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# Production of phycocyanin by *Leptolyngbya* sp. in desert environments

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ABSTRACT

*Leptolyngbya* sp. QUCCCM 56 was investigated as a possible alternative to *A. platensis*, for the production of phycocyanin-rich biomass under desert conditions. Under elevated temperatures and light intensities, of up to 40 °C and 1800 $\mu mol \cdot m^{-2} \cdot s^{-1}$, the strain's biomass productivity was up to 45% higher as compared to reported productivities for *A. platensis*, with comparable phycocyanin content. Increasing temperatures were found to improve the biomass productivity and phycocyanin content, which, at 40 °C, were 1.09 ± 0.03 $g_X \cdot L^{-1} \cdot d^{-1}$ and 72.12 ± 3.52 $mg_{PC} \cdot g_X^{-1}$, respectively. The optimum biomass productivity was found at a light intensity of 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$, with higher light intensities causing a decrease of 15%. Furthermore, of the various phycocyanin extraction methods tested, bead-beating in phosphate buffer had the highest combined phycocyanin yield (169.9 ± 3.6 $mg_{PC} \cdot g_X$) and purity (7.37 ± 0.16) for *Leptolyngbya* sp. For *A. platensis*, this extraction method also resulted in the highest extract purities (3.78 ± 0.04). The extract purities obtained for *Leptolyngbya* sp. are considerably higher than other reported phycocyanin purities, and further investigation is recommended to study the scale-up of both *Leptolyngbya* sp. and bead-beating for commercial scale high-grade phycocyanin production under desert conditions.

## 1. Introduction

Phycocyanin is a water-soluble pigment-protein complex unique to cyanobacteria and eukaryotic algae, which functions as light-harvesting complex that absorbs light in regions of the visible spectrum that are poorly absorbed by chlorophyll. Applications of phycocyanin in biotechnological processes, as well as in the food and pharmaceutical industries, are increasing as it is a natural source of bioactive-pigment, with antioxidant, anticancer, and anti-inflammatory effects [1–3].

Presently, the main source of commercial phycocyanin is *Arthrospira platensis*, which generally contains around 7% phycocyanin (dry weight basis), however values of up to 18% have also been reported [4]. Furthermore, volumetric biomass productivities of *A. platensis* have been reported up to 0.32 and 1.59 $g_X \cdot L^{-1} \cdot d^{-1}$ for open raceway ponds and novel tubular reactors, respectively [5,6]. Even though *A. platensis* is widely cultivated, limitations still exist under elevated temperatures and light intensities [7]. This is especially a concern for production in desert climates, where temperatures and light intensities can reach extreme levels, but also in more temperate regions, when cultivating in closed reactors, in which temperatures can increase significantly. 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 [8].

*Leptolyngbya*, a member of the *Oscillatoriales* order, is one of the most common cyanoprokaryotic organisms, and has been found in an extreme diverse range of ecological habitats, ranging from desert environments to hot springs and even the coastal waters of Antarctica [9–11]. At present, 158 species have been taxonomically classified to the genus [12]. Despite its abundant global presence, which would signify the genera's highly competitive edge over other cyanoprokaryotic strains, there is limited research into the genus' commercial potential. The research on applications is limited to identification of the strain as an interesting candidate for bioremediation of $CO_2$ streams and biofuel production [9,13–15] and as a possible candidate for wastewater treatment [16,17]. Furthermore, the strain has been identified as a potential producer of phycobiliproteins (amongst which

*Abbreviations:* $C_{PC}$, phycocyanin concentration $mg_{PC} \cdot L^{-1}$; $C_X$, biomass concentration $g_X \cdot L^{-1}$; *EP*, extract purity; $F_H$, harvest volume $L \cdot d^{-1}$; *I*, light intensity $\mu mol \cdot m^{-2} \cdot s^{-1}$; $P_{PC}$, phycocyanin productivity $mg_{PC} \cdot L^{-1} \cdot d^{-1}$; $P_X$, biomass productivity $g_X \cdot L^{-1} \cdot d^{-1}$; *T*, temperature °C; $X_{PC}$, phycocyanin content $mg_{PC} \cdot g_X^{-1}$; $V_R$, reactor volume L

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phycocyanin) [18,19], as well as a potential alternative to *Arthrospira*, possessing advantageous characteristics in terms of high biomass productivity, protein and lipid content, under a wide range of temperatures (10–40 °C) and salinities (0–80 ppt) [20,21]. Nonetheless, no studies have been found which look into the commercial application for the production of phycocyanin from *Leptolyngbya*.

The aim of this study is to quantify and optimize biomass and phycocyanin productivity of *Leptolyngbya* sp. QUCCCM 56, a thermotolerant marine cyanobacteria isolated from Qatar [9]. Focus was specifically on assessing the strain's performance under desert conditions, with temperatures and light intensities of up to 45 °C and 1800 $\mu mol \cdot m^{-2} \cdot s^{-1}$ respectively. Furthermore, the optimal phycocyanin extraction protocol for *Leptolyngbya* sp. was investigated, to allow for rapid phycocyanin extraction with high yields and purities. The effectiveness of the various phycocyanin extraction protocols was compared with *A. platensis* in order to verify the methods as well as compare extract yields and purities between the two strains.

## 2. Materials and methods

### 2.1. Cultures

*Leptolyngbya* sp. QUCCCM 56 was obtained from the Qatar University Culture Collection of Cyanobacteria and Microalgae (QUCCCM, Doha, Qatar). *Arthrospira platensis* UTEX 1940 was obtained from the UTEX® Culture Collection of Algae (University of Texas, Austin, USA). Stock cultures were maintained in 250 mL conical flasks with a working volume of 100 mL, in Zarrouk medium [22], and pH was not controlled. Flasks were kept in an environmental incubator (Snijders Scientific®; Micro Clima-Series; Economic Lux Chamber) at 30 °C and 25 °C, for *Leptolyngbya* sp. QUCCCM 56 and *A. platensis*, respectively, under a 12:12 h light:dark cycle with a light intensity of 85 ± 5 $\mu mol \cdot m^{-2} \cdot s^{-1}$, and the culture was agitated using a flask shaker set at 150 rpm (Heidolph Instruments® Rotamax 120).

### 2.2. Effects of light intensity and temperature on biomass productivity, and phycocyanin content and productivity

*Leptolyngbya* sp. QUCCCM 56 cultures were inoculated in flat-panel airlift photobioreactors (Algaemist, Technical Development Studio, Wageningen University, the Netherlands) with a working volume of 0.4 L ($V_R$), an optical depth of 14 mm, and one-sided illumination by six broad spectrum LEDs (BXRA W1200, Bridgelux, USA), over an illumination area of 0.028$m^2$ under a 12:12 h light:dark cycle [23]. Aeration was set at 200 ± 20 $mL \cdot min^{-1}$ with $CO_2$ added to maintain a pH of 9.0 ± 0.1. Cultures were initiated as batch, and after reaching a biomass concentration of 1.0 $g_X \cdot L^{-1}$ operation mode was set to turbidostat and a constant biomass concentration was maintained. The optical density (750 nm) and harvest volume ($F_H$, $L \cdot d^{-1}$) were measured every 24 h, and biomass dry weight determinations were performed every 48 h. Biomass productivity and phycocyanin content were evaluated for six light intensities (80, 160, 300, 700, 1000 and 1800 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and six temperatures (20, 25, 30, 35, 40, and 45 °C). When not under investigation, standard temperature and light intensity set-points of 30 °C and 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$ were used, respectively. Samples were taken for 3 consecutive days once a steady state was reached (stable biomass concentration, $C_X$, $g_X \cdot L^{-1}$, and harvest volume for 2 consecutive days) from duplicate reactors (n = 6), and the harvest volume, biomass concentration, and phycocyanin content ($X_{PC}$, $mg_{PC} \cdot g_X^{-1}$) were determined. Biomass productivities ($P_X$, $g_X \cdot L^{-1} \cdot d^{-1}$) and phycocyanin productivities ($P_{PC}$, $mg_{PC} \cdot L^{-1} \cdot d^{-1}$) were calculated as per Eqs. (1) and (2), respectively.

$$P_X = \frac{F_H \bullet C_X}{V_R} \tag{1}$$

$$P_{PC} = P_X \bullet X_{PC} \tag{2}$$

### 2.3. Phycocyanin extraction optimization & measurements

Biomass aliquots containing 5 mg and 10 mg of biomass of *Leptolyngbya* sp. QUCCCM 56 and *A. platensis* were taken from batch cultures cultivated in flasks (as described in 2.1). Biomass was separated from the media by centrifugation (30 min at 4200 RCF at 4 °C), after which pellets were resuspended in 1.25 mL of either a) Phosphate Buffer (0.1 M 6.0 pH), b) Calcium Chloride (10 $g \cdot L^{-1}$), or c) Milli-Q water. Samples were subjected to either i) freeze-thawing (incubated at −20 °C until solid, followed by thawing for 24 h at 4 °C in the dark), ii) bead-beating (3 cycles of 25 s at 2500 rpm, Bertin® Precellys 24 and Lysing Matrix Tubes, Lysing Matrix E, 2 mL tubes, containing 1.4 mm ceramic spheres, 0.1 mm silica spheres, and one 4 mm glass bead, mpbio®), or iii) sonication (5 s pulses of 8 W over 30 s, on ice, Sonics® VCX 130 Ultrasonic processor). After all treatments, the samples were centrifuged (20,238 RCF for 30 min at 4 °C), and the pellet and supernatant were separated. Phycocyanin was determined in the supernatant, and the pellet was resuspended in an equal volume of fresh extraction buffer and incubated for an additional 24 h at 4 °C in the dark. The process of centrifugation and resuspension was repeated twice more (48 h and 96 h), or until no significant amount of phycocyanin was extracted during subsequent incubation times. For freeze-thawing, no direct measurements were performed due to the nature of the treatment, requiring at least 24 h incubation time. Phycocyanin concentrations ($C_{PC}$, $mg_{PC} \cdot L^{-1}$) were determined as per Lawrenz et al. [24,25] (Eq. (3)).

$$C_{PC} = \frac{Abs_{620} - Abs_{750}}{\varepsilon d} \bullet M_w \bullet \frac{V_{buffer}}{V_{sample}} \bullet 10^6 \tag{3}$$

In which $Abs_{620}$ and $Abs_{750}$ are the measured absorbances of the phycocyanin extract at 620 and 750 nm, respectively, which were determined using a Hach-Lange DR 6000 spectrophotometer. $\varepsilon$, $d$ and $M_w$ are the molar extinction coefficient of phycocyanin ($1.9 \cdot 10^6$ $L \cdot mol^{-1} \cdot cm^{-1}$), path length of the cuvette (1 cm), and the molecular weight of phycocyanin (264,000 $g \cdot mol^{-1}$), respectively. $V_{buffer}$ and $V_{sample}$ are the volume of the buffer and sample. The phycocyanin content ($X_{PC}$, $mg_{PC} \cdot g_X^{-1}$) was then determined as per Eq. (4).

$$X_{PC} = \frac{C_{PC}}{C_X} \tag{4}$$

In which $C_X$ is the concentration of biomass in $g_X \cdot L^{-1}$. In addition to absorbance measurements at 620 and 750 nm, the absorbance at 280 nm was determined to calculate the extract purity (*EP*) as per Eq. (5) [26]:

$$EP = \frac{Abs_{620}}{Abs_{280}} \tag{5}$$

In which $Abs_{620}$ and $Abs_{280}$ are the measured absorbance of the phycocyanin extract at 620 nm and 280 nm respectively. In Fig. 1 an overview is given of the different extraction buffers, cell disruption techniques and incubation times tested. Each combination of biomass quantity, extraction buffer and cell disruption method was performed in triplicate (n = 3).

As per the results of the phycocyanin extraction optimization, in all reactor experiments investigating the light and temperature effects on *Leptolyngbya* sp. QUCCCM 56 as described in 2.2, phycocyanin was determined using aliquots containing 5 mg biomass, phosphate buffer and bead-beating as the extraction buffer and cell-disruption method, respectively, followed by direct measurements of the extract (no incubation time).

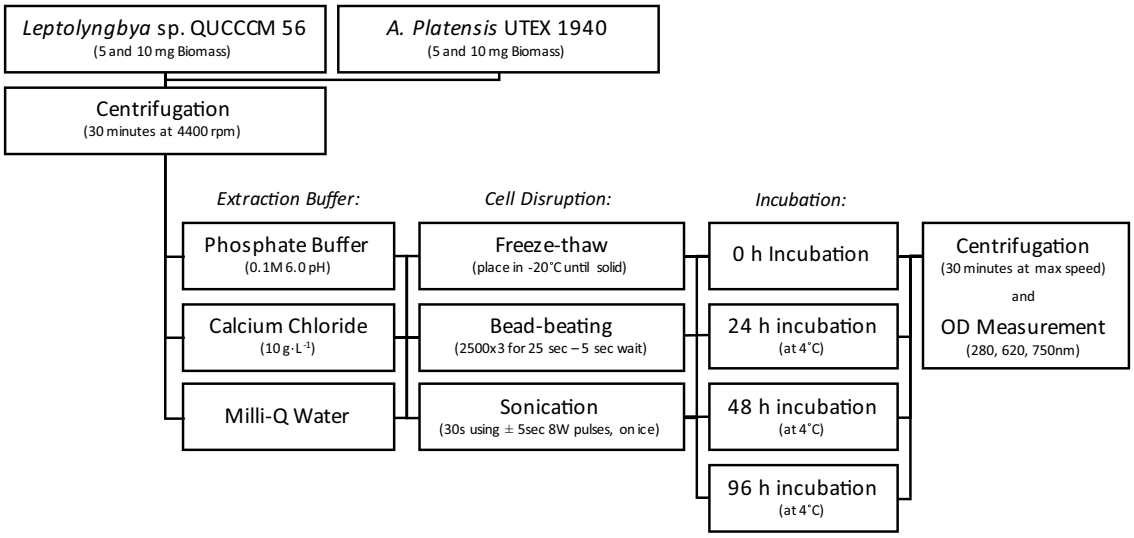


**Fig. 1.** Overview of extraction buffers, cell disruption methods, and incubation times tested for phycocyanin extraction from *Leptolyngbya* sp. QUCCCM 56 and *A. platensis* UTEX 1940.

### 2.4. Biomass dry weight

For biomass dry weight determination, duplicate biomass samples of 2–15 mL were diluted 5 times with ammonium formate (0.5 M) prior to being filtered through pre-dried (24 h, 95 °C), pre-weighed, and washed with 0.5 M ammonium formate, glass microfiber filters (Whatman GF/F™ Ø 55 mm) under a constant vacuum. The filters were then washed with a double volume of 0.5 M Ammonium Formate, dried (24 h, 95 °C), cooled in a desiccator (> 2 h) and weighed. The biomass dry weight was determined as the difference between the weight of the dried filters prior to and after biomass filtration and drying.

### 2.5. Statistical analysis

The reported values are the mean of all individual samples, whilst the error bars represent the standard deviation. For the effect of light and temperature on the biomass productivity and phycocyanin content, one-way ANOVA was used to determine significance difference between the means of independent conditions (n = 6). Variable effects were deemed significant if $p < 0.05$. Furthermore, correlations between light, temperature, biomass productivity, phycocyanin content, and extract purity, were tested using Pearson Correlation Analysis. For the extraction protocol development, the effect of the different variables (biomass amount, extraction buffer and cell disruption method) on phycocyanin content and extract purity was analyzed using a General Linear Mixed Model with Gamma Regression and Linear Regression, respectively. The effect of the variables on both phycocyanin content and extract purity simultaneously was analyzed using a regression factor representing both values with equal weight. This factor was computed through a dimension reduction factor analysis applying principle components analysis (PCA). Subsequently, a 3-way General Linear Mixed Model was applied with a Linear Regression. All statistical analyses were performed using SPSS 26 (SPSS, Chicago, IL, USA).

## 3. Results & discussion

### 3.1. Effect of temperature and light intensity on biomass productivity and phycocyanin content

Under desert climate conditions, as can be found in Qatar, ambient temperatures and light intensities can reach up to 49.8 °C and over 2200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ [27]. In open raceway ponds, average culture temperatures during summer are generally around 7–8 °C below ambient temperatures, which is still considerably higher than the 10–30 °C temperature range appropriate for most algal species [28]. In order to investigate the potential of *Leptolyngbya* sp. QUCCCM 56 under such desert conditions, the effects of light intensities up to 1800 $\mu mol \cdot m^{-2} \cdot s^{-1}$, and temperatures up to 45 °C on biomass productivity, and phycocyanin content, phycocyanin productivity, and extract purity, were investigated under continuous turbidostat cultivations. Results are shown in Fig. 2.

Both temperature and light intensity were found to have a significantly effect on biomass productivity, phycocyanin content, phycocyanin productivity, and extract purity ($p < 0.05$) (Appendix A). Increasing temperatures showed a strong positive correlation with the biomass productivity and phycocyanin content of the strain (r = 0.921 and 0.977, respectively), with the highest biomass productivity and phycocyanin content of 1.09 ± 0.03 $g_X L^{-1} \cdot d^{-1}$ and 72.12 ± 3.52 $mg_{PC} \cdot g_X^{-1}$ found at 40 °C. This phycocyanin content is on par with the average content of *A. platensis*, however there are reports of higher concentrations for *A. platensis* up to 184 $mg_{PC} \cdot g_X^{-1}$ [4,29]. At 45 °C, cell death occurred, which is concurrent with previous results obtained [9]. In terms of temperature effect, the number of studies on the effect of temperature of phycocyanin content and productivity are limited [8]. In temperature studies done on a number of different strains, such as *A. platensis*, *Anabena* sp. and *Lyngbya* sp., generally a peak in phycocyanin content is found for temperatures between 30 and 36 °C, with higher temperatures reducing the phycocyanin content [30–33]. For *A. platensis*, temperature optima for both biomass productivity and phycocyanin content are reported ranging from 27 to 35 °C (Table 1).

Our study shows a similar effect in terms of increasing temperatures leading to an increased phycocyanin content, however unlike the other studies, the optimum for both biomass productivity and phycocyanin content lies at a higher temperature (40 °C), and the maximum biomass productivity was 45% higher than reported biomass productivities for *A. platensis* (Table 1). This higher optimum temperature could give *Leptolyngbya* sp. QUCCCM 56 a competitive edge over other commonly cultivated strains for phycocyanin production, for cultivation both in desert climates, as well as in closed photobioreactors in temperate regions, where in summer cooling is generally required to reduce culture temperatures [39]. Furthermore, the higher optimum temperature could also indicate that phycocyanin from *Leptolyngbya* sp. QUCCCM 56 could be more thermostable as compared to that isolated from other strains with lower temperature optima [2].

In regard to light intensity, the optimal for biomass productivity was

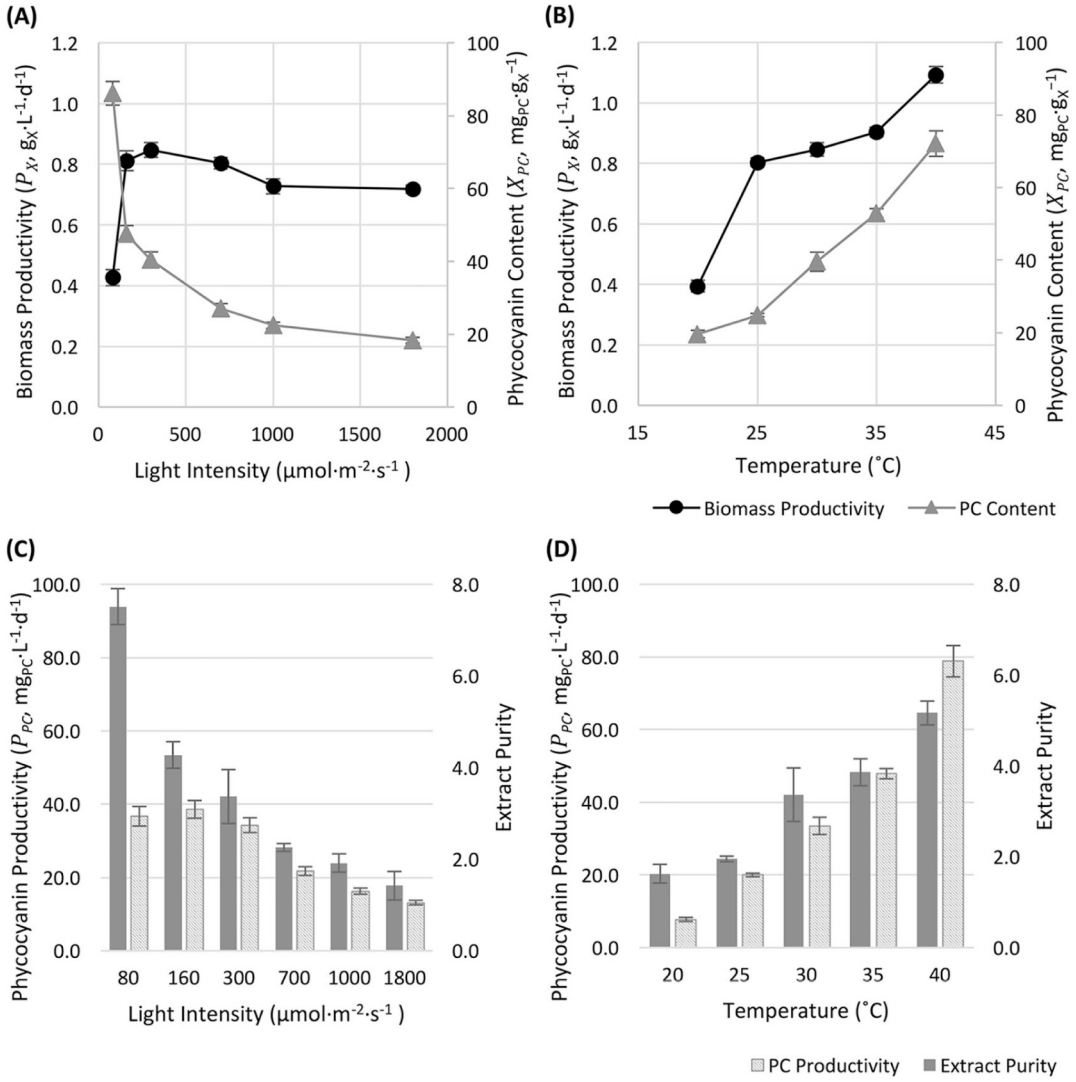


**Fig. 2.** (A & B) Biomass Productivities (● $P_X$, $g_X \cdot L^{-1} \cdot d^{-1}$) and Phycocyanin Content (▲ $X_{PC}$, $mg_{PC} \cdot g_X^{-1}$) of 1 $g \cdot L^{-1}$ cultures, operated under continuous turbidostat cultivation, with different light intensities (80–1800 µmol·m$^{-2}$·s$^{-1}$ at 30 °C) and temperatures (20–40 °C at 300 µmol·m$^{-2}$·s$^{-1}$), respectively; (C & D) Phycocyanin Productivity (▨ $P_{PC}$, $mg_{PC} \cdot L^{-1} \cdot d^{-1}$) and Phycocyanin Extract Purity (■ *EP*) for different light intensities and temperatures, respectively. Data shown is the mean ± stdev (n = 6).

**Table 1**
Comparison of performance of *Leptolyngbya* sp. QUCCCM 56 and *A. platensis* from referenced studies, in terms of biomass productivity ($P_X$), phycocyanin content ($X_{PC}$), and phycocyanin productivity ($P_{PC}$) for various temperatures (T) and light intensities (I).

| Strain | Operational conditions | | Optima for $P_x$ | | | | Optima for $X_{PC}$ | | | | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Light: dark | | | T | I | $P_x$ | T | I | $X_{PC}$ | $P_{PC}$ | |
| | Reactor type | Cultivation mode | h | °C | µmol·m$^{-2}$·s$^{-1}$ | $g_X \cdot L^{-1} \cdot d^{-1}$ | °C | µmol·m$^{-2}$·s$^{-1}$ | $mg_{PC} \cdot g_X^{-1}$ | $mg_{PC} \cdot L^{-1} \cdot d^{-1}$ | |
| *A. platensis* | Glass Vessel (ø 9.5 cm) | Batch | 24:0 | 28 | 300[a] | 0.436 | 28 | 75[a] | 184 | 40.0 | [29] |
| | | Fed-batch | 24:0 | 28 | 300 | 0.588 | – | – | 161 | 94.8 | |
| *S. platensis* | Flat-PBR (depth n/a) | Batch | 24:0 | 30 | 700[a] | 0.75 | 30 | 100[a] | 140 | 110 | [34] |
| *S. platensis* | Flasks (500 mL) | Batch | 12:12 | 35[a] | ± 27[a] | 0.023[b] | 35[a] | ± 27[a] | 77 | 1.79[b] | [32] |
| *S. platensis* | PBR (ø 3.4 cm) | Batch | 14:10 | 30 | 200 | 0.39[b] | – | – | 168[b] | 66.1[b] | [35] |
| *A. platensis* | Flasks (1000 mL) | Batch | 24:0 | 31 | 150[a] | 0.104 | 31 | 150[a] | 93 | 9.62[b] | [36] |
| *A. platensis* | U-shaped water basin | Batch | 16:8 | 27 | 800[a] | 0.110 | 27 | 70[a] | 130 | 5.4 | [37] |
| *A. platensis* | Vessel w. top lighting (depth 9.8 cm) | Continuous fixed dilution | 12:12 | 30 | 403[a] | 0.30[b] | 30 | 124[a] | 92.3 | 8.3 | [38] |
| *Leptolyngbya* sp. QUCCCM 56 | Flat-panel PBR (depth 14 mm) | Continuous | 12:12 | 40[a] | 300[a] | 1.09 | 40[a] | 80[a] | 86 | 78.8 | This study |

[a] Optimized temperature/light intensity for biomass productivity ($P_X$) or phycocyanin content ($X_{PC}$).
[b] Calculated based on referenced data.

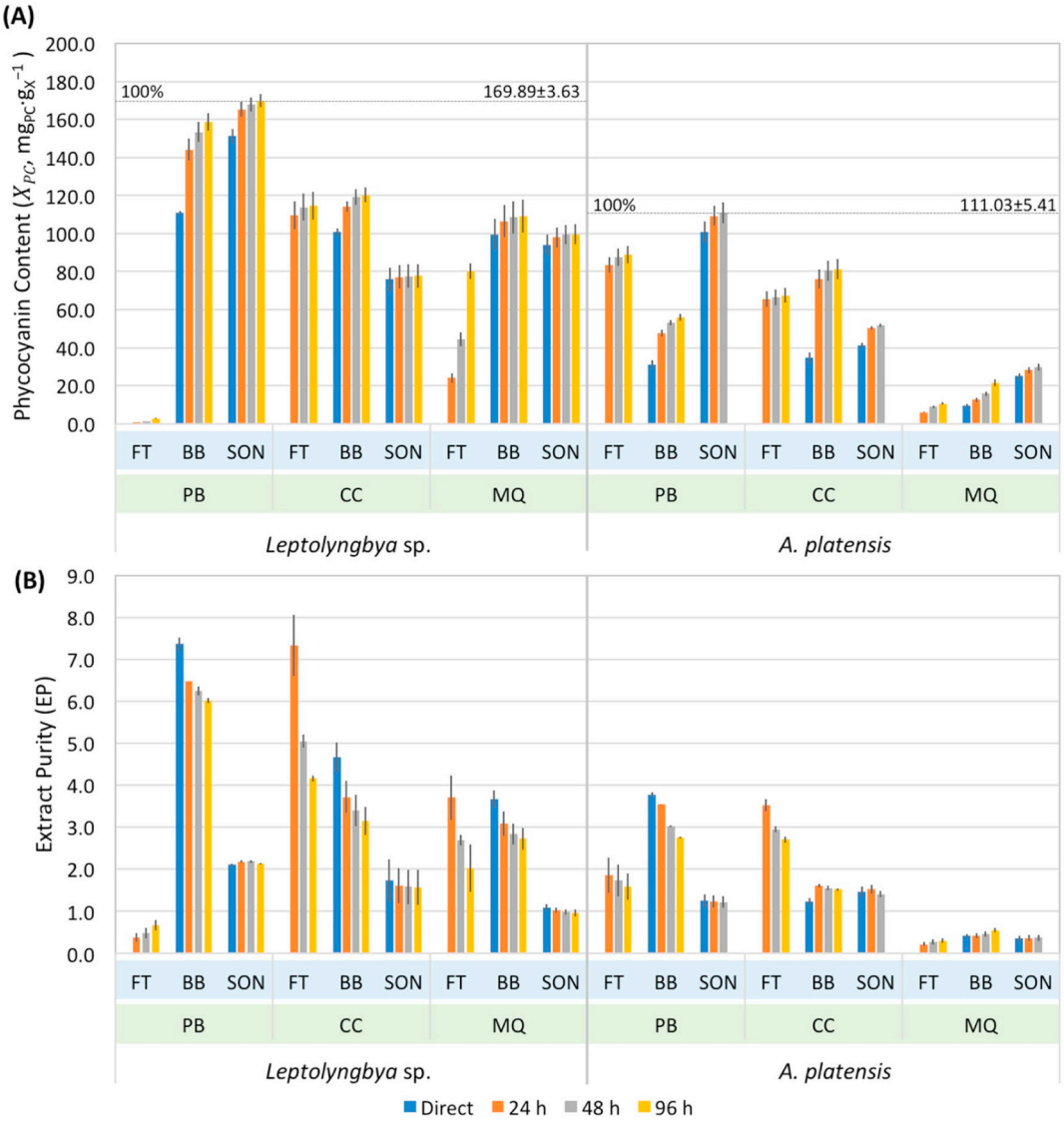


**Fig. 3.** (A) Phycocyanin Content ($X_{PC}$ $mg_{PC}·g_X^{-1}$) and (B) Extract Purity (*EP*) from *Leptolyngbya* sp. QUCCCM 56 and *A. platensis* UTEX 1940 using different cell-disruption methods (FT: Freeze-Thawing, BB: Bead-Beating, SON: Sonication), extraction buffers (PB: Phosphate Buffer, CC: Calcium Chloride, MQ: Milli-Q Water), and incubation times, each sample containing 5 mg of biomass (10 mg data is provided in Appendix B). Dashed line indicates the max. obtained phycocyanin assumed to be 100% extraction. Data shown is the mean ± SD, n = 3.

found at 300 $\mu mol·m^{-2}·s^{-1}$. At higher light intensities, biomass productivities decreased slightly (up to 15%), however the biomass productivity of 0.72 ± 0.01 $g_X·L^{-1}·d^{-1}$ even at 1800 $\mu mol·m^{-2}·s^{-1}$ is still on par with reported productivities of *A. platensis* under optimal conditions (Table 1). The results indicate that the strain is capable of maintaining high biomass productivities under a wide range of light intensities, even under dilute culture conditions (1.0 $g_X·L^{-1}$ and 14 mm culture depth). This could be very beneficial for cultivation in open raceway ponds in desert environments, where daily light intensities can fluctuate significantly. However, as has been reported for many other strains, including *A. platensis* [8], the optimal light intensity for phycocyanin content was found at low light intensities (80 $\mu mol·m^{-2}·s^{-1}$), and showed a significant decrease of 53.0% and 78.7% for increasing light intensities of 300 and 1800 $\mu mol·m^{-2}·s^{-1}$, respectively. Furthermore, even under the optimal conditions, the phycocyanin content of *Leptolyngbya* sp. QUCCCM 56 found was in the lower range (86.1 ± 3.3 $mg_{PC}·g_X$) as compared to reported values for *A. platensis* (up to 184 $mg_{PC}·g_X$, Table 1). Nonetheless, overall phycocyanin productivities, which are a combination of both phycocyanin content and biomass productivity, were on par with that of *A. platensis*, and the maximum extract purity found for *Leptolyngbya* sp. (7.51 ± 0.39) was considerably higher than generally reported for other strains, significantly increasing the value of the extract [40,41].

In this work, the biomass concentration, light:biomass ratio, as well as nitrogen availability, were kept constant for each condition studied, through applying a turbidostat cultivation regime with a fixed biomass concentration of 1.0 $g·L^{-1}$. A continuous culture permits the maintenance of cultures very close to the maximum growth rate, thereby increasing the biomass productivities, but also limiting the effects of nutritional limitations and changes in biomass concentration, allowing for the investigation into the effects of process parameters independently [7]. To the best of the authors' knowledge, there are no known studies in which the effect of light intensity and temperature on phycocyanin productivity have been investigated under such continuous culture regimes, and all referenced works researching these effects have been performed in (fed-)batch cultures. However, under batch cultivation conditions, the light:biomass ratio, as well as the availability of nitrogen and other nutrients, will change over the duration of the experiment. This causes the biomass productivity and phycocyanin content to be dependent not only on the process

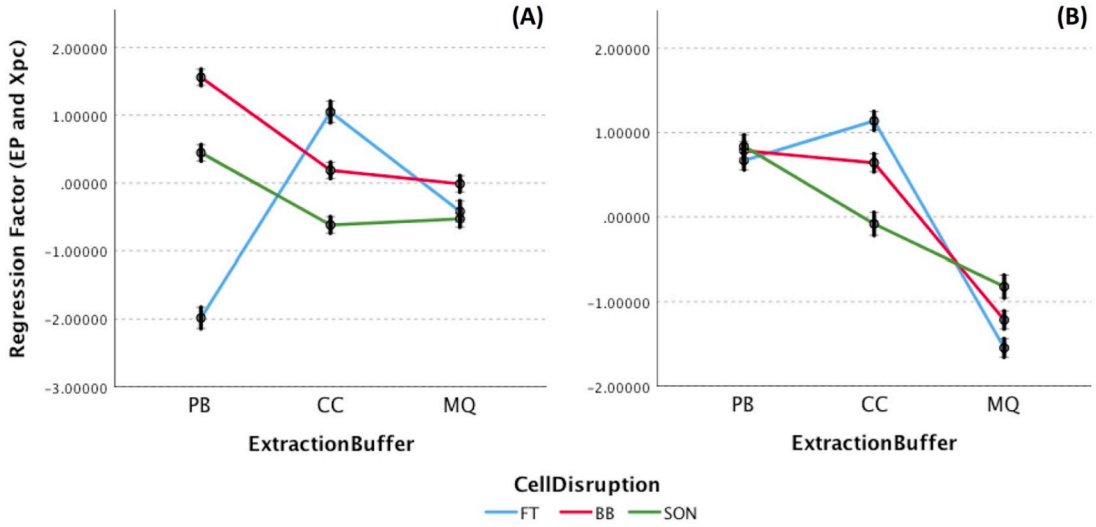


**Fig. 4.** Estimated means charts for significant ($p < 0.05$) two-way effects of extraction buffer and cell disruption method on both extract purity (***EP***) and Phycocyanin Content ($\boldsymbol{X_{PC}}$ $mg_{PC} \cdot g_X^{-1}$) for (A) *Leptolyngbya* sp. QUCCCM 56 and (B) *A. platensis* UTEX 1940, represented by a combined regression factor (see text for more details). FT: Freeze-Thawing, BB: Bead-Beating, SON: Sonication, PB: Phosphate Buffer, CC: Calcium Chloride, MQ: Milli-Q Water.

parameters under investigation, such as temperature and light intensity, but also on the cultivation stage, and related biomass and nitrogen concentrations. For example, both Chen et al. and Xie et al. found that the maximum phycocyanin content during batch cultures coincided with nitrogen depletion and high biomass concentrations, and subsequent low light:biomass ratios [29,34]. The lower phycocyanin contents found in this work are therefore hypothesized to be due to the applied cultivation regime and set biomass density, in which neither (near) nitrogen depletion nor very high biomass concentrations occurred. Especially the latter would result in higher light:biomass ratios in continuous cultures as compared to batch cultures, which has a significant negative effect on the phycocyanin content. This was also suggested during the extraction assays, in which *Leptolyngbya* sp. QUCCCM 56 from batch cultures (flasks) was used. There, phycocyanin contents of 160 $mg_{PC} \cdot g_X$ were found, which were near double compared to the values found for the continuous cultures of the cultivation assays. More investigation is required to see how the phycocyanin content of the strain can be improved, for example by applying a higher biomass density, whilst maintaining the high biomass productivities of a continuous culture, thereby improving the overall phycocyanin productivity.

### 3.2. Phycocyanin extraction optimization

The efficient extraction of phycocyanin from the biomass is essential to accurately determine the phycocyanin content and productivities. 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 [42–45]. Furthermore, the optimal extraction method can differ from strain to strain [43], and as this is the first known study of *Leptolyngbya* sp. for phycocyanin production, the most effective method of extraction was investigated and compared to *A. platensis*. Extraction yields and purities were analyzed for three different cell disruption methods (bead-beating, freeze-thawing and sonication), in combination with either phosphate buffer, milli-Q water or calcium chloride as extraction buffer. Furthermore, two biomass-buffer ratios were tested, and 4 incubation times, ranging from 0 to 96 h. The results for both *Leptolyngbya* sp. QUCCCM 56 and *A. platensis* UTEX 1940 for each treatment are shown in Fig. 3.

The effect of extraction buffer and cell disruption method on the phycocyanin content and extract purity were found to be significant for all treatments tested for both strains ($p < 0.05$). No significant effect of biomass concentration was found on the phycocyanin yield of *Leptolyngbya sp.* ($p = 0.359$), nor on for the extract purity of *A. platensis* ($p = 0.898$). Nonetheless, increasing biomass quantities from 5 to 10 mg were found to have a significant negative effect on the extracted phycocyanin content for *A. platensis*, and a significant positive effect on the extract purity for *Leptolygnbya* sp. As the phycocyanin content found with 5 mg biomass for both *Leptolyngbya* sp. and *A. platensis* were either similar or higher as compared to higher biomass concentrations (10 mg), further statistical analyses were limited to the lower biomass concentration (5 mg).

Sonication in phosphate buffer showed the highest extraction yields, with phycocyanin contents' of 169.89 ± 3.63 and 111.03 ± 5.41 $mg_{PC} \cdot g_X^{-1}$, after 96 and 48 h incubation, for *Leptolyngbya* sp. and *A. platensis*, respectively. Freeze-thawing in phosphate buffer was the second-best extraction method for *A. platensis* (80% extraction as compared to sonication), however unexpectedly performed the least for *Leptolyngbya* sp., giving a phycocyanin content of only 2.73 ± 0.10 $mg_{PC} \cdot g_X^{-1}$. Bead-beating with phosphate buffer performed very well for *Leptolyngbya* sp., with 93% extraction yields as compared to sonication. In terms of extract purity, for both strains, bead-beating in phosphate buffer with direct measurement resulted in the highest purities, of 7.37 ± 0.16 and 3.78 ± 0.04 for *Leptolyngbya* sp. and *A. platensis*, respectively. Overall, a decrease of purity was found for increasing incubation times, whilst the extraction yield increased slightly.

In order to determine which treatment yielded both the highest extraction yield and purity, a regression factor was computed representing both values with equal weight for each strain. The estimated means on the regression factor for significant two-way effects (extraction buffer and cell disruption) were computed and are shown in Fig. 4.

Fig. 4 shows that when assessing the best method in terms of both extraction yield and purity, and taking into account interactions between the extraction buffer and the cell disruption method, bead-beating in phosphate buffer had the highest regression factor value for *Leptolyngbya* sp. For *A. platensis*, freeze-thawing with calcium chloride was the best performing, which was the second-best method for *Leptolyngbya* sp. The differences between Fig. 4A and B clearly show how strain dependent the optimal phycocyanin extraction method is, as the results vary significantly between the two strains.

Cell disruption is one of the key factors for the extraction of phycocyanin with high yields and purities; inadequate disruption results in reduced extraction of phycocyanin, whilst excessive cell disruption can

lead to release of other undesired biomolecules in addition to phycocyanin, thereby reducing the extract purity and possibly denaturing the phycocyanin [46]. Bead-beating, a method commonly applied for cell disruption for lipid extraction [47,48], was not found to be described previously for phycobiliprotein extraction. In combination with phosphate buffer, however, the method had the highest combined extract yields and purities for *Leptolyngbya* sp., and the highest extract purity for *A. platensis*. As bead-beating allows for multiple variations from the currently applied protocol (number and duration of cycles, RPM, bead-size), it is hypothesized that this method could be optimized even further to increase the extract yield with direct measurement for both strains, without compromising the extract purity. Sonication resulted in the highest phycocyanin content values, which is supportive of results found by Lawrenz et al. [24]. However, the extract purity was low compared to other cell-disruption methods, suggesting that excessive cell disruption occurred, a factor which was not taken into account by Lawrenz et al. The effectiveness of freeze-thawing for phycocyanin extraction from *Leptolyngbya* sp. QUCCCM 56 was found to be dependent on the extraction buffer used. With phosphate buffer, no significant phycocyanin amounts were extracted, which is surprising as it is one of the most commonly used methods for phycocyanin extraction from cyanobacteria [49,50], however with calcium chloride, the method performed well. Calcium chloride has been reported as an efficient salt for the extraction of cell wall proteins [51] and even though phycocyanin does not exist within the cell-wall membrane itself, it does forms clusters that adhere to the membrane. This could be a possible explanation as to why, when coupled to the least disruptive cell-disruption method (freeze-thawing), calcium chloride gives the highest extract yield and purity as compared to other extraction buffers combined with freeze-thawing.

The purity of the extracted phycocyanin significantly influences its commercial value, with analytical grade phycocyanin (purities of 4.0 and higher) having prices of 15 US$ per mg or more, compared to 0.13US$ and 1–5 US$ for food grade (0.7) and reagent grade (3.9), respectively [41]. Extract purities can differ from strain to strain, but are also dependent on the applied extraction methods, and additional purification steps are generally applied to increase the extracts' purity [43]. In this study, both strains showed the highest purities with bead-beating in phosphate buffer, indicating that this method of cell-disruption could be more effective for high-purity extraction as compared to other, more commonly applied methods. Furthermore, the highest extract purity found for *Leptolyngbya* sp. QUCCCM 56 (7.37 ± 0.16) was significantly higher than obtained for *A. platensis* (3.78 ± 0.04). Even phycocyanin extract purities reported from other studies for *A. platensis* were found ranging from 1.43 to maximum 6.69 after additional extract purification steps [4,40,43]. It is therefore hypothesized that not only the improved extraction method, but also the physiology of the strain, contributes to obtaining such high extract purities, and further investigation is recommended to study the scale-up of both *Leptolyngbya* sp. QUCCCM 56 and bead-beating for commercial scale high-grade phycocyanin production.

## 4. Conclusions

*Leptolyngbya* sp. showed to be able to grow well under elevated temperatures and light intensities, with an optimal biomass productivity found at 40 °C. Furthermore, bead beating was shown to be an effective and fast way to extract high-purity phycocyanin from both *Leptolyngbya* sp. and *A. platensis*. The obtained extract purities from *Leptolyngbya* sp. were higher than previously reported for any other strain. The improved productivities of both biomass and phycocyanin at higher temperatures, as well as the high purity of the obtained extract, suggest that the strain is an interesting candidate for commercial phycocyanin production in desert environments.

## CRediT authorship contribution statement

**Kira Schipper:** Conceptualization, Methodology, Supervision, Formal Analysis, Writing – Original Draft, Funding acquisition. **Filippo Fortunati:** Methodology, Investigation, Validation, Formal Analysis, Writing – Review & Editing. **Pieter C. Oostlander:** Methodology, Investigation, Supervision, Writing – Review & Editing. **Mariam Al Muraikhi:** Conceptualization, Methodology, Writing – Review & Editing. **Hareb S.J. Al Jabri:** Conceptualization, Supervision, Writing – Review & Editing, Funding acquisition. **René H. Wijffels:** Conceptualization, Supervision, Writing – Review & Editing. **Maria J. Barbosa:** Conceptualization, Supervision, Methodology, Writing – Review & Editing

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Statistical analysis data for light and temperature experiments

Table A.1
One-way ANOVA for temperature data.

| | | Sum of squares | df | Mean square | F | Sig. |
|---|---|---|---|---|---|---|
| $P_x$ | Between groups | 4.691 | 4 | 1.173 | 3010.844 | 0.000 |
| | Within groups | 0.033 | 85 | 0.000 | | |
| | Total | 4.724 | 89 | | | |
| $X_{PC}$ | Between groups | 32,856.962 | 4 | 8214.240 | 1344.261 | 0.000 |
| | Within groups | 519.401 | 85 | 6.111 | | |
| | Total | 33,376.363 | 89 | | | |
| EP | Between groups | 150.443 | 4 | 37.611 | 232.812 | 0.000 |
| | Within groups | 13.732 | 85 | 0.162 | | |
| | Total | 164.174 | 89 | | | |

Table A.2
One-way ANOVA for light intensity data.

| | | Sum of squares | df | Mean square | F | Sig. |
|---|---|---|---|---|---|---|
| $P_x$ | Between groups | 2.113 | 5 | 0.423 | 868.416 | 0.000 |
| | Within groups | 0.050 | 102 | 0.000 | | |
| | Total | 2.163 | 107 | | | |
| $X_{PC}$ | Between groups | 56,347.360 | 5 | 11,269.472 | 2204.928 | 0.000 |
| | Within groups | 521.326 | 102 | 5.111 | | |
| | Total | 56,868.686 | 107 | | | |
| EP | Between groups | 451.527 | 5 | 90.305 | 317.925 | 0.000 |
| | Within groups | 28.973 | 102 | 0.284 | | |
| | Total | 480.500 | 107 | | | |

Table A.3
Pearson Correlation for temperature data.

| | | Temperature | $P_x$ | $X_{PC}$ | EP |
|---|---|---|---|---|---|
| Temperature | Pearson Correlation | 1 | 0.921** | 0.977** | 0.941** |
| | Sig. (2-tailed) | | 0.000 | 0.000 | 0.000 |
| $P_x$ | Pearson Correlation | 0.921** | 1 | 0.850** | 0.832** |
| | Sig. (2-tailed) | 0.000 | | 0.000 | 0.000 |
| $X_{PC}$ | Pearson Correlation | 0.977** | 0.850** | 1 | 0.940** |
| | Sig. (2-tailed) | 0.000 | 0.000 | | 0.000 |
| EP | Pearson Correlation | 0.941** | 0.832** | 0.940** | 1 |
| | Sig. (2-tailed) | 0.000 | 0.000 | 0.000 | |

** Correlation is significant at the 0.01 level (2-tailed).

Table A.4
Pearson Correlation for light data.

| | | Light intensity | $P_x$ | $X_{PC}$ | EP |
|---|---|---|---|---|---|
| Light Intensity | Pearson Correlation | 1 | 0.162 | −0.755** | −0.745** |
| | Sig. (2-tailed) | | 0.094 | 0.000 | 0.000 |
| $P_x$ | Pearson Correlation | 0.162 | 1 | −0.721** | −0.689** |
| | Sig. (2-tailed) | 0.094 | | 0.000 | 0.000 |
| $X_{PC}$ | Pearson Correlation | −0.755** | −0.721** | 1 | 0.971** |
| | Sig. (2-tailed) | 0.000 | 0.000 | | 0.000 |
| EP | Pearson Correlation | −0.745** | −0.689** | 0.971** | 1 |
| | Sig. (2-tailed) | 0.000 | 0.000 | 0.000 | |

** Correlation is significant at the 0.01 level (2-tailed).

## Appendix B. Results of phycocyanin extraction protocol development

Table B.1
Phycocanin content ($X_{PC}$) for different treatments and incubations times for 5 mg *Leptolyngbya* sp. QUCCCM 56 and *A. platensis* UTEX 1940.

| Strain | Buffer | Cell disruption | Direct | 24 h | 48 h | 96 h |
|---|---|---|---|---|---|---|
| *Leptolyngbya* sp. QUCCCM 56 | Phosphate buffer | Freeze-thawing | | 0.80 ± 0.08 | 1.36 ± 0.08 | 2.73 ± 0.10 |
| | | Bead-beating | 110.79 ± 1.07 | 144.14 ± 5.82 | 153.24 ± 5.32 | 158.62 ± 4.71 |
| | | Sonication | 151.24 ± 3.73 | 165.30 ± 3.74 | 167.90 ± 3.77 | 169.89 ± 3.63 |
| | Calcium chloride | Freeze-thawing | | 109.65 ± 7.33 | 113.87 ± 7.00 | 114.61 ± 7.24 |
| | | Bead-beating | 100.75 ± 2.00 | 114.10 ± 2.78 | 119.11 ± 4.17 | 120.27 ± 4.02 |
| | | Sonication | 75.84 ± 6.22 | 77.17 ± 6.15 | 77.58 ± 6.10 | 77.69 ± 6.08 |
| | Milli-Q | Freeze-thawing | | 23.97 ± 2.64 | 44.39 ± 3.81 | 80.07 ± 4.04 |
| | | Bead-beating | 99.52 ± 8.07 | 106.55 ± 8.34 | 108.45 ± 8.52 | 109.09 ± 8.54 |
| | | Sonication | 94.01 ± 5.23 | 97.89 ± 5.12 | 99.44 ± 5.16 | 99.64 ± 5.19 |
| *A. platensis* | Phosphate buffer | Freeze-thawing | | 83.35 ± 3.93 | 87.38 ± 4.64 | 88.74 ± 4.67 |
| | | Bead-beating | 31.09 ± 2.35 | 47.37 ± 1.86 | 53.09 ± 1.58 | 55.85 ± 1.65 |
| | | Sonication | 100.77 ± 5.75 | 109.31 ± 5.39 | 111.03 ± 5.41 | |
| | Calcium chloride | Freeze-thawing | | 65.33 ± 4.12 | 66.43 ± 3.99 | 67.50 ± 4.06 |
| | | Bead-beating | 34.82 ± 2.78 | 76.08 ± 5.03 | 80.42 ± 5.30 | 81.20 ± 5.31 |
| | | Sonication | 41.18 ± 1.16 | 50.30 ± 0.98 | 51.69 ± 1.12 | |
| | Milli-Q | Freeze-thawing | | 6.00 ± 0.22 | 8.82 ± 0.57 | 10.49 ± 0.59 |
| | | Bead-beating | 9.31 ± 1.21 | 12.53 ± 1.30 | 15.71 ± 1.12 | 21.44 ± 1.67 |
| | | Sonication | 24.99 ± 1.65 | 28.18 ± 1.64 | 29.53 ± 1.76 | |

Table B.2
Phycocanin content ($X_{PC}$) for different treatments and incubations times for 10 mg *Leptolyngbya* sp. QUCCCM 56 and *A. platensis* UTEX 1940.

| Strain | Buffer | Cell disruption | Direct | 24 h | 48 h | 96 h |
|---|---|---|---|---|---|---|
| *Leptolyngbya* sp. QUCCCM 56 | Phosphate buffer | Freeze-thawing | | 1.55 ± 0.66 | 2.20 ± 0.72 | 3.50 ± 0.79 |
| | | Bead-beating | 90.21 ± 9.94 | 125.83 ± 11.19 | 138.12 ± 12.80 | 149.86 ± 12.25 |
| | | Sonication | 152.40 ± 6.35 | 165.92 ± 8.04 | 169.67 ± 8.30 | 172.13 ± 8.10 |
| | Calcium chloride | Freeze-thawing | | 84.35 ± 6.57 | 117.70 ± 7.22 | 117.98 ± 7.16 |
| | | Bead-beating | 76.62 ± 3.02 | 86.85 ± 3.07 | 92.63 ± 2.60 | 93.03 ± 2.57 |
| | | Sonication | 79.66 ± 10.49 | 81.27 ± 10.09 | 81.57 ± 10.06 | 81.68 ± 10.04 |
| | Milli-Q | Freeze-thawing | | 31.48 ± 3.08 | 62.41 ± 6.99 | 95.21 ± 3.76 |
| | | Bead-beating | 58.54 ± 5.59 | 65.75 ± 6.38 | 67.94 ± 6.65 | 71.21 ± 6.29 |
| | | Sonication | 94.30 ± 5.39 | 98.18 ± 5.76 | 100.34 ± 6.01 | 101.77 ± 5.93 |
| *A. platensis* | Phosphate buffer | Freeze-thawing | | 56.34 ± 1.54 | 64.83 ± 2.45 | 67.46 ± 2.20 |
| | | Bead-beating | 17.53 ± 1.03 | 27.94 ± 1.75 | 32.59 ± 2.18 | 35.46 ± 2.19 |
| | | Sonication | 64.96 ± 0.80 | 79.70 ± 1.90 | 82.30 ± 1.68 | |
| | Calcium chloride | Freeze-thawing | | 53.18 ± 1.34 | 53.98 ± 1.34 | 54.74 ± 1.26 |
| | | Bead-beating | 23.69 ± 3.89 | 62.07 ± 3.47 | 68.58 ± 2.86 | 69.59 ± 2.86 |
| | | Sonication | 40.01 ± 1.76 | 53.60 ± 5.30 | 54.69 ± 5.36 | |
| | Milli-Q | Freeze-thawing | | 6.57 ± 1.47 | 9.42 ± 2.39 | 11.34 ± 2.55 |
| | | Bead-beating | 6.69 ± 0.80 | 12.06 ± 1.37 | 18.49 ± 0.72 | 21.52 ± 0.81 |
| | | Sonication | 23.43 ± 1.10 | 27.17 ± 1.05 | 28.76 ± 0.94 | |

Table B.3
Extract Purity (EP) for different treatments and incubations times for 5 mg *Leptolyngbya* sp. QUCCCM 56 and *A. platensis* UTEX 1940.

| Strain | Buffer | Cell disruption | Direct | 24 h | 48 h | 96 h |
|---|---|---|---|---|---|---|
| *Leptolyngbya* sp. QUCCCM 56 | Phosphate buffer | Freeze-thawing | | 0.38 ± 0.11 | 0.49 ± 0.12 | 0.67 ± 0.13 |
| | | Bead-beating | 7.37 ± 0.16 | 6.48 ± 0.00 | 6.25 ± 0.11 | 6.02 ± 0.06 |
| | | Sonication | 2.10 ± 0.03 | 2.17 ± 0.03 | 2.19 ± 0.03 | 2.13 ± 0.02 |
| | Calcium chloride | Freeze-thawing | | 7.34 ± 0.73 | 5.05 ± 0.15 | 4.16 ± 0.07 |
| | | Bead-beating | 4.66 ± 0.36 | 3.72 ± 0.38 | 3.39 ± 0.38 | 3.15 ± 0.34 |
| | | Sonication | 1.73 ± 0.49 | 1.60 ± 0.41 | 1.58 ± 0.41 | 1.57 ± 0.42 |
| | Milli-Q | Freeze-thawing | | 3.70 ± 0.53 | 2.69 ± 0.13 | 2.03 ± 0.57 |
| | | Bead-beating | 3.68 ± 0.20 | 3.08 ± 0.29 | 2.83 ± 0.26 | 2.72 ± 0.26 |
| | | Sonication | 1.09 ± 0.07 | 1.02 ± 0.06 | 0.98 ± 0.06 | 0.96 ± 0.08 |
| *A. platensis* | Phosphate buffer | Freeze-thawing | | 1.86 ± 0.41 | 1.73 ± 0.38 | 1.58 ± 0.31 |
| | | Bead-beating | 3.78 ± 0.04 | 3.54 ± 0.01 | 3.02 ± 0.02 | 2.75 ± 0.02 |
| | | Sonication | 1.25 ± 0.16 | 1.24 ± 0.14 | 1.21 ± 0.14 | |
| | Calcium chloride | Freeze-thawing | | 3.53 ± 0.14 | 2.95 ± 0.06 | 2.71 ± 0.07 |
| | | Bead-beating | 1.23 ± 0.08 | 1.60 ± 0.05 | 1.55 ± 0.05 | 1.52 ± 0.03 |
| | | Sonication | 1.46 ± 0.13 | 1.52 ± 0.11 | 1.41 ± 0.08 | |
| | Milli-Q | Freeze-thawing | | 0.22 ± 0.04 | 0.27 ± 0.06 | 0.30 ± 0.05 |
| | | Bead-beating | 0.41 ± 0.05 | 0.42 ± 0.07 | 0.46 ± 0.06 | 0.55 ± 0.05 |
| | | Sonication | 0.35 ± 0.07 | 0.36 ± 0.07 | 0.37 ± 0.07 | |

Table B.4
Extract Purity (EP) for different treatments and incubations times for 10 mg *Leptolyngbya* sp. QUCCCM 56 and *A. platensis* UTEX 1940.

| Strain | Buffer | Cell disruption | Direct | 24 h | 48 h | 96 h |
|---|---|---|---|---|---|---|
| *Leptolyngbya* sp. QUCCCM 56 | Phosphate buffer | Freeze-thawing | | 0.77 ± 0.26 | 0.74 ± 0.10 | 0.82 ± 0.09 |
| | | Bead-beating | 7.63 ± 0.79 | 6.93 ± 0.89 | 6.59 ± 0.92 | 6.44 ± 0.76 |
| | | Sonication | 2.24 ± 0.48 | 2.33 ± 0.47 | 2.32 ± 0.44 | 2.30 ± 0.39 |
| | Calcium chloride | Freeze-thawing | | 7.91 ± 0.49 | 5.92 ± 0.71 | 5.20 ± 0.57 |
| | | Bead-beating | 3.64 ± 0.49 | 3.15 ± 0.40 | 2.89 ± 0.33 | 2.74 ± 0.26 |
| | | Sonication | 1.89 ± 0.59 | 1.80 ± 0.54 | 1.76 ± 0.52 | 1.72 ± 0.52 |
| | Milli-Q | Freeze-thawing | | 5.04 ± 0.36 | 5.11 ± 0.28 | 2.55 ± 0.08 |
| | | Bead-beating | 4.63 ± 0.48 | 3.54 ± 0.33 | 3.24 ± 0.30 | 2.97 ± 0.28 |
| | | Sonication | 1.48 ± 0.60 | 1.31 ± 0.44 | 1.22 ± 0.36 | 1.16 ± 0.31 |
| *A. platensis* | Phosphate buffer | Freeze-thawing | | 1.54 ± 0.01 | 1.46 ± 0.03 | 1.39 ± 0.02 |
| | | Bead-beating | 3.54 ± 0.26 | 3.10 ± 0.11 | 2.85 ± 0.09 | 2.54 ± 0.01 |
| | | Sonication | 1.15 ± 0.01 | 1.12 ± 0.02 | 1.10 ± 0.02 | |
| | Calcium chloride | Freeze-thawing | | 3.13 ± 0.24 | 2.77 ± 0.19 | 2.49 ± 0.13 |
| | | Bead-beating | 1.61 ± 0.23 | 1.86 ± 0.31 | 1.85 ± 0.26 | 1.83 ± 0.25 |
| | | Sonication | 0.84 ± 0.04 | 0.95 ± 0.07 | 0.94 ± 0.07 | |
| | Milli-Q | Freeze-thawing | | 0.29 ± 0.08 | 0.37 ± 0.09 | 0.40 ± 0.08 |
| | | Bead-beating | 0.46 ± 0.11 | 0.55 ± 0.08 | 0.71 ± 0.06 | 0.72 ± 0.05 |
| | | Sonication | 1.79 ± 0.01 | 1.31 ± 0.02 | 1.18 ± 0.01 | |

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