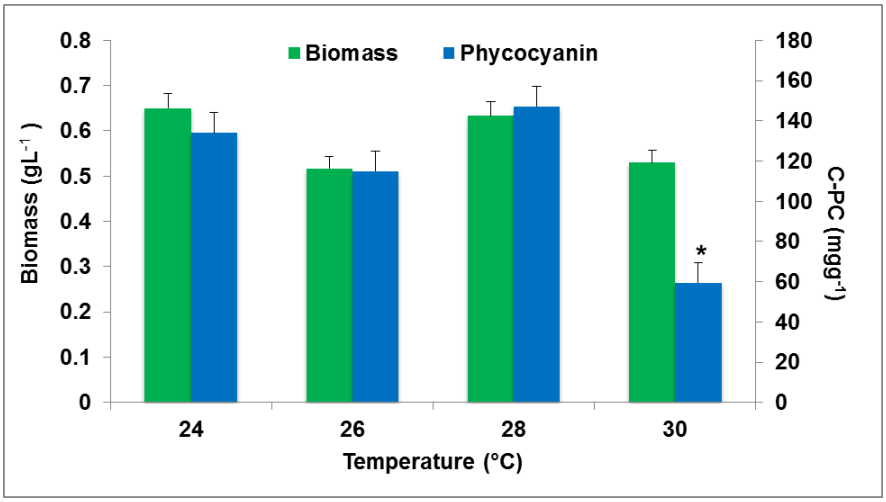
Fig. 3 Effect of different light quality on biomass and C-PC yield. C-PC is presented as mg per g of dry biomass. *Statistically significant difference as compared to control (white light, P- value is <0.05)



Temperature

By growing the culture at temperatures used in our experiments, the biomass yield was not affected (Fig. 4), while the C-PC production was. The highest C-PC yield was achieved at the temperature of 28°C. Further increase of the temperature (30°C) suppressed C-PC production.

Fig. 4. Effect of temperature on biomass and C-PC yield. C-PC is presented as mg per g of dry biomass. *Statistically significant difference as compared to control (24°C).



Effect of selenium

Supplementation of BG-11 medium with Se resulted in an increased production of C-PC (Tab. 1). For example, when grown in the medium with 250 mgL^{-1} of Na_2SeO_3, the biomass contained 38% more C-PC than that of the control. Free radical scavenging activity (FRSA) of C-PC isolated from the same biomass had 3.1 times higher activity and contained 144 times more Se than that isolated from the control biomass (Tab. 1).

Tab. 1. Effect of Se on biomass and C-PC yield, free radical scavenging activity (FRSA) of C-PC, and Se- content in C-PC. The biomass was harvested and analyzed after 13 days of cultivation. Standard BG-11 medium was used as control or supplemented with different concentrations of Na_2SeO_3.

Na_2SeO_3 (mgL^{-1})	Biomass yield (gmL^{-1})	C-PC (mgg^{-1} dry biomass)	FRSA (%)	Se-content in C-PC (ppb)
BG11-Control	0.70±0.03	94.77±8.0	13.71±1.0	11.35
200	0.53±0.02	94.32±8.0	37.31±3.0	N/D
250	0.65±0.03	130.02±11.0	42.77±4.0	1584.70
300	0.74±0.03	97.86±9.0	40.59±4.0	N/D

Discussion

With an increased interest in phycocyanin applications, it is desirable to obtain more productive strains and determine culture conditions that will result in higher C-PC production. In this work we have identified growth conditions for improved biomass and C-PC production in *Limnothrix* sp. 37-2-1, which in our earlier work (Gantar et al. 2012a) was shown to be a good source of C-PC.

There is a large number of different media available for cultivation of cyanobacteria. However, there is limited information on how media composition and other factors can affect biomass or product yield in a specific strain. In this work we used a standard BG-11 medium and assessed how the change of nitrate and phosphate concentrations affect biomass and C-PC yield. Our results showed that increasing nitrate concentrations in media to 20mM resulted in a significant enhancement of both biomass (37%) and C-PC (23%) yield when compared to control. Further increase of nitrate concentration (22mM) suppressed biomass yield while still stimulating C-PC accumulation. Since decrease of a biomass yield would eventually result in a decreased C-PC yield, we suggest 20mM as the most favorable concentration of nitrates. Similar effect of C-PC-enhanced accumulation in a medium with an increased nitrogen concentration was also reported for other cyanobacteria such as *Geitlerinema sulphureium* (Kenekar and Deodhar, 2013) and *Spirulina* (Urek and Tarhan, 2012; Abd, 2003). This phenomenon is consistent with the fact that C-PC serves as a nitrogen source during nitrogen starvation (Boussiba and Richmond, 1980) and explains the decline of C-PC on 17^{th} day of cultivation in media with lower concentrations of nitrate (14mM, 16mM, and 17.6 mM) (Fig. 1 a,b.c – supplement). Since increasing or decreasing the phosphate concentration from that defined in a control medium (BG-11), did not result in a significant improvement in biomass or C-PC yield, we suggest 0.25mM of K_2HPO_4 as the optimal phosphate concentration.

Both light intensity and light quality play significant roles in C-PC production. As it was shown in previous research, cyanobacterial accumulation of C-PC is stimulated with lower rather than higher light intensities (Kenekar and Deodhar, 2013; Mohite, and Wakte, 2011; Takano et al., 1995; Oliveira et al., 2014). The cyanobacterial strategy of reducing the content of phycobiliproteins at high light intensity is consistent with prevention of photo-oxidative damage that can be caused by free radicals. Our strain of *Limnothrix* sp. 37-2-1 had the highest C-PC content at the light intensity of 25 $\mu mol^{-2}s^{-1}$. The same optimal light intensity for C-PC accumulation was reported for *Synechococcus* (Takano et al., 1995). Our data are in agreement with data of Oliveira et al. (2014) who found that light intensities greater than 30 $\mu mol^{-2}s^{-1}$ progressively decrease phycobiliprotein production.

Chromatic adaptation in cyanobacteria is a known and well explained phenomenon in which cells adapt to the available light quality through synthesis of specific pigments (Tandeau de Marsac, 1977). In our experiments we have grown the culture of *Limnothrix* sp. 37-2-1 initially under white light and then at the day 13, exposed the cultures to different light qualities. This strategy was used in order to test the effect of different light qualities only after the biomass reached the stationary phase; an approach potentially applicable in a technology of large-scale production. Under our experimental

conditions, the highest C-PC content was obtained when the culture remained under white light, while the red light had an inhibitory effect. This is contrary to what was reported by Takano et al. (1995) for *Synechococcus* sp. NKBG 042902, where the highest C-PC content was obtained in the culture exposed to red light.

Temperature is another growth factor that determines C-PC production. Our results showed that there were no significant differences in the biomass yield at used temperatures, however the temperature of 28°C was identified as the optimal for C-PC production. Further increase of temperature (30°C) resulted in suppression of C-PC production. Similar results on C-PC suppression with higher temperatures were reported for the cyanobacteria *Geitlerinema sulphureum* (Kenekar and Deodhar, 2013) and *Arthrospira platensis* (Mohite and Wakte, 2011).

In order to obtain the optimal production of C-PC, the right time of a growth phase for biomass harvesting needs to be determined. Under our standard cultivation conditions, between 15th and 17th day of cultivation suggests the optimal time for harvest. Appropriate harvest time depends not only on cultivation conditions but also on the organism used. For example, in the case of five strains of *Spirulina*, it was reported (Soundarapandian and Vasanthi, 2008) that the highest content of C-PC was found at the 30^{th} of cultivation.

Selenium is considered one of the most important trace dietary nutrients involved in the cellular protection against oxidative damage (Margaret, 2000). It is known that food supplementation with organic Se is safer than that with inorganic source. To obtain the organic food-form of Se, microorganisms such as yeast cells (Schrauzer, 2006) and *Spirulina* have been used (Huang et al., 2001; Chen et al. 2006). By growing *Limnothrix* sp. 37-2-1 in Se-enriched medium
(250mgL^{-1}), we have shown that the Se content in C-PC was increased 14 times compared to control. Based on our data and data by Huang et al. (2007) who used *S. platensis*, it can be concluded that C-PC serves as an important protein for Se binding. Even though the addition of Na_2SeO_3 to the growth medium somewhat suppressed the growth of *Limnorthix* sp. 37-2-1, surprisingly it stimulated C-PC accumulation, at least at the concentration of 250mgL^{-1}. In addition, it also improved its antioxidative properties; C-PC from the Se-grown culture had 3.2 times higher activity compared to that in control.

In conclusion, by changing cultivation conditions it is possible to improve C-PC accumulation in *Limnothrix* sp. 37-2-1, however compromising between the conditions that provide the best growth and C-PC yield is needed. When using *Limnothrix* sp. 37-2-1, the best conditions for C-PC production are identified as follows: BG-11 medium amended with 20mM of $NaNO_3$, light intensity of 25µmol photons $m^{-2}s^{-1}$, exposure to white light, temperature of 28°C, Se_2O_3 concentration of 250mgL^{-1}, and the most favorable harvesting time is between 15 and 17 days.

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