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RESEARCH ARTICLE

Phycocyanin extraction from frozen and freeze-dried biomass of *Pseudanabaena* sp. by using mild cell disruption methods

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ABSTRACT

Phycocyanin is a precious, natural, blue coloured pigment-protein complex that has commercial value and wide application in cosmetics, food, and pharmaceutical industries. In the present study, we performed various cell disruption methods (ultrasonication, homogenization, freeze/thaw and $CaCl_2$ extraction) for phycocyanin extraction from different forms of biomass of a thermophilic *Pseudanabaena* sp. that has a high potential to produce high-quality phycocyanin. Using potassium phosphate buffer and ultrasonic bath method, we achieved the highest phycocyanin yield (345 mgPC.g$^{-biomass}$) from freeze-dried biomass and we obtained increased yield as the duration of application increases. Phycocyanin yields were calculated as 345 mgPC.g$^{-biomass}$, 255 mgPC.g$^{-biomass}$ and 220 mgPC.g$^{-biomass}$ for 5, 10 and 15 min, respectively. In this study, cell disruption methods have determined significantly more effective on freeze-dried biomass rather than frozen biomass. Phycocyanin content of freeze-dried biomass was analysed after six months of storage and dramatic decrement was observed in the phycocyanin content of the cells.

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Introduction

Pseudanabaena species are non-heterocystous cyanobacteria which are characterized by trichomes and cells that are smaller than 2-4 μm in size (Tamburaci, 2009). Some species of *Pseudanabaena* possess chromatic adaptation mechanism that allows microorganisms to regulate their pigment composition depending on the exposed light quality (intensity, colour etc.) that results in quick adaptation to their environment (Acinas et al., 2009). In the literature, phycobiliprotein production capacity of *Pseudanabaena* sp. has been widely investigated in the aspect of phycoerythrin, and in some studies, the phycocyanin production capacity of the species was also reported (Tamburaci, 2009; Khan et al., 2019).

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Phycobiliproteins are highly fluorescent pigment-protein complexes that are produced in cyanobacteria species as an accessory pigment of chlorophyll. Having distinct spectral characteristics and bioactive properties (anti-inflammatory, anti-viral, anti-oxidant, etc.) are the main features that make phycobiliproteins widely-applicable natural substances in cosmetics, food, and pharmaceutical industries (Puzorjov & McCormick, 2020). Phycocyanin (PC), Phycoerythrin (PE), and Allophycocyanin (APC) are the subtypes of phycobiliproteins that have distinct spectral characters and commercial value depending on their purity and stability.

Phycocyanin is a blue-coloured water-soluble natural pigment that shows the major absorption peak at 620 nm. It is highly produced by cyanobacterium *Arthrospira* sp. (commercial name Spirulina) (Eriksen, 2008) and widely applied as a natural colourant instead of carcinogenic chemical colour additives (İlter et al., 2018). It has remarkable bioactive properties such as antioxidant, anti-inflammatory, hepatoprotective, and ROS scavenging, and depending on the purity ratio and stability, finds applications in different fields. The current PC industry highly depends on *Arthrospira* sp. since this species produce a high amount of PC and the cultivation process is not laborious. However, PC extracted from *Arthrospira* sp. has considerable drawbacks in terms of stability that impact its potential applications, especially when increased temperature and pH are desired (Liang et al., 2018, Liang et al., 2019; Klepacz-Smółka et al., 2020).

As PC has become commercially important product, efficient, cost-effective, environmentally friendly extraction of it has been emerged as a new challenge. All microalgae and cyanobacteria species have a rigid, tightly organized cell wall structure making liberation of intracellular molecules a great challenge while preserving their functionality and structure. Therefore, the applied cell disruption method highly influences the final product, its application, and the market value. For this reason, selecting the proper cell disruption method should be done considering the cell wall structure of the microorganism and the quality of the desired product. In terms of phycobiliproteins, since these compounds are sensitive to light and temperature, gentle disruption of the cells is vital to promote the liberation of proteins from the cell while preserving the functionality and bioactivity of the molecules (Phong et al., 2018).

Among various cell disruption methods, a feasible one for protein extraction should be selected considering the mildness of the method. Harsh conditions such as high pressure, high temperature and pH level should be avoided since they might cause structural changes in protein and later loss of activity. For this reason, mild cell disruption methods are mostly preferred for protein extraction from biomass. Bead milling, ultrasonication, enzymatic disruption, and ionic liquids are the main categories of existing mild cell disruption technologies (Phong et al., 2018). In the aspect of engineering, each method has its own advantages and limitations that should be taken into consideration in terms of cost and sustainability (Günerken et al., 2015).

This study was primarily aimed to determine the most effective PC extraction method for two different forms of the biomass of thermophilic *Pseudanabaena* sp. and to reveal the potential using of *Pseudanabaena* sp. as source of PC. For this purpose, frozen and freeze-dried biomasses were utilized, and selected mild cell disruption methods were compared in terms of PC extraction yields.

Materials and Methods

Materials and Chemicals

Pseudanabaena EGE MACC 40 was used as a biomass source and supplied from the Microalgae Culture Collection of Ege University (Ege Macc). Originally, the cyanobacterium was isolated from the thermal source in Turkey, Denizli-Sarayköy (Tamburaci, 2009).

BG 11 medium (Behle, 2019) with the addition of 22 g/L sea salt was used as a growth medium and at the end of the production, cells were harvested by centrifuge (6000 rpm, 5 min) and washed with distilled water. Following harvesting, half of the biomass was freeze-dried while the remaining was kept at -20°C. Freeze-dried biomass was stored at 4°C, preserving from humidity and light.

0.1 M potassium phosphate buffer (pH 7) was used as an extraction buffer. Buffer was prepared following the recipe of online tool (Centre for Proteome Research, Liverpool).

Extraction Procedure

Different phycocyanin extraction methods were performed by using freeze-dried and frozen biomass of *Pseudanabaena* sp.; all experiments were conducted by using 0.1 M potassium phosphate buffer (pH 7). Frozen biomass was thawed in 25°C prior to experiments. The freeze/thaw process was repeated twice. To explore the best cell disruption using frozen biomass, all samples were prepared resuspending 0.02 g (paste biomass) cell in 5 ml of 0.1 M potassium phosphate buffer (pH 7). Five different cell disruption methods were performed as freeze/thaw, homogenization (10 min, 8000 rpm), ultrasonic bath (HYDRA, 10 min, 45 kHz), ultrasonic probe (BADELIN, 2 min and 4 min, 9 cycles, 50% P, 20 kHz), and chemical extraction ($CaCl_2$ extraction). After each method, samples were centrifuged at 3500 rpm for 5 min and supernatant were

subjected to spectroscopic measurement (ULTROSPEC 1100 PRO). $CaCl_2$ extraction was performed as described by (İlter et al., 2018).

Extraction from freeze-dried biomass was performed using the 0.02 g (freeze-dried biomass) of cell and 5 ml of 0.1 M potassium phosphate buffer (pH 7). Ultrasonic bath (5 min, 10 min, and 15 min, 50%P), ultrasonic probe (1 min and 4 min, 50% P, 15-sec cycle), and chemical extraction ($CaCl_2$ extraction) methods were implemented for cell disruption. After each method, the sample was centrifuged, and the supernatant was collected for spectroscopic measurements. Chlorophyll-*a* analyses were performed as described by Mishra et al. (2012). Briefly, the pellet was collected and extracted with acetone (80%) incubating 1 h in dark at 4°C. Following, the supernatant was measured, and chlorophyll-*a* amount was calculated using the equation given below (Eq. 1).

Phycocyanin (PC), Allophycocyanin (APC) and Phycoerythrin (PE) concentrations were calculated approximately by using Bennet & Bogobad's equations (Eq. 2, 3, 4) (Bennett & Bogobad, 1973) where A indicates the measured absorbance values at given wavelengths.

$$Chlorophyll - a(\mu g/ml) = 12.7(A_{663}) - 2.1(A_{645}) \quad (1)$$

$$PC(mg\ /ml) = [A_{620} - 0.474\ A_{652}]/5.34 \quad (2)$$

$$APC(mg/ml) = [A_{652} - 0.208A_{620}]/5.09 \quad (3)$$

$$PE(mg\ /ml) = [A_{568} - 2.41PC - 0.849APC]/9.62 \quad (4)$$

Results

Phycocyanin extraction from frozen biomass was performed by implementing different mild cell disruption methods including ultrasonication, homogenization, and freeze/thaw, and $CaCl_2$ extraction (not shown) (Table 1). Calculated PC yields showed that the ultrasonic probe was the most effective method for PC extraction from frozen biomass while the freeze/thaw method had almost no visible effect on the cells. PC extraction yields were calculated as 43.75 mgPC.g$^{-biomass}$ and 2.375 mgPC.g$^{-biomass}$ for ultrasonic probe and freeze/thaw methods, respectively.

As the most efficient method was determined as an ultrasonic probe, results showed that duration of application is an important parameter in terms of obtained PC content. 4 min ultrasonic probe application resulted in 25% increased PC content compared to 2 min application.

Microscopic images and colour values of extracts (Figure 1 and Figure 2) further confirmed the degree of cell disruption in the samples. According to microscopy images, after homogenization and freeze/thaw applications there was no cell disruption in the samples and the supernatants were colourless while slightly disrupted cells could be observed after 15 min ultrasonic bath. Images supported that the ultrasonic probe method was the most efficient one, therefore almost all cells were disrupted in the sample. The supernatant of the sample after overnight extraction with $CaCl_2$ was colourless and no further measurement was performed for the sample.

Table 1. Phycocyanin content and phycocyanin yield of frozen biomass of *Pseudanabaena* sp. obtained by performing different cell disruption methods

Frozen Biomass	*PC (mg/ml)*	*PC Yield (mg/g_{biom})*
Freeze/Thaw	0.0095	2.375
Ultrasonic Bath (15 min)	0.0339	8.475
Homogenization (10 min)	0.0178	4.45
Sonic Probe (2 min)	0.130	32.5
Sonic Probe (4 min)	0.175	43.75

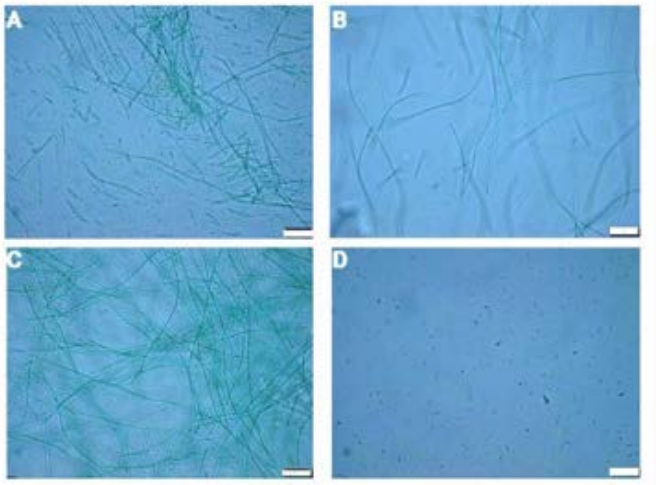

Figure 1. Microscopic images (10x) of frozen biomass of *Pseudanabaena* sp. after cell disruption by A) Ultrasonic bath method; B) Homogenization method; C) Freeze/thaw method; D) Ultrasonic Probe method. The morphology of the untreated cells is expected to be closer to the C.

PC extraction using freeze-dried biomass was performed by ultrasonication (ultrasonic bath and ultrasonic probe) and $CaCl_2$ extraction methods (Table 2). The highest PC yield was obtained by using an ultrasonic bath even though this method was not efficient for fresh cells, the best results were obtained by this method for freeze-dried cells. Figure 3 shows a comparison of obtained PC amounts from frozen and freeze-dried cells by ultrasonic method. The ultrasonic bath was performed for 5, 10, and 15 min, and compared to other methods, obtained PC amounts were significantly higher and increased by a longer duration of application. PC yields were calculated as 345 mgPC.g$^{-biomass}$, 255 mgPC.g$^{-biomass}$ and 220 mgPC.g$^{-biomass}$ for 5, 10 and 15 min, respectively.

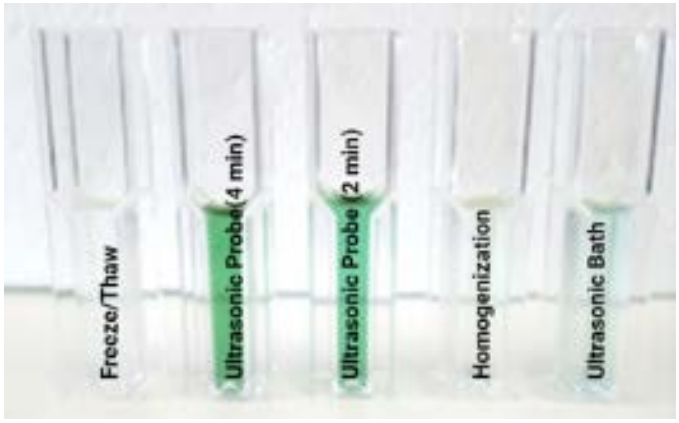

Figure 2. The supernatant of frozen biomass collected after performing cell disruption by freeze/thaw, ultrasonic probe, homogenization, and ultrasonic bath methods.

Table 2. Phycocyanin content and Chlorophyll-*a* content of freeze-dried biomass of *Pseudanabaena* sp. obtained by performing different cell disruption methods. n/a: not available

Freeze-dried Biomass	PC (mg/ml)	Chl-*a* (mg/ml)	PC Yield (mg/g_{biom})
Sonic Probe (1 min)	0.24	0.0015	120.36
Sonic Probe (4 min)	0.22	0.0040	110.16
$CaCl_2$	0.21	0.014	109.64
Ultrasonic Bath (15 min)	0.69	n/a	345
Ultrasonic Bath (10 min)	0.51	n/a	255
Ultrasonic Bath (5 min)	0.44	n/a	220

The ultrasonic probe was performed for 1 min and 4 min. The results showed that obtained PC amounts following 1 min and 4 min ultrasonic probe and $CaCl_2$ extraction were the same while chlorophyll-*a* contents were varied. The highest chlorophyll-*a* content was observed following cell disruption by using an ultrasonic probe (4 min) while the lowest was observed after 1 min. Chlorophyll-*a* contents were observable by the colour values of the samples. After 1 min ultrasonication, the sample was brilliant blue while after 4 min it was green (Figure 4). According to the results, for freeze-dried cells, a longer duration of application (ultrasonic probe) caused an increment in undesired chlorophyll-*a* content while obtained PC amount was the same. After $CaCl_2$ extraction, the same amount of PC was obtained while 10-fold higher chlorophyll-*a* content was found in the sample.

PC content of freeze-dried cells was analysed after five and six months following the lyophilization. After five months, PC concentration was calculated as 1.5-fold lower while it was dramatically decreased after the fifth month.

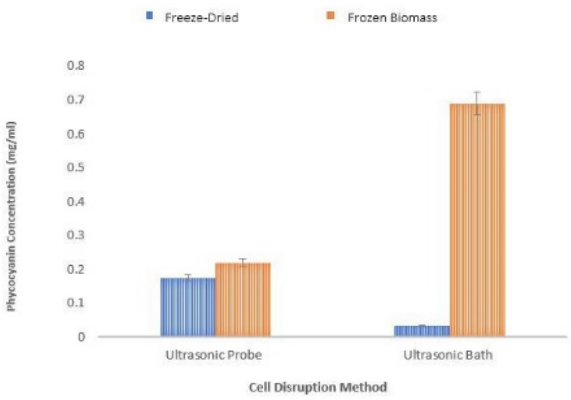

Figure 3. Phycocyanin concentration obtained by ultrasonic bath (15 min) and ultrasonic probe (4 min) methods from different forms (freeze-dried and frozen) of biomass of *Pseudanabaena* sp.

Figure 4. Raw protein extracts prepared from freeze-dried biomass of *Pseudanabaena* sp. A) The supernatant collected after cell disruption by ultrasonic probe (4 min) method; B) The supernatant collected after cell disruption by ultrasonic probe (1 min)

Discussion

In the present study, protein extraction was performed by using different forms of biomass (frozen (wet) and freeze-dried (dried)) and various cell disruption techniques were performed to reveal the most efficient PC extraction method. The maximum yield of PC was obtained by using freeze-dried biomass and the ultrasonic bath method. Obtained yield value showed that *Pseudanabaena* sp. produced a remarkable amount of PC considering the commonly used *Arthrospira* species that have yield values ranging from 100 to 380 $mgPC.g^{-biomass}$ (Leu et al., 2013; Lima et al., 2018; Prates et al., 2018; Hsieh-Lo et al., 2019).

Calculated PC concentration and yields for frozen biomass were critically lower than freeze-dried biomass (Table1 and Table 2). These findings show that extraction yield and

obtained amount of PC were highly affected by the form of biomass. Cell disruption methods were more efficient on freeze-dried cells. These results are considered to be associated with the water content of the cells that affects cell wall strength and enhances cell disruption ratio. Pan-utai & Iamtham (2019) reported varying extraction yields in *Arthrospira platensis* and purity values depending on the method of drying, and freeze-drying is the most suitable method to maintain cell composition. In the literature, freeze-drying method is recommended to preserve the heat-sensitive content of cells without damaging the cell wall (Güroy et al. 2017; Tavanandi & Raghavarao, 2019).

In the present study, the PC content of freeze-dried biomass was analysed after five months and revealed significant decrease. This situation may be related to the storage conditions of the samples. Throughout this period, samples were exposed to light and humidity, even for a short time, during sample preparation for experiments.

The freeze/thaw method and homogenization for phycobiliprotein extraction from frozen and freeze-dried biomass had no effect on cells, according to the literature, freeze/thaw method is widely used for phycobiliprotein extraction from fresh biomass. Cano-Europa et al. (2010), used the freeze/thaw method for phycobiliprotein extraction in *Pseudanabaena tenuis,* and they reported 2% phycocyanin and 89% phycoerythrin content. In the present study, freeze/thaw method was not enough to achieve cell disruption therefore, it was used to increase the efficiency of combined methods. Moraes et al. (2011) reported similar results in their comparative study with *A. platensis*, they obtained the lowest extraction yield after freeze/thaw and homogenization.

Ultrasonication method (ultrasonic bath and ultrasonic probe) was applied as a mild cell disruption method which is widely used for protein extraction. This method causes cell disruption exposing the cells to the high-intensity ultrasonic waves that create mechanical stress and ultimately allow releasing cell content into the extraction medium (Safi et al., 2014; Phong et al., 2018). During ultrasonication process, keeping sample cool to prevent overheating is highly important to maintain the functionality of the proteins (Lee et al., 2012). Therefore, sonication processes were performed using ice and water.

The biomass in frozen samples; the best result was obtained by ultrasonic probe and results showed that duration of application is an important parameter in terms of PC content. Increased duration of application resulted in 25% increased phycocyanin content for the ultrasonic probe. Furuki et al. (2003) reported increased PC content by applying longer ultrasonication, and they indicated that the proper application time is an important factor in terms of protein functionality.

The biomass in freeze-dried samples; the ultrasonic bath was the most effective method even though the same observation was not valid for frozen biomass. The PC extraction yield was remarkably increased compared to other methods. Considering other mild cell disruption methods, ultrasonication method promotes pore enlargement by cavitation and it disrupts cell walls more efficiently (Vinatoru, 2001). Similar to ultrasonic probe, longer duration of application of ultrasonic bath caused an increment in obtained PC amount without degradation. Longer ultrasonic bath application resulted in a 2-fold higher PC concentration.

Varying chlorophyll-*a* contents were observed depending on the duration of application of the ultrasonic probe. Chlorophyll-*a* contents were observable by the colour values of the samples. After 1 min ultrasonication, the sample was brilliant blue while after 4 min ultrasonication it was green. The same situation was detected by İlter et al. (2018), who reported that the highest PC content from *Spirulina* sp. was obtained after ultrasonication and the increment in green colour was observed which is attributed to chlorophyll.

Conclusion

In the current study, PC extraction yield was significantly affected by the form of the biomass. All applied cell disruption methods showed better results for the freeze-dried biomass in terms of yield, and the most efficient method was determined as the ultrasonic bath. Results revealed that *Pseudanabaena* sp. can produce a significant amount of PC compared to commonly used species and its thermophilic nature may be advantageous in the aspect of stability (Ferraro et al., 2020).

Compliance With Ethical Standards

Authors' Contributions:

This study was performed under supervision of MCD and all experiments were designed and performed by BK and ZD. Manuscript was written by BK and all authors read and approved the final manuscript.

Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical Approval

For this type of study, formal consent is not required.

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