

# Ultrasound-Assisted Extraction of Chlorophylls and Phycocyanin from *Spirulina platensis*

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**Abstract:** *Spirulina platensis* found enormous applications in agriculture, food industry, pharmacy, cosmetics, medicine, mainly because of its high nutritional content and health-promoting properties. Therefore, the extraction of bioactive compounds from it still remains a challenge. The aim of the current study was to extract photosynthetic pigments from different *Spirulina platensis* samples and to evaluate the yield and purity of phycocyanin using ultrasonic irradiation at two different frequencies. The content of chlorophylls and phycocyanin was determined in three samples of *Spirulina platensis* (dry, lyophilized and frozen). The highest values of total chlorophyll were found in SP 1 (*Spirulina platensis* dry biosample) – 9080 µg/g dw. For the extraction of phycocyanin the classical and the ultrasound-assisted extractions were performed with distilled water under two frequencies. The highest yield of phycocyanin (2.5 mg/g dw) and purity 0.8 were obtained for 3 hours at 35 kHz sonification. This study remained the main advantage of using ultrasonic power as green extraction for the highest phycocyanin yield and purity, reducing both processing times and costs.

**Keywords:** *Spirulina platensis*; chlorophylls; phycocyanin; ultrasonic extraction.

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## 1. Introduction

Nowadays, the interest in cyanobacteria *Spirulina platensis* constantly increases in different areas as agriculture [1], food, cosmetics, medicine, pharmacy, and technics [2-7]. *Spirulina* is labeled as "super food" by The World Health Organization (WHO) [2] and also used in space food for astronauts [6] because of high nutritional value. The great attention is paid as a food supplement, because of its valuable composition that comprises as proteins (50-70% from the dry weight) [3], unsaturated fatty acids, polysaccharides, vitamins, minerals as selenium, calcium, chromium, iron, manganese, zinc, and magnesium [2,5], phenolics, flavonoids, chlorophylls, carotenoids, and phycobiliproteins [2,3,5-7]. Chlorophyll content in *Spirulina* varied between 6.8 to 11 g.kg<sup>-1</sup>, while carotenoids are presented mainly from beta carotene and xanthophylls at a concentration of 1.0 g.kg<sup>-1</sup>, and its concentration depends upon the species and the environmental conditions [8-11]. Phycobiliproteins (PBP) are a group of colored proteins, mainly present in cyanobacteria (blue-green algae) and red algae, as components of photosynthetic light-harvesting (phycobilisome) antenna complexes [12-13]. Phycobiliproteins are divided into three main groups: phycoerythrins, phycocyanin, and allophycocyanins [2,8,12]. In general, the above-mentioned proteins are divided into two

groups according to their colors and absorption spectra, such as phycoerythrin (PE, bright pink, red  $\lambda$  max = 490–570 nm) and phycocyanin (PC, blue  $\lambda$  max = 610–625 nm). In particular, the phycocyanins are included: C-phycocyanin (C-PC), R-phycocyanin (R-PC), and allophycocyanin (APC), which differ in their spectral properties, structural composition, and color [12]. *Spirulina platensis* is an excellent source of phycocyanin, as the protein fraction may contain up to 20% of phycocyanin [2,4-5]. Phycocyanin is used not only as an important commercially available water-soluble blue food colorant used in foods (chewing gums, dairy products, gellies, etc.), cosmetics (lipstick and eyeliners), with fluorescent properties, but it is also considered anticoagulation [16] as anti-inflammatory, anticancer agent [2,4,14], anti-diabetes [2,15], hepatoprotective [17], antioxidant [2,4,5,18] and anti-obesity activities, antimicrobial [2], and used treatment of many diseases such as Alzheimer's, Parkinson's and Huntington's diseases [5]. Therefore, due to valuable food coloring compounds as chlorophylls, carotenoids, and phycocyanin, *Spirulina platensis*. Many reports demonstrated that the pigment content in *Spirulina platensis* depends on different environmental parameters (pH, temperature and light intensity) [3,8,19], extraction conditions [17, 18, 20] and used solvents [2,7,14,17].

The extraction of chlorophyll and C-PC rain challenges because of their biological effects, such as anticancer, anti-aging, anti-inflammatory, and antioxidant activity effects [21]. Nowadays, different procedures were performed for accelerated extraction based on the "green chemistry" principles. Contrary to conventional methods for phycocyanin and chlorophyll extractions that include time-consuming and low efficient maceration, freezing, and thawing, the "green" method for extraction (including pressurized liquid extraction (PLE), supercritical fluid extraction (SFE), supercritical CO<sub>2</sub>, low-temperature extraction process using high-pressure homogenization (HP) [7], microwave extraction (MW), ultrasound-assisted (US) extraction assure low cost, environmentally friendly solvents, high efficiency during extraction, reduce time and higher yield [6,7,12,16,17,22]. However, the purity of phycocyanins isolated by different ultrasonic frequencies is not studied in detail.

The aim of the current study was to extract photosynthetic pigments from different *Spirulina platensis* samples (dry, lyophilized and frozen biomass) and to evaluate the yield and purity of C-phycocyanin using ultrasonic irradiation at two different frequencies, comparing with classical extraction.

## 2. Materials and Methods

### 2.1. Materials.

Three samples of *Spirulina platensis* were used in the current study. The first sample (SP1) is a commercially available dry biosample obtained from the Zoya on-line market. The second (SP2) and third samples (SP3) were kindly donated from a producer of *Spirulina platensis* cultivated in Bulgaria. The second sample was lyophilized, while the third one was used as frozen biomass.

### 2.2. Pigment analysis.

For the analyses of chlorophyll a (Ca), chlorophyll b (Cb), and the total carotenoids (Cx+b), each of three *Spirulina* samples were extracted with 100% acetone in solid ratio to the solvent to (1:50 w/v) extract in duplicate. The extraction procedure was carried out in an ultrasonic bath VWR (Malaysia) with frequency 45 kHz, power 30W at 40°C for 20 min. The extraction procedure was repeated twice; the acetone extracts were filtered through a filter

paper. The absorbance of the combined final extracts was measured at three wavelengths 662, 645, and 470 nm. The amount of these pigments was calculated according to equations (from 1 to 3) reported by Lichtenthaler and Wellburn [23].

$$\text{Ca, } \mu\text{g/ml} = 11.75 \times A_{662} - 2.35 \times A_{645} \quad (1)$$

$$\text{Cb, } \mu\text{g/ml} = 18.61 \times A_{645} - 3.96 \times A_{662} \quad (2)$$

$$\text{Cx+b, } \mu\text{g/ml} = [1000 \times A_{470} - 2.27 \times \text{Ca} - 81.4 \times \text{Cb}] / 227 \quad (3)$$

### 2.3. Extraction of phycocyanin.

#### 2.3.1. Conventional extraction.

*Spirulina* samples (2 g) were weighed in 50 ml centrifuge tubes, and they were extracted with distilled water (1:25 w/v) on a magnetic stirrer with a hot plate and under constant stirring for 1, 2, 3, 24, and 48 hours at temperature 35° C. This extraction was listed as classical extraction (CA). Then the samples were filtered and used for further analysis.

#### 2.3.2. Ultrasound-assisted extraction.

*Spirulina* samples (2 g) were weighed 50 ml centrifuge tubes with screw caps, and the samples were extracted with distilled water (1:25 w/v) in duplicate in ultrasonic baths operating at different condition, as follows:

- 1) An ultrasonic bath VWR (Malaysia) with frequency 45 kHz, power 30W - (UAE 45 kHz)
- 2) An ultrasonic bath SIEL (Bulgaria) with frequency 35 kHz, power 300W - (UAE 35 kHz)

The ultrasound-assisted extraction was done under two frequencies for 1, 2, 3, 24, and 48 hours at temperature 35° C. The extracts were filtered through a paper filter and then used for further analyses.

### 2.4. Phycocyanin analysis.

After filtration, the absorbance of extracts was measured by spectrophotometer Camspec M107 (Spectronic-Camspec Ltd., Leeds, UK) at two wavelengths - 615 and 652 nm, respectively. Phycocyanin concentration (PC) was calculated using equation (1), according to Bennett and Bogorad [24]:

$$\text{Phycocyanin, mg/mL} = \frac{A_{615 \text{ nm}} - 0.474 \times A_{652 \text{ nm}}}{5.34} \quad (1)$$

The purity of phycocyanin extract was monitored spectrophotometrically by the A615/A280 ratio [25].

$$\text{Yield} = \frac{\text{PC} \times \text{V}}{\text{DB}}, \quad (2)$$

Where PC is the crude C-phycocyanin in mg/ml, V is the volume of extract used in ml, and DB is the dry biomass in grams.

### 2.5. Statistical analysis.

All analyses were performed in triplicate. The data obtained were expressed as the mean ± standard deviation, considering a level of 95% confidence (p<0.05).

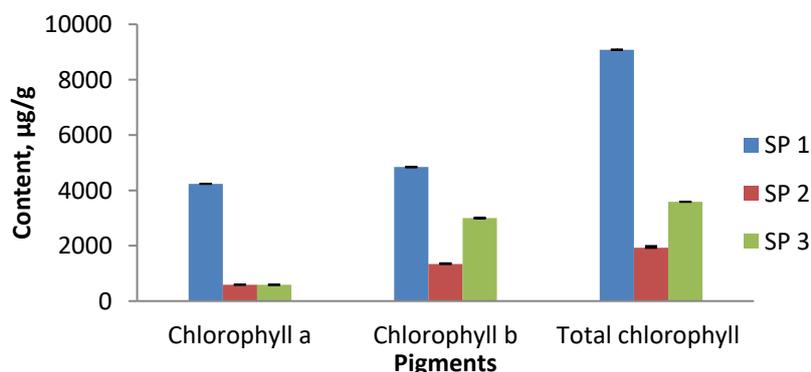
### 3. Results and Discussion

#### 3.1. Chlorophylls and carotenoids content.

It is known that *Spirulina* provides the pure form of chlorophyll an at the lowest production costs since most of the chlorophyll (approximately 90%) that exists in *Spirulina* is in the form of chlorophyll a [6,7]. In particular, the demand for chlorophyll a has recently increased because its biological activity, such as antioxidant activity, is considered to be higher than that of other chlorophylls, such as chlorophyll b or c [7].

The chlorophyll content found in *Spirulina* samples was presented in Figure 1. However, in this study, the total carotenoids were not detected at all in these samples. The presented results demonstrated that chlorophyll b dominated in all samples in comparison with chlorophylls a. The highest values of chlorophyll b were found in SP 1 (*Spirulina platensis* dry biosample) – 4843  $\mu\text{g/g dw}$ . The values of chlorophyll a was between 589 and 4237  $\mu\text{g/g dw}$ . Near to our results were the findings of Marzorati *et al.* [22] for chlorophyll a chlorophyll b content obtained by CO<sub>2</sub> supercritical extraction from dry *Spirulina* -  $5.7 \pm 0.2 \text{ mg g}^{-1}$  and  $3.4 \pm 0.3 \text{ mg g}^{-1}$  weights, respectively.

The total chlorophyll content was in the range from 1925 to 9080  $\mu\text{g/g dw}$  (Figure 1). The lowest values were detected in lyophilized samples 1925  $\text{mg/g dw}$ . The domination of chlorophyll b was also detected in the infusions [26], while in dry *Spirulina platensis* samples, chlorophyll a were reported to be 0.6  $\text{mg/g dw}$  [5]. In our study, the content of chlorophyll an obtained by ultrasonic extraction at 45 kHz from the dry *Spirulina* samples was near to the previous reports for acetone extraction by sonification (between 2.6–4.7  $\text{mg/g}$ ) [21] and for 24 h extraction (4.91 - 5.27  $\text{mg/g dw}$ ) [6,7]. However, its values were twice times lower than the results obtained by a high-pressure homogenization process at 650 bar with a shear stress of approximately 20,000 (1/s) - 9.85  $\text{mg/g}$  [6]. According to Choi and Lee [7], the optimal ultrasonic extraction conditions for chlorophyll a with ethanol was 20.52 kHz, 32.59 °C, for 4.91 h and yield 17.98  $\text{mg/g}$ . The increase in extraction temperature and time could be caused by the breakdown of the chlorophylls in *Spirulina* at higher temperatures since chlorophylls are known to be heat sensitive [7].



**Figure 1.** Chlorophyll content in three samples *Spirulina platensis*, where SP1- dry biosample, SP2- lyophilized sample and SP3-fresh biomass

In addition, chlorophyll content depends on cultivation media and environmental factors [8,10,19].

3.2. Influence of different extraction conditions on the phycocyanin extraction.

The results for phycocyanin content in water extracts and its purity during different extractions and forms of biomass were summarized in Table 1.

**Table 1.** Comparison of different methods for extraction of phycocyanin from three samples *Spirulina platensis* (mean values  $\pm$ SD).

Sample <sup>1</sup>	Extraction methods	Time, h	Phycocyanin, mg/ml	Purity ratio (A <sub>615/280</sub> )	References	
SP1	Classical extraction	1	2.04	0.25	In this study	
		2	2.47	0.55		
		24	2.94	0.79		
		48	3.97	0.80		
	Ultrasound-assisted extraction at 45 kHz	1	1.13	0.63		
		2	3.08	0.75		
		3	2.90	0.81		
	Ultrasound-assisted extraction at 35 kHz	1	0.37	0.77		
		2	2.35	0.79		
		3	3.25	0.86		
	SP2	Classical extraction	1	0.38		0.22
			2	1.57		0.24
24			1.42	0.26		
48			1.20	0.25		
Ultrasound-assisted extraction at 45 kHz		1	0.18	0.24		
		2	0.94	0.20		
		3	1.16	0.22		
Ultrasound-assisted extraction at 35 kHz		1	0.09	0.55		
		2	1.18	0.32		
		3	1.43	0.22		
SP3		Classical extraction	1	0.50	0.29	
			2	0.53	0.35	
	24		0.53	0.39		
	48		0.32	0.36		
	Ultrasound-assisted extraction at 45 kHz	1	0.39	0.29		
		2	0.57	0.30		
		3	0.68	0.32		
	Ultrasound-assisted extraction at 35 kHz	1	0.24	0.23		
		2	0.50	0.30		
		3	0.65	0.32		
	<i>Spirulina platensis</i> dry powder	Cold maceration 4°C	24	0.57 $\pm$ 0.05	0.149 $\pm$ 0.07	[16]
		Sonification 40 kHz	40 min	0.26 $\pm$ 0.05	0.07 $\pm$ 0.02	
<i>Spirulina platensis</i>	Aqueous extraction	24	0.01 $\pm$ 0.00	Absent	[5]	
			0.34 $\pm$ 0.00			
			3.73 $\pm$ 0.12	0.4-0.5	[27]	

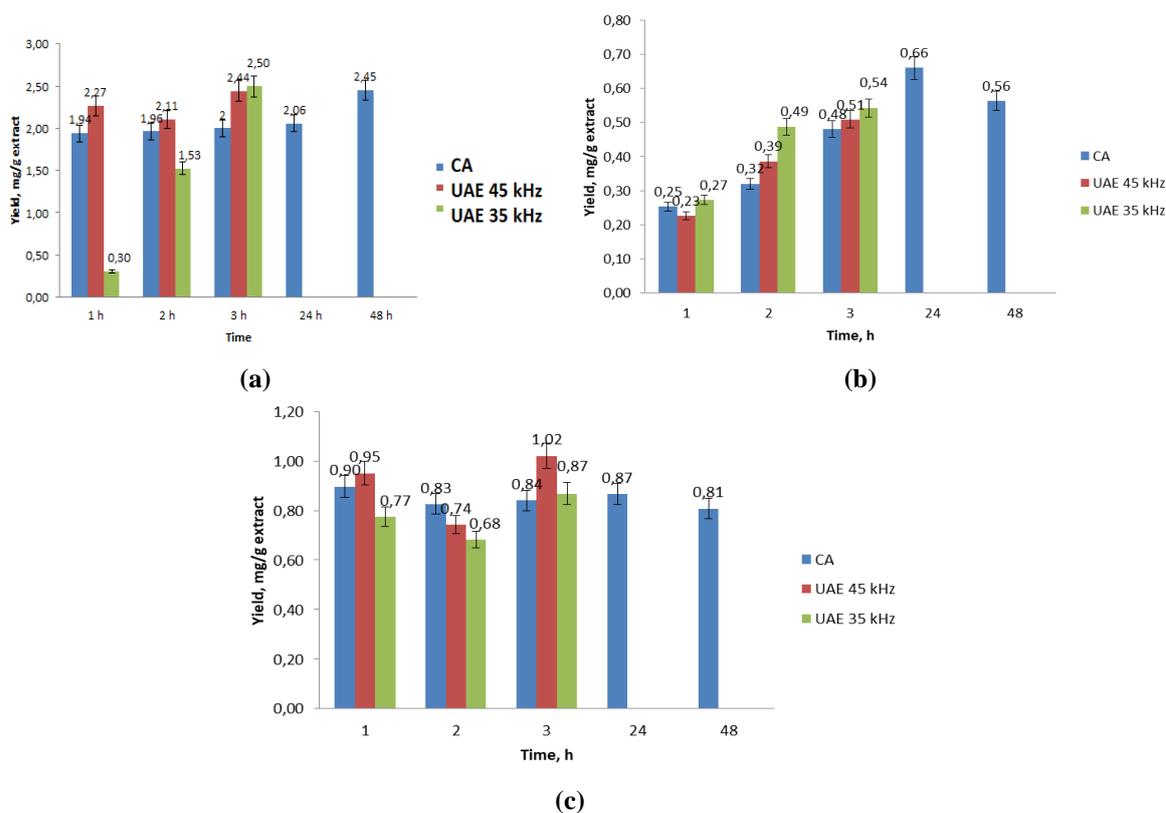
<sup>1</sup> SP1- dry biosample, SP2- lyophilized sample, and SP3-fresh biomass.

Our results for phycocyanin yield from dry *Spirulina* samples reached 3.25 mg/ml and were close to the reports of [14, 27], who reported C-PC yield of 3.27 $\pm$ 0.09 mg/ml. In our case, the yield of phycocyanin from the dry sample after 24 h was 2.94 mg/ml (Table 1) that was near to the values reported by the same extraction conditions [27]. The phycocyanin purity was calculated as the phycocyanin absorbance at 620 nm (A<sub>620</sub>) were divided on the absorbance from aromatic amino acids in all proteins at 280 nm (A<sub>280</sub>)[21]. It was clearly observed that with increasing the extraction time, the concentration and purity of phycocyanin also increased. The purity varied between 0.2 to 0.86. However, the highest yield and good purity (0.86) were obtained from dry *Spirulina* (SP1) by UAE for 3 hours at 30 °C (Table 1).

In lyophilized *Spirulina* (SP2), the purity did not exceed 0.6. Our results were higher than some reports with water as a solvent [5,16]. However, in literature, after extraction with salts, ultrafiltration, dialyzes can be obtained purity 2.2 [16, 17, 20, 21].

According to Rito-Palomares *et al.* [28], phycocyanin preparations with A620/A280 lower than 0.7 are suitable for food grade. However, those with A620/A280 in the range from 0.7 to 3.9 are reagent grade, while those with A620/A280 greater than 4.0 are the analytical grade. Moreover, companies sell C-PC, with purities between 0.5 to 1.5 for use as a food dye, between 1.50 and 2.50 for use as a cosmetic dye, between 2.5 and 3.5 for use as a biomarker, and purity greater than 4 for use in biomedical applications and as a therapeutic agent [29]. Therefore, all obtained extracts in our study at 1 hour are suitable for food-grade purposes. All UAE extracts from dry biosample *S. platensis* were with purities (0.6-0.8) and can be considered as food coloring purposes (Tabel 1).

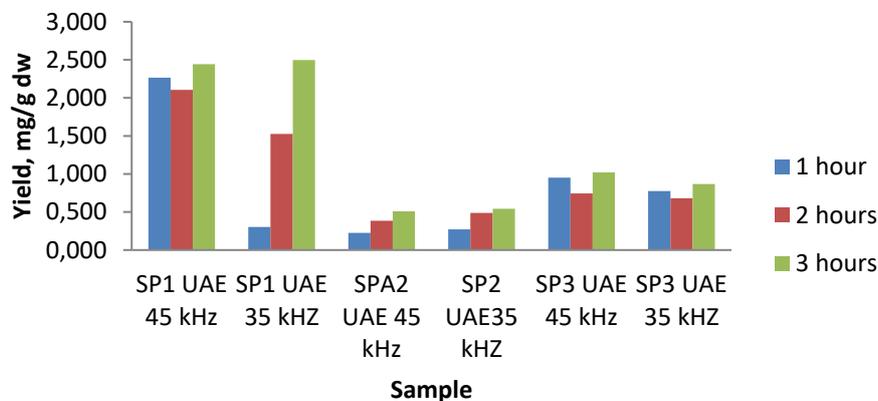
The yield of phycocyanin from different *S. platensis* (dry biosample, lyophilized sample, and fresh biomass) using different extraction techniques were presented in Figure 2.



**Figure 2.** Extraction yield of phycocyanin from different *S. platensis* where (a) SP1- dry biosample, (b)SP2- lyophilized sample and (c) SP3-fresh biomass using different extraction techniques (CA-classical extraction, UAE – ultrasound-assisted extraction)

The highest yield of phycocyanin was obtained from a dry bio *S. platensis* sample (SP1) for 3 hours at 35 kHz - 2.5 mg/g dw (Figure 2 a). For all *S. platensis* samples, the results showed that ultrasonic extraction reduced the time for extraction, as the yield of phycocyanin at first hour was comparable with yields obtained by classical extraction for 24 and 48 hours. The best extraction yield for frozen sample (Figure 2 c) was found at 45 kHz UAE – 1.2 mg/g dw. In case with dry bio and frozen samples (Figure 2 a and c) the extraction of phycocyanin was obtained in good yields for 1 hour at 45 kHz. However, in most of the cases for 3 hours at 35 kHz sonification, the highest yield was detected (Figure 3) -2.4-2.5 mg/g dw with the highest purity 0.8 (Table 1). The optimum yield of 8.25 mg/g dry biomass and purity of 0.6 were obtained using phosphate buffer as the solvent at 37 kHz for 25 minutes [30]. The lowest yields were obtained from lyophilized samples – 0.2 mg/g. Enhanced extraction yields obtained from

UAE can also be attributed to the fact that the cavitation breaks down the cell walls and facilitates the washing out of the cell content [17]. In some previous reports, the extraction using an ultrasonic bath at 50 kHz was the most efficient method. The phycocyanin yields varied between 0.57 mg.g<sup>-1</sup> (sonication) and 43.75 mg.g<sup>-1</sup> with a C-phycocyanin concentration of 0.21 mg.mL (sonication with glass pearls), 56% higher than using freezing and thawing (the method most frequently used) [31].



**Figure 3.** Phycocyanin yield obtained by ultrasound-assisted extraction from three samples *Spirulina platensis*, where SP1- dry biosample, SP2- lyophilized sample and SP3-fresh biomass

In other study, the highest C-phycocyanin concentration of 6 mg ml<sup>-1</sup> was obtained from freeze-dried samples extracted by 0.01 M sodium phosphate buffer (pH 7) and the biomass-solvent ratio of 1:15, with extract purity and yield of 0.6 and 60 mg g<sup>-1</sup> [32]. The content of and purity of phycocyanin depended not only on the extraction procedure[17,31], but also from growing conditions [33, 34].

#### 4. Conclusions

In the current study, chlorophylls and phycocyanin from *Spirulina platensis* in the different physical forms of biomass (dried, lyophilized, and frozen) were determined. Additionally, the purity and yield of phycocyanin using various extraction methods (classical and ultrasonic irradiation at two frequencies) were evaluated. In conclusion, phycocyanin extraction from dry *S. platensis* biomass was found as more suitable than frozen and lyophilized biomasses. In general, the highest yield and purity of phycocyanin were obtained from dry bio *Spirulina platensis* after 3 hours and 35 kHz. Hence, the physical form of the biomass extraction methods should be determined considering the targets in the extraction process.

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#### Conflicts of Interest

The authors declare no conflict of interest.

## References

1. Godlewska, K.; Michalak, I.; Pacyga, P.; Baśladyńska, S.; Chojnacka, K. Potential applications of cyanobacteria: *Spirulina platensis* filtrates and homogenates in agriculture. *World J Microbiol Biotechnol* **2019**, *35*, 1-18, <https://doi.org/10.1007/s11274-019-2653-6>.
2. Shao, W.; Ebaid, R.; El-Sheekh, M.; Abomohra, A.; Eladel, H. Pharmaceutical applications and consequent environmental impacts of *Spirulina* (Arthrospira): An overview. *Grasas Aceites* **2019**, *70*, <https://doi.org/10.3989/gya.0690181>.
3. Michael, A.; Kyewalyanga, M.S.; Lugomela, C.V. Biomass and nutritive value of *Spirulina* (*Arthrospira fusiformis*) cultivated in a cost-effective medium. *Ann Microbiol* **2019**, *69*, 1387–1395, <https://doi.org/10.1007/s13213-019-01520-4>.
4. Reddy, M.C.; Subhashini, J.; Mahipal, S.V.K.; Bhat, V.B.; Reddy, P.S.; Kiranmai, G.; Madyastha, K.M.; Reddanna, P. C-Phycocyanin, a selective cyclooxygenase-2 inhibitor, induces apoptosis in lipopolysaccharide-stimulated RAW 264.7 macrophages. *Biochem Biophys Res Commun*. **2003**, *304*, 385–392, [https://doi.org/10.1016/s0006-291x\(03\)00586-2](https://doi.org/10.1016/s0006-291x(03)00586-2).
5. Gabr, G.A.; El-Sayed, S.M.; Hikal, M.S. Antioxidant Activities of Phycocyanin: A bioactive compound from *Spirulina platensis*. *J Pharm Res Inter*. **2020**, *32*, 73-85, <https://doi.org/10.9734/jpri/2020/v32i230407>.
6. Choi, W.Y.; Lee, H.Y. Enhancement of neuroprotective effects of spirulina platensis extract from a high-pressure homogenization process. *Appl Sci* **2018**, *8*, <https://doi.org/10.3390/app8040634>.
7. Choi, W.Y.; Lee, H.Y. Enhancement of Chlorophyll a Production from marine *Spirulina maxima* by an optimized ultrasonic extraction process. *Appl Sci* **2018**, *8*, <https://doi.org/10.3390/app8010026>.
8. Marrez, D.A.; Naguib, M. M.; Sultan, Y.Y.; Daw, Z.Y.; Higazy, A.M. Impact of culturing media on biomass production and pigments content of *Spirulina platensis*. *Inter J Adv Res* **2013**, *1*, 951-961.
9. Danesi, E.D.G.; Rangel-Yagui, C.O.; Carvalho, J.C.M.; Sato, S. Effect of reducing the light intensity on the growth and production of chlorophyll by *Spirulina platensis*. *Biomass Bioenergy* **2004**, *26*, 329–335, [https://doi.org/10.1016/S0961-9534\(03\)00127-2](https://doi.org/10.1016/S0961-9534(03)00127-2).
10. Soni, R.A.; Sudhakar, K.; Rana, R.S. Comparative study on the growth performance of *Spirulina platensis* on modifying culture media. *Energy Reports* **2019**, *5*, 327-336, <https://doi.org/10.1016/j.egy.2019.02.009>.
11. Pawar, S.T.; Puranik, P.R. C-phycocyanin production by halotolerant cyanobacteria. *PHYKOS—Off. J. Phycol. Soc. India* **2014**, *44*, 25–32.
12. Ruiz-Domínguez, M.C.; Jáuregui, M.; Medina, E.; Jaime, C.; Cerezal, P. Rapid green extractions of c-phycocyanin from *Arthrospira maxima* for functional applications. *Appl Sci* **2019**, *9*, <https://doi.org/10.3390/app9101987>.
13. Puzorjov, A.; McCormick, A.J. Phycobiliproteins from extreme environments and their potential applications, *J Exp Bot* **2020**, *71*, 3827–3842, <https://doi.org/10.1093/jxb/eraa139>.
14. Safaei, M.; Maleki, H.; Soleimanpour, H. Development of a novel method for the purification of C-phycocyanin pigment from a local cyanobacterial strain *Limnothrix* sp. NS01 and evaluation of its anticancer properties. *Sci Rep* **2019**, *9*, <https://doi.org/10.1038/s41598-019-45905-6>.
15. Okechukwu, P.N.; Ekeuku, S.O.; Sharma, M.; Nee, C.P.; Chan, H.K.; Mohamed, N.; Anisah Froemming, G.R. *In vivo* and *in vitro* antidiabetic and antioxidant activity of Spirulina. *Phcog Mag* **2019**, *15*, 17-29.
16. Kamble, S.P.; Gaikar, R.B.; Padalia, R.B.; Shinde, K.D. Extraction and purification of C-phycocyanin from dry Spirulina powder and evaluating its antioxidant, anticoagulation and prevention of DNA damage activity. *J Appl Pharm Sci* **2013**, *3*, 149-153.
17. İlter, I.; Akyıl, S.; Demirel, Z.; Koç, M.; Conk-Dalay, M.; Kaymak-Ertekin, F. Optimization of phycocyanin extraction from *Spirulina platensis* using different techniques. *J Food Compos Anal* **2018**, *70*, 78–88, <https://doi.org/10.1016/j.jfca.2018.04.007>.
18. Rame, R.; Nilawati, D.S.; Novarina, I.H.; Agus, P.; Harjanto, G.R.D. Cell-wall disruption and characterization of phycocyanin from microalgae: *Spirulina platensis* using Catalytic ozonation. *E3S Web Conf*. **2018**, *73*, 1-4, <https://doi.org/10.1051/e3sconf/20187308010>.
19. Abdul-Adel, E.; Saleh, M.M.; Salman, J.M. Production of photosynthesis pigments by *Spirulina platensis* under different NaCl concentrations. *Plant Archives* **2019**, *19*, 3254–3258.
20. Purohit, A.; Kumar, V.; Chownk, M.; Yadav, S.K. Processing-independent extracellular production of high purity C-Phycocyanin from *Spirulina platensis*, *ACS Biomater Sci & Eng* **2019**, *5*, 3237-3245, <https://doi.org/10.1021/acsbiomaterials.9b00370>.
21. Park, W.S.; Kim, H.J.; Li, M. Two Classes of pigments, carotenoids and c-phycocyanin, in spirulina powder and their antioxidant activities. *Molecules* **2018**, *23*, <https://doi.org/10.3390/molecules23082065>.
22. Marzorati, S.; Schievano, A.; Idà, A.; Verotta, L. Carotenoids, chlorophylls and phycocyanin from *Spirulina*: supercritical CO<sub>2</sub> and water extraction methods for added value products cascade. *Green Chem* **2020**, *22*, 187-196, <https://doi.org/10.1039/C9GC03292D>.
23. Lichtenthaler, H.K.; Wellburn, A.R. Determination of total carotenoids and chlorophylls a and b of leaf in different solvents. *Biochem Soc Transact* **1983**, *11*, 591-592, <https://doi.org/10.1042/bst0110591>.
24. Bennett, A.; Bogorad, L. Complementary chromatic adaptation in a filamentous bluegreen alga. *J Cell Biol*. **1973**, *58*, 419–435, <http://dx.doi.org/10.1083/jcb.58.2.419>.

25. Abalde, J. Purification and characterization of phycocyanin from the marine cyanobacterium *Synechococcus* sp. 109201. *Plant Sci.* **1998**, *136*, 109–120, [http://dx.doi.org/10.1016/S0168-9452\(98\)00113-7](http://dx.doi.org/10.1016/S0168-9452(98)00113-7).
26. Milkova Tomova, I.; Minchev, I.; Radusheva, P.; Nikolova, K.; Denev, P.; Buhalova, D.; Bodurova, I. Fizikohimichna charakteristika na infuzii ot morski I sladkovodni vodorasli. Collection of articles from the National Scientific Conference “15 Years of Pharmacy in Medical University – Plovdiv”, SPA Hotel “Orpheus” Devin, Bulgaria, 1–3 June **2018**; pp. 221–226.
27. Silveira, S.T.; Burkert, J.D.M.; Costa, J.A.V.; Burkert, C.A.V.; Kalil, S.J. Optimization of phycocyanin extraction from *Spirulina platensis* using factorial design. *Bioresource Technol.* **2007**, *98*, 1629–1634, <https://doi.org/10.1016/j.biortech.2006.05.050>.
28. Rito-Palomares, M.; Nunez, L.; Amador, D. Practical application of aqueous two-phase systems for the development of a prototype process for c-phycocyanin recovery from *Spirulina maxima*. *J Chem Technol. Biotechnol.* **2001**, *76*, 1273–1280, <https://doi.org/10.1002/jctb.507>.
29. Figueira, F.S.; Moraes, C.C.; Kalil, S.J. C-phycocyanin purification: multiple processes for different applications. *Technology and Biotechnology* **2001**, *76*, 1273–1280, <https://doi.org/10.1590/0104-6632.20180353s20170160>.
30. Dianursanti; Indraputri, C.M.; Taurina, Z. Optimization of phycocyanin extraction from microalgae *Spirulina platensis* by sonication as antioxidant. *AIP Conference Proceedings of 2nd Biomedical Engineering's Recent Progress in Biomaterials, Drugs Development, and Medical Devices* **2018**, *1933*, <https://doi.org/10.1063/1.5023960>.
31. Moraes, C.C.; Sala, L.; Cerveira, G.P.; Kalil, S.J. C-phycocyanin extraction from spirulina platensis wet biomass. *Braz J Chem Engin* **2011**, *28*, 45–49.
32. Panutai, W.; Iamtham, S. Physical extraction and extrusion entrapment of C-phycocyanin from *Arthrospira platensis*, *Journal of King Saud University-Science* **2019**, *31*, 1535–1542, <https://doi.org/10.1016/j.jksus.2018.05.026>.
33. Manirafasha, E.; Murwanashyaka, T.; Ndikubwimana, T.; Ahmed, N.R.; Liu, J.; Lu, Y.; Jing, K. Enhancement of cell growth and phycocyanin production in *Arthrospira (Spirulina) platensis* by metabolic stress and nitrate fed-batch. *Biores Technol.* **2018**, *255*, 293–301, <https://doi.org/10.1016/j.biortech.2017.12.068>.
34. Mehar, J.; Shekh, A.; Nethravathy, M.U.; Sarada, R.; Chauhan, V.; Mudliar, S. Automation of pilot-scale open raceway pond: A case study of CO<sub>2</sub>-fed pH control on *Spirulina* biomass, protein and phycocyanin production. *Journal of CO<sub>2</sub> Utilization* **2019**, *33*, 384–393, <https://doi.org/10.1016/j.jcou.2019.07.006>.