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ORIGINAL PAPER

Enhancing phycocyanin yield from *Spirulina sp.* under salt stress using various extraction methods

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Abstract

Phycocyanin, a blue-coloured pigment, predominantly found and derived from *Spirulina sp.,* has gained researchers' interest due to its vibrant hues and other attractive properties like antioxidant and anti-microbial. However, the lack of reliable and sustainable phycocyanin extraction strategies without compromising the quality has hindered the scaling up of its production processes for commercial purposes. Here in this study, phycocyanin was extracted from wet and dry biomass *Spirulina sp.*, using three different physical cell disruption methods (ultrasonication, homogenization, and freeze–thaw cycles) combined with two different buffers (phosphate buffer and acetate buffer) and water (as control). The result showed that the freeze–thaw method combined with acetate buffer produced the highest yield (25.013 ± 2.572 mg/100 mg) with a purity ratio of 0.806 ± 0.079. Furthermore, when subjected to 30% $_{w/v}$ salt stress, 1.9 times higher phycocyanin yield with a purity ratio of 1.402 ± 0.609 was achieved using the previously optimized extraction method.

Keywords Phycocyanin · Natural pigments · *Spirulina* sp. · Extraction · Physical cell disruption methods · Salt-stress

Introduction

Spirulina sp., a filamentous microalga belonging to the cyanobacterium (blue-green algae) family, has gained recognition as a superfood due to its high protein content (up to 70%), along with other valuable nutrients (Grosshagauer et al. 2020; Soni et al. 2017). Due to its high nutritional value and easy digestibility, this microalgal species has been cultivated extensively for over four decades, as an alternative food source. Moreover, being a source of different photosynthetic pigments like phycocyanin, which has diverse applications in the food- or textile industry, has garnered the interest of researchers (Park et al. 2018).

Phycocyanin is a blue-coloured protein-pigment complex within cyanobacteria that serves as an accessory photosynthetic pigment. This oligomeric pigment consists of two dissimilar subunits, namely α (alpha) and β (beta), with molecular weights of 18 and 20 kilodaltons (kDa), respectively (De Morais et al. 2018; Glazer 1989). The primary function of this complex is to harvest the sunlight in the photosystem II reaction center (Pradeep and Nayak 2019). Beyond its primary function as an accessory photosynthetic pigment, phycocyanin has ecological appeal due to its environmentally friendly nature. This water-soluble pigment has several interesting bio-active properties, including antibacterial, antioxidant, anti-inflammatory, hepatoprotective, and neuroprotective properties, which have been extensively investigated for their possible application as a food additive, colorant, and nutraceutical (Jaeschke et al. 2019; Safari et al. 2020). In addition to these applications, phycocyanin is also used as a fluorescent marker in various scientific disciplines for its broad excitation spectrum and fluorescence with a high quantum yield (Fernández-Rojas et al. 2014). Despite the numerous advantages and increasing market demand, the process of scaling up phycocyanin production for commercial purposes is currently facing multiple obstacles including, limited stability (towards the light, temperature, and pH) (Jespersen et al. 2005; Mishra et al. 2008; Sarada et al. 1999), high extraction and purification cost, and low yield (Furuki et al. 2003; Tomaselli et al. 1997).

The first step in extracting phycocyanin, an intracellular pigment, involves disruption of the cell wall. Four-layered

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cyanobacterial cell wall, which consists of fibril, peptidoglycan, and proteins (like gram-negative bacteria) makes the first step of the extraction process challenging as it impacts both the yield and purity of the extract (Wachda et al. 2019). Furthermore, the susceptibility of phycocyanin to extreme pH, temperature, or light introduces the risk of phycobiliprotein loss during the extraction process, resulting in a low extraction purity. In the search for an efficient phycocyanin extraction method that offers selective release of the target molecule with high energy efficiency various physical-, and chemical- methods have been explored in the last two decades (Pez Jaeschke et al. 2021).

However, both the physical- (such as ball milling and high-pressure techniques) and the chemical methods (such as acid, alkali, salt, or detergent extraction), which are commonly used for phycocyanin extraction, have their limitations. For instance, the physical methods can achieve higher yield by breaking down the cell walls but lack specificity, thereby potentially compromising the quality and purity of the resulting extracts. Whereas the chemical cell disruption method demonstrates better selectivity for target biomolecules, but at the expense of reduced yield and higher extraction costs (Pez Jaeschke et al. 2021). Therefore, to overcome these constraints associated with individual techniques, combinations of physical and chemical methods for phycocyanin extraction have recently been explored by researchers, where various physical methods of cell wall disruption were performed (to release the intracellular phycocyanin) followed by chemical extraction methods (to separate the released phycocyanin from other cellular components) (Wang et al. 2023). For instance, extraction of phycocyanin from *Spirulina platensis* using various physical extraction strategies such as freeze–thaw, ultrasonication, and shearing, combined with phosphate buffer led to a high phycocyanin yield of 9.07% w/w (Yu 2017). Pott (2019) also reported a phycocyanin yield of 170 mg/g using a combination of bead milling with sodium acetate buffer. For instance, a study by Chentir et al. (2018b) reported a phycocyanin yield of 217.18 ± 21.47 mg/g using 20 mM sodium acetate and 50 mM sodium chloride (pH 5.1) buffer, combined with the freeze–thaw method from the dry *Spirulina* sp. biomass. It was observed from the reported literature that most of the extraction methods have used phosphate buffer for enhancing the extraction efficiency, whereas other high-yielding buffers like acetate buffer were less explored.

In addition to developing an optimized extraction technique for achieving better purity with a higher yield, various strategies were explored to boost phycocyanin production. One such strategy involved subjecting the microalgal biomass to different abiotic stress conditions during cultivation. One such study by Chentir et al. (2018a) used a two-stage cultivation system with multiple stress factors to enhance phycocyanin production. The study reported a 16% increase in the phycobiliprotein yield, when cultivated under a low luminous intensity of 10 µmol photons m^{-2} s^{-1} along with media alteration (11.76 g/L of sodium chloride, 0.5 g/L of sodium nitrate, and 2.68 g/L of potassium hydrogen phosphate). Bachchhav et al. (2017) reported a maximum phycocyanin yield of 380 mg from one gram of dry biomass of *Spirulina platensis* using light-emitting diodes (LEDs) as the light source. Another study reported by Devi et al. (2023) produced a maximum yield of 2.04 mg/L/day when the culture was subjected to pH stress (pH below 7). Here it is noteworthy that most of these stress-induced yield enhancement studies have used distilled water or phosphate buffer to extract the phycocyanin, which could be further improved by using other high-yielding extraction buffers. Moreover, lack of a combined optimization study of both the upstream (*i.e.,* enhancing phycocyanin production through the application of various abiotic stresses) and downstream (*i.e.,* increasing extraction efficiency using various physical- and chemical- methods) part of the extraction process, was also observed, which could help generate a higher yield with a higher purity of phycocyanin.

Therefore, to bridge this knowledge gap, the present study employed a singular stress factor approach to augment the production of phycocyanin coupled with an optimized extraction method to achieve a higher yield. The whole study was divided into two sections. Firstly, to optimize the phycocyanin extraction process three distinct physical cell-disruption methods: freeze–thaw cycles, ultrasonication, and homogenization, combined with two high-yielding buffers: 0.1 M phosphate buffer (pH 7), 20 mM sodium acetate and 50 mM sodium chloride buffer (pH 5.1), were assessed and water was used as control. Once the optimal extraction method was determined, the subsequent part of the study aimed to enhance the phycocyanin yield by subjecting the microalgal biomass to different concentrations of salt stress (NaCl). To the best of the author's knowledge, this is the foremost study reported so far to integrate the optimization and enhancement of phycocyanin yield by using both dry and wet biomass of the *Spirulina sp*. This study emerges as the foundation in maximizing the utilization of valuable microalgal biomass by improving the extraction efficiency while maintaining high purity levels, through successful integration of optimized extraction method with selective stress applications.

Materials and methods

Microalgal cultivation

Spirulina sp. cultivation for the extraction process optimization study

Spirulina sp. strain was procured from Dr. M.G.R. Fisheries College and Research Institute, Ponneri, Tamil Nadu, and was cultivated in a modified Zarrouk medium as described by Rajasekaran et al. (2016) with a slight modification in the composition, $NaHCO_3$- 10 g/L, NaCl- 5 g/L, Urea- 0.25 g/L, K_2SO_4- 0.625 g/L, $MgSO_4$- 0.16 g/L, phosphoric acid- 2.5 mL and $FeSO_4$ solution- 2.5 mL. The phosphoric acid stock was prepared by adding 2.5 mL to 100 ml of deionised water and for $FeSO_4$ stock, 0.1 g of $FeSO_4$ was added to a 10 ml of HCl solution, where water and HCl were added in a 1:1(v/v) ratio. The microalgal strain was cultivated at an ambient temperature of 30 ± 4 ℃ with a 12-h photoperiod and light intensity of 60 µmol photons m^{-2} s^{-1}. The pH of the medium was maintained between 8.8 and 11 throughout the cultivation period. The culture was initially inoculated in a 1 L Erlenmeyer flask. For checking the microalgal growth absorbance reading was taken at 680 nm on every fourth day and a growth curve was plotted. After reaching a stationary growth phase, a plankton mesh of 200–300 µm was used to harvest biomass, which was weighed and divided into two equal parts: one was the wet biomass, and the other was freeze-dried to obtain dried biomass (≈10% of wet biomass was constantly obtained). Later, the extraction process optimization study was carried out from both the wet and dry biomasses.

Spirulina sp. cultivation under various salt stresses for phycocyanin yield enhancement study

Spirulina sp. culture was acclimatized to the salt-stressed (up to 30% $_{w/v}$) environment through repeated sub-culturing, and stable growth was achieved after four rounds of sub-culturing. For the phycocyanin yield enhancement study, the microalgal strain was cultivated in the previously mentioned modified Zarrouk medium with an additional NaCl concentration of 0.5 g/L (10% $_{w/v}$), 1.0 g/L (20% $_{w/v}$), and 1.5 g/L (30% $_{w/v}$), in 1 L Erlenmeyer flasks. Other environmental conditions like photoperiod, light intensity, temperature, and pH were kept same as in the previous studies. For checking the microalgal growth, absorbance reading was taken at 680 nm on every fourth day and was compared with the non-stressed. After achieving a stationary growth phase, cultures were harvested using plankton mesh and freeze-dried for phycocyanin extraction.

Extraction of phycocyanin

Preparation of buffers

Two buffers were used for the phycocyanin extraction: (i) 20 mM sodium acetate and 50 mM NaCl buffer (pH 5.1) (acetate buffer) prepared based on the protocol by Chentir et al. (2018b) and (ii) 0.01 M phosphate buffer (pH 7) (phosphate buffer) was prepared based on the protocol by Pan-utai et al. (2018) and water was used as control solvent.

Extraction of crude phycocyanin

The phycocyanin was extracted from the *Spirulina sp*. using three physical cell disruption techniques: ultrasonication, freeze–thaw, and homogenization. For the extraction, the biomass: solvent ratio was kept at 1:25, and all three solvents (acetate buffer, phosphate buffer, and water) were taken in the same ratio. For ease of calculation, approximately 1 g (≈100 mg of dry biomass) of wet biomass and 100 mg of dry biomass were taken. Extraction from both the wet and the dry microalgal biomass was done simultaneously (Fig. 1), in triplicates.

For the freeze–thaw cycle, biomass was frozen for three hours at – 20 ℃, followed by thawing the sample for 10 min at room temperature (Chentir et al. 2018b). This whole cycle was repeated three times, followed by centrifugation

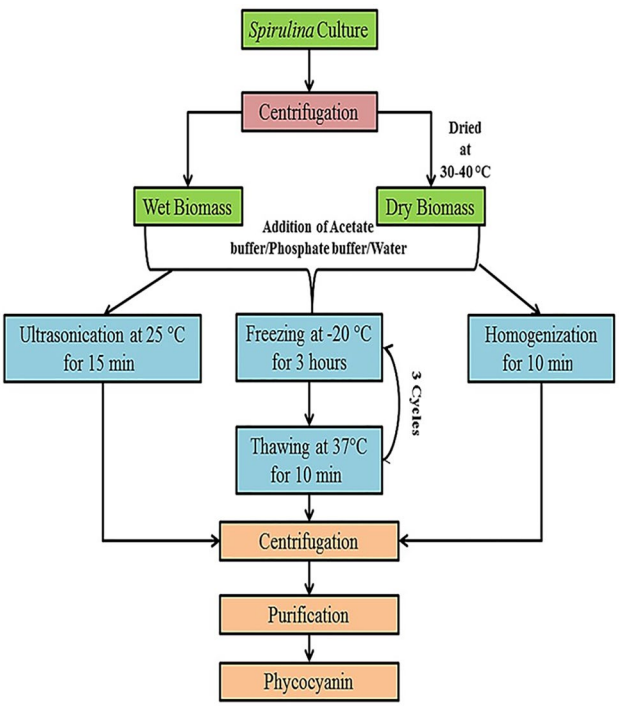

Fig. 1 Workflow for the extraction of phycocyanin from the *Spirulina sp*

at 8000 rpm for 20 min at 4 ℃. For further analysis, the supernatant was collected and stored.

For the ultrasonication method, sonication was done for 15 min at 25 ℃ in an ultrasonicator bath manufactured by Labman Scientific Instruments followed by 30 min of centrifugation at 4500 rpm at 4 ℃ (Pan-utai et al. 2018). For further analysis, the supernatant was collected and stored.

For the homogenization method, the samples were homogenized for 10 min using a homogenizer probe manufactured by Remi Electrotechnik Limited, followed by 10 min of centrifugation at 6000 rpm at 4 ℃. For further analysis, the supernatant was collected and stored (Chentir et al. 2018b).

Purification of phycocyanin

Phycocyanin was purified with activated charcoal following the protocol developed by Aoki et al. (2021) with a few modifications. The supernatant from the previous experiment (consisting of phycocyanin along with other impurities) was taken and 70 g/L $_{w/v}$ activated charcoal was added at room temperature. After 20 min, the phycocyanin-charcoal mixture was centrifuged at 6000 rpm for 10 min. The supernatant was collected, filter sterilized by 0.45µ filter, and stored at – 20 ℃ for further analysis.

Characterization of the extracted phycocyanin

Spectroscopic analysis

The absorbance values from a UV spectrophotometer at 280 nm, 620 nm, and 652 nm were used to calculate the concentration, purity, and yield of the phycocyanin by Eqs. 1, 2, 3 (Berns and MacColl 1989)

$$\text{Concentration of phycocyanin (mg/mL)} = \frac{A_{620} - 0.474 * A_{652}}{5.34} \quad (1)$$

$$\text{Purity of phycocyanin} = \frac{A_{620}}{A_{280}} \quad (2)$$

$$\text{Phycocyanin yield(mg/100mg)} = \frac{\text{Concentration of phycocyanin} * \text{Volume of solvent}}{\text{Biomass weight}} \quad (3)$$

The wavelength scan for the phycocyanin was also taken to verify the purity.

FTIR analysis

The major functional groups present in the sample (phycocyanin) were identified using FTIR manufactured by Microteknik Pvt.Ltd and the results were compared to standard phycocyanin data. The freeze-dried phycocyanin samples were mixed with potassium bromide (KBr) and converted into pellets for the FTIR analysis, KBr was used as the control. The spectra were scanned between the range of 400–4000 cm^{-1} at the resolution of 4 cm^{-1}.

Colour analysis

The colorimetric analysis of the unstressed and stressed *Spirulina sp.* as well as the phycocyanin extracted from them was done using a refractive spectrocolorimeter manufactured by X-rite spectrometer CI7600. The color measurements were expressed by lightness (L*) which includes the chromaticity parameters a* from green (a–) to red (a +) and b* from blue (b–) to yellow (b +).

Statistical analysis

All the extraction experiments were performed in triplicates and the yield and purity ratio were expressed as means ± standard deviation. Sigmastat software was used for the statistical analysis. The comparison was done between the extraction buffers, and methods and between the extracted yield and the purity with a significance value of 0.01 ($p < 0.01$), 0.05 ($p < 0.05$), and 0.001 ($p < 0.001$).

Results and discussion

Growth analysis of unstressed *Spirulina sp.*

Spirulina sp. was cultivated in modified Zarrouk's media for 32 days. Microalgal growth was evaluated from the increase in algal concentration over time, which was measured by the increasing optical density (OD). After taking the culture's OD at 680 nm on every fourth day, a 4-day lag phase, followed by a log phase was observed (see the supplementary file 1). After 24 days of cultivation, a stationary growth phase was observed. After 32 days of cultivation, the microalgal biomass was harvested using plankton

mesh, and a biomass yield of 1.021 ± 0.108 g/L (wet wt.) and 0.120 ± 0.010 g/L (dry wt.) [the biomass contains approximately 90% of moisture] was constantly obtained, which was further used for the extraction experiment. At each stage of growth, microscopic analysis revealed an increase in the number of spirals and the length of the *Spirulina sp.*

Extraction of phycocyanin and its optimization

For the optimization study, phycocyanin extraction from *Spirulina sp*. was carried out using three different buffers along with three different extraction methods, from the dry and wet biomasses. Physical parameters like concentration, yield, and purity were calculated using previously mentioned Eqs. 1, 2, and 3. The highest phycocyanin yield obtained was 25.013 ± 2.572 mg/100 mg with a purity ratio of 0.806 ± 0.079, obtained by freeze-thawing the dried biomass in a buffer solution of 20 mM sodium acetate and 50 mM sodium chloride (pH 5.1). This yield was higher than the previously reported yield of 217.18 ± 21.47 mg/g with a purity ratio of 0.77 ± 0.08 by Chentir et al. (2018b) from dried biomass of *Spirulina sp.* using the acetate buffer. Another similar study by Pan-utai et al. (2018) reported a yield of 105.31 ± 7.63 mg/g with a purity ratio of 0.62 ± 0.03 from the dried *Spirulina sp*. biomass using phosphate buffer.

The phycocyanin yield obtained from the wet biomass of *Spirulina sp*. was reported to be 146 ± 0.265 mg/g with a purity ratio of 3.2 by using phosphate buffer (Saran et al. 2016). Another similar study by Moraes et al. (2011) reported a phycocyanin yield of 43.75 mg/g with a concentration of 0.21 mg/mL from the wet *Spirulina platensis* biomass. When cultivated under normal conditions, *Spirulina sp.* contains up to 25% $_{w/w}$ of phycocyanin of its total biomass weight (Dianursanti et al. 2018). In the present study, the freeze–thaw extraction method was able to achieve the utmost extraction efficiency for the case of dry biomass (Fig. 2) whereas, for wet biomass, only 10% $_{w/w}$ of phycocyanin of the total biomass weight was obtained with the same extraction technique. Overall exhaustive data on comparing the yield, and purity ratio of the extracted phycocyanin from the wet and dry biomass is depicted in Table 1.

Purification of phycocyanin

Depending on the purity ratio phycocyanin can be used for different purposes. For example, a purity ratio (≥ 0.7) is usually a food grade, whereas a purity ratio (≤ 3.9) is a reagent grade, and (> 4.0) is the analytical grade phycocyanin (Gorgich et al. 2020). Due to this reason, after extraction generally, a series of purification steps are followed to increase the purity of the phycocyanin. For example, one study by

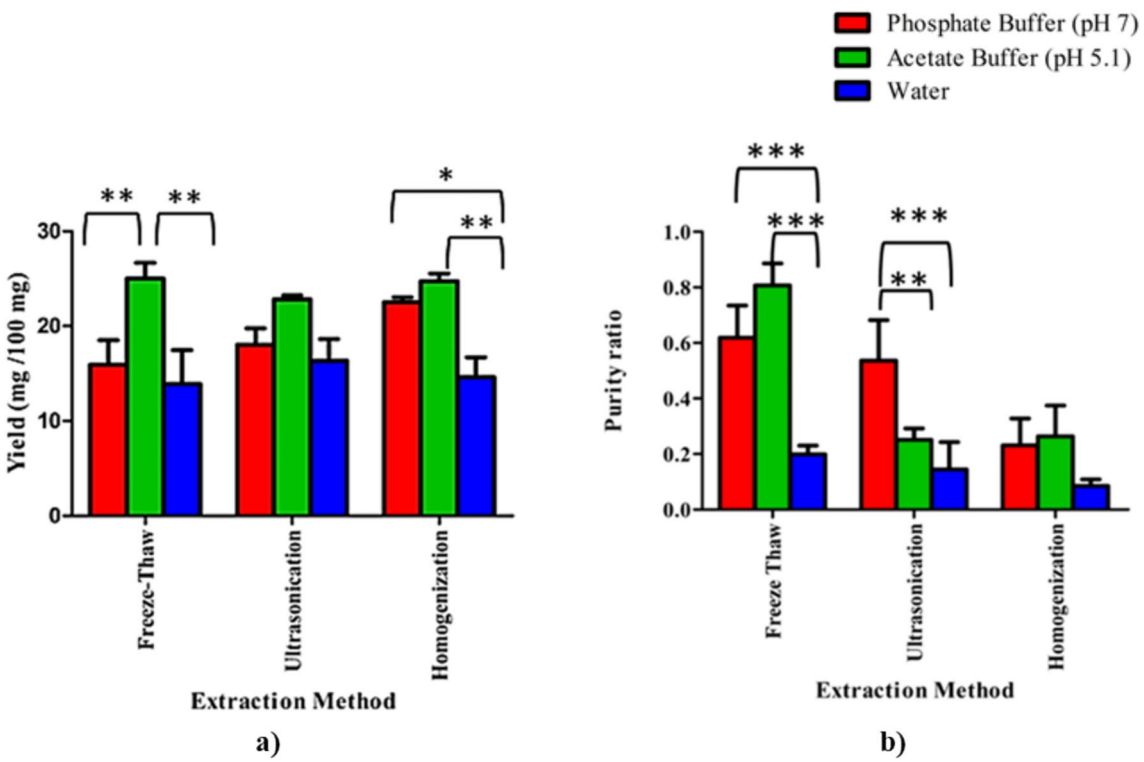

Fig. 2 Comparison of (**a**) yield, (**b**) purity ratio of phycocyanin from dry *Spirulina sp.* (*P < 0.05, **P < 0.01, ***P < 0.001)

Table 1 The yield and purity of phycocyanin extracted from dry and wet *Spirulina sp.* biomass

Method	Type of biomass	Buffer /solvent	Purity ratio	Yield (mg/100 mg)
Freeze–Thaw	Dry	Phosphate buffer$_{(pH\ 7)}$	0.618 ± 0.103	15.933 ± 3.997
		Acetate buffer$_{(pH\ 5.1)}$	0.806 ± 0.079	25.013 ± 2.572
		Water	0.198 ± 0.031	13.924 ± 5.500
	Wet	Phosphate buffer$_{(pH\ 7)}$	0.552 ± 0.140	4.557 ± 1.479
		Acetate buffer$_{(pH\ 5.1)}$	0.992 ± 0.347	6.481 ± 1.272
		Water	0.359 ± 0.152	4.508 ± 1.530
Ultrasonication	Dry	Phosphate buffer$_{(pH\ 7)}$	0.536 ± 0.145	18.036 ± 2.662
		Acetate buffer$_{(pH\ 5.1)}$	0.251 ± 0.036	22.817 ± 0.668
		Water	0.144 ± 0.087	16.331 ± 3.584
	Wet	Phosphate buffer($_{pH\ 7)}$	0.733 ± 0.268	5.171 ± 1.149
		Acetate buffer$_{(pH\ 5.1)}$	0.685 ± 0.189	6.894 ± 1.316
		Water	0.270 ± 0.028	5.649 ± 1.689
Homogenization	Dry	Phosphate buffer$_{(pH\ 7)}$	0.231 ± 0.086	20.550 ± 0.749
		Acetate buffer$_{(pH\ 5.1)}$	0.263 ± 0.098	24.741 ± 1.247
		Water	0.085 ± 0.021	14.612 ± 3.240
	Wet	Phosphate buffer$_{(pH\ 7)}$	0.118 ± 0.007	3.193 ± 1.514
		Acetate buffer$_{(pH\ 5.1)}$	0.096 ± 0.011	5.635 ± 1.078
		Water	0.084 ± 0.005	3.607 ± 1.902

Minkova et al. (2007) used ammonium sulfate precipitation and column chromatography for phycocyanin purification, where a purity ratio of 0.87 ± 0.08 was achieved. The same purification strategy was followed by Bhaskar et al. (2005) in which the purity ratio of the phycocyanin was around 0.97. In another study by Pan-utai et al. (2022), ultrafiltration was used to purify the phycocyanin, and a purity ratio of 0.611 ± 0.10 was obtained. A study by (Chen et al. 2018) used an integrated approach for the extraction and purification of phycocyanin using a stirred fluidized bed combined with hydrophobic interaction chromatography and reached a maximum purity ratio of 3.9. A similar study was performed with a few alterations by the same research group, the hydrophobic interaction chromatography was replaced with ion exchange chromatography and reached a maximum purity ratio of 3.0 (Chen et al. 2019). Liao et al. (2011) used a series of purification steps which included purification by chitosan followed by activated charcoal, 10 mM potassium phosphate buffer, ultrafiltration, and ion-exchange chromatography, resulting in a purity ratio of around 4.3. The maximum purity ratio of phycocyanin reported so far is 6.17 ± 0.075, where purification was done by ammonium sulfate precipitation and column chromatography (Purohit et al. 2019)**.**

For purity enhancement, the use of activated charcoal, as a low-cost alternative to chromatography techniques was also reported in a few studies. A study conducted by Aoki et al. (2021) used activated charcoal to purify the phycocyanin extracted from the two *Cyanobacterium* strains: *Pseudanabaena sp.* ABRG5-3 and *Limnothrix sp.* SK1-2-1, where a purity ratio of 3.10 and 2.14 was obtained from the two respective microalgal strains. In another study, Shaochen (2016) used activated charcoal to purify phycocyanin from the *Spirulina sp*., and the purity ratio obtained was 0.987. In the present study, extracted crude phycocyanin was purified using the activated charcoal purification method, which resulted in a maximum purity of 0.806 ± 0.079. A wavelength scan of the purified sample showed only two major peaks at 620 nm and 260 nm (see supplementary file 2), which further confirms the purity of the phycocyanin (Aoki et al. 2021). Therefore, it can be concluded that the result obtained after the purifying process, was a food-grade one, and this can further be enhanced by combining the method with other low-cost purification techniques.

Physicochemical characterization of phycocyanin extracted from unstressed *Spirulina sp.*

FTIR analysis of the extracted phycocyanin by freeze–thaw method with acetate and phosphate buffer was done (see supplementary file 3). The peaks in the FTIR were analyzed and compared with the standard peak of phycocyanin documented in the literature. At 1654 cm^{-1} a peak was observed, which is a major peak reported for phycocyanin (Seyed Yagoubi 2017; Seyed Yagoubi et al. 2018; Sonia & Ravindran 2019). Other predominant peaks at 563 cm^{-1}, 1045 cm^{-1}, and 1028 cm^{-1} correspond to the functional groups of nitriles and ether, respectively (El-Naggar et al. 2017). A broad peak at 3310 cm^{-1} which corresponds to the amine group, was obtained in both the acetate and phosphate

buffer samples (Seyed Yagoubi 2017). Other than these several sharp and narrow peaks at 2924 cm^{-1}, 1654 $cm^{-1}$and 534 cm^{-1}, were observed in the FITR, which corresponds to C–H, C–O, and C–C–N groups, respectively (Sonia and Ravindran 2019). Overall, the intensity of the peaks in the phycocyanin sample extracted with phosphate buffer was found to be higher than the acetate ones which indicates a higher bond intensity in the acetate buffer sample.

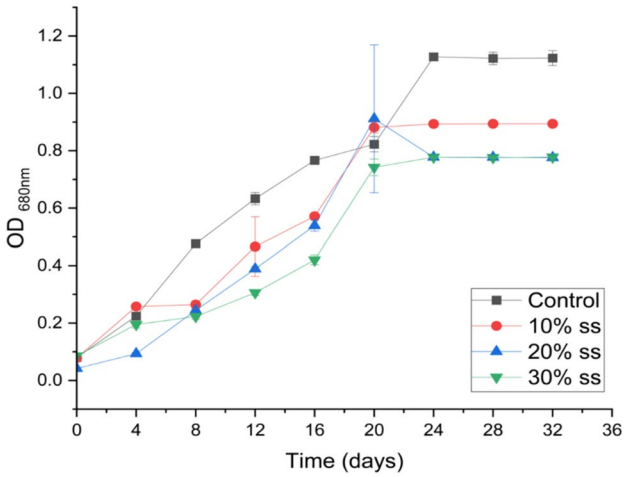

Fig. 3 Growth curve of the *Spirulina sp.* cultured in un-stressed (control) and salt-stressed (ss) conditions

Growth analysis of the salt-stressed *Spirulina sp.*

For the yield enhancement study, *Spirulina sp.* was subjected to salt stress at varying concentrations from 10 to 30% $_{w/v}$. The culture was first acclimatized to the salt-stressed environment through repeated sub-culturing, and stable growth was achieved only after four rounds of sub-culturing. All the stressed cultures reached a stationary growth phase after 20 days of cultivation (Fig. 3). Compared to the stressed culture, the control (unstressed culture) showed a more prolonged log phase. This indicates that for phycocyanin extraction, *Spirulina sp.* cultivated under salt-stressed conditions can be harvested earlier than the unstressed culture, as it showed an early stationary phase. In terms of biomass, the highest yield (0.921 ± 0.108 g/L) was obtained from the control or the unstressed culture. Among all the stressed cultures, 20% $_{w/v}$ salt stress gave the highest biomass yield of 0.626 ± 0.020 g/L followed by 10% $_{w/v}$ and 30% $_{w/v}$ stressed

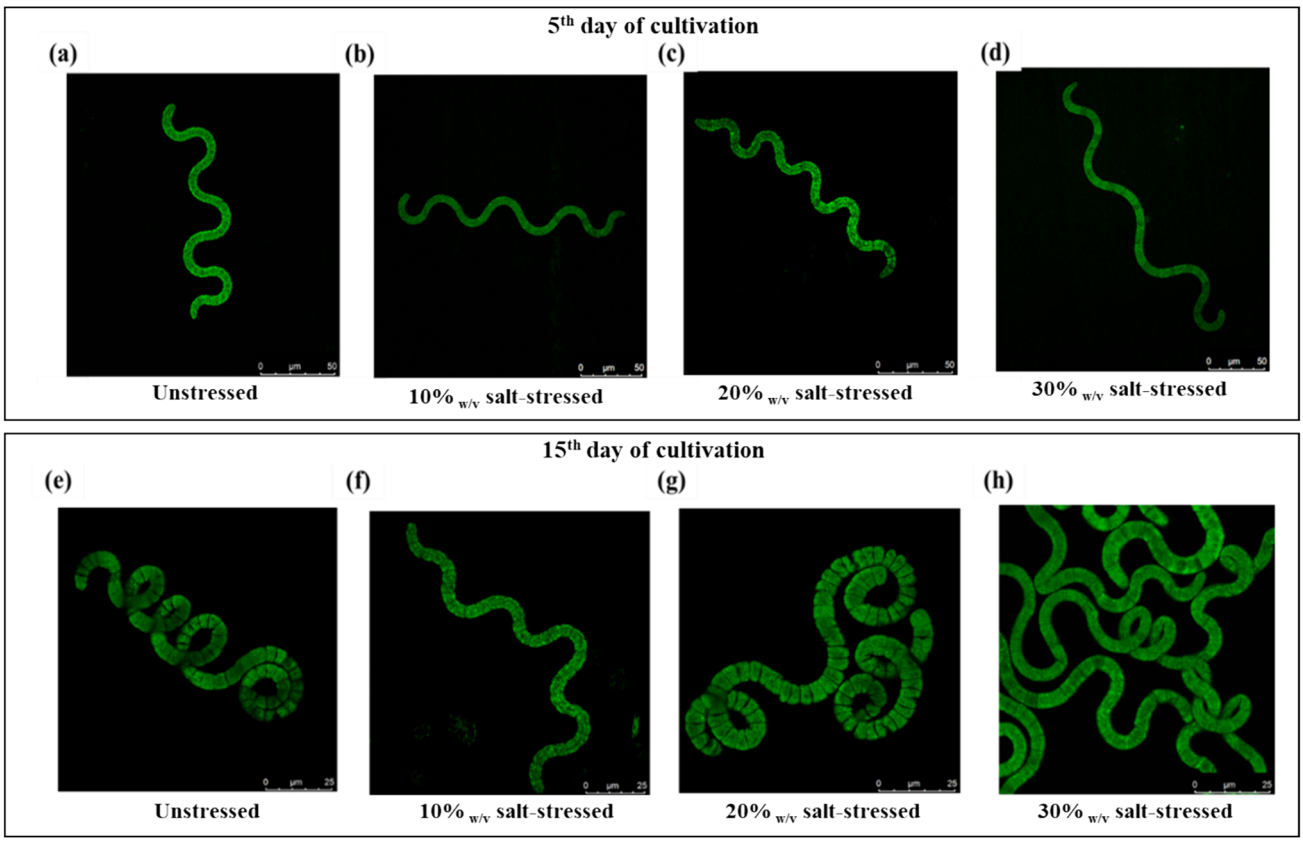

Fig. 4 Confocal microscopic images of unstressed and various salt-stressed *Spirulina sp.* at different cultivation periods

Table 2 Hue analysis of the unstressed and the salt-stressed *Spirulina sp*

Spirulina sp. Sample	L*	a*	b*	hº
Unstressed	45.27 ± 0.01	− 15.80 ± 0.12	17.56 ± 0.05	123.91 ± 0.07
10% salt-stressed	48.01 ± 0.03	− 13.27 ± 0.02	13.81 ± 0.04	133.84 ± 0.14
20% salt-stressed	41.88 ± 0.02	− 14.72 ± 0.09	17.44 ± 0.01	130.18 ± 0.21
30% salt-stressed	50.17 ± 0.01	− 11.11 ± 0.02	10.32 ± 0.02	137.11 ± 0.10

cultures with a biomass yield of 0.528 ± 0.026 g/L and 0.514 ± 0.023 g/L, respectively.

Confocal images of the cultures taken at two different times: 5 days and 15 days (Fig. 4), showed that the fragments within the *Spirulina* culture were visible during the 15th day of cultivation. It was also observed from the confocal microscopic images that as the stress percentage increases, *Spirulina sp.* loses its typical spiral coil-like structure, which was very predominant in the unstressed one.

Colorimetric analysis of the unstressed and the salt-stressed *Spirulina sp.*

The colorimeter's color index interprets the L* value as a measure of brightness. Positive and negative a* values signify the extent of redness and greenness, respectively, while positive and negative b* values indicate the degree of yellowness and blueness in the sample, and hº is the hue angle of the sample. The angles from 100-150º indicate the depth of greenness in the sample and angles from 160-270º indicate the depth of blueness in the sample (Zhuxin et al. 2023). The colorimetric analysis of the *Spirulina sp.* (Table 2) revealed that the 30% $_{w/v}$ salt-stressed *Spirulina sp.* has the highest L* value. Moreover, the lowest negative a* value of the sample indicates its deeper green color. The other two salt-stressed *Spirulina sp.* (*i.e.,* 10% $_{w/v}$ and 20% $_{w/v}$) also showed a similar trend to the 30% $_{w/v}$ salt-stressed one: higher L* and hue (hº) values and lower negative a* values indicating their deeper and brighter green color compared to the unstressed one. All these results indicate the fact that salt-stressed *Spirulina sp.* contains a higher amount of photosynthetic pigment along with phycocyanin, compared to the non-stressed one.

Extraction and purification of phycocyanin from the salt-stressed *Spirulina sp.*

Phycocyanin yield enhancement by subjecting the *Spirulina sp.* to various abiotic stresses like temperature stress (Chentir et al. 2018b), light stress (Bachchhav et al. 2017), and media alteration (Chentir et al. 2018b), has been reported in several works of literature over the last decade. In the present study media alteration approach was taken, where the different concentration of NaCl (i.e., 10–30% $_{w/v}$) was used for the phycocyanin yield enhancement. Phycocyanin was extracted from the salt-stressed *Spirulina sp.* using the previously optimized extraction method (*i.e.,* freeze–thaw

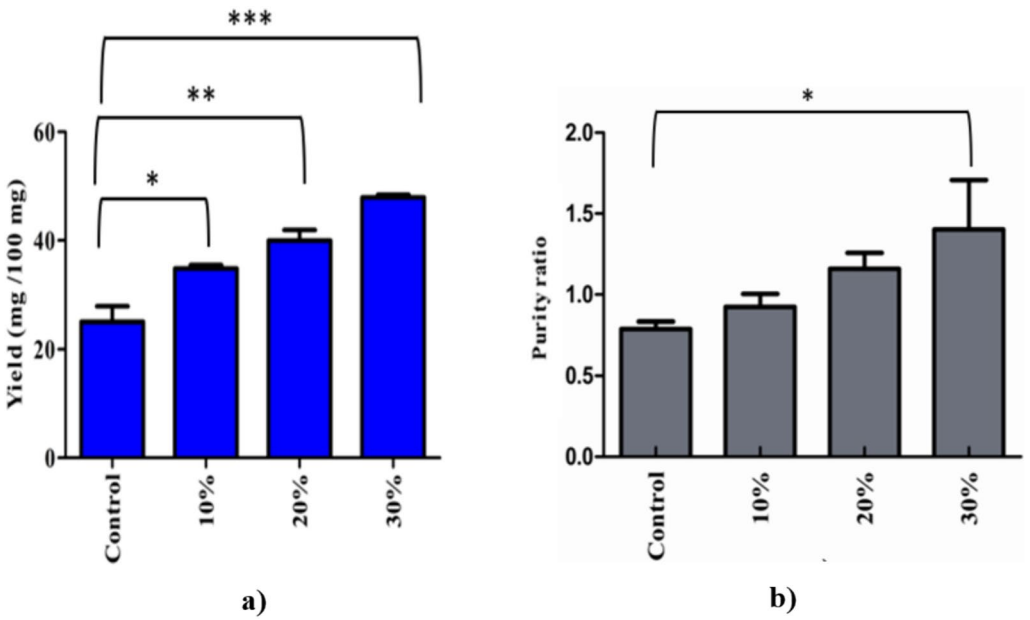

Fig. 5 **a** yield and **b** purity ratio of the phycocyanin extracted from unstressed and salt-stressed *Spirulina sp*

method combined with the acetate buffer). The impurities in the crude phycocyanin have been adsorbed using activated charcoal during the process of purification. The highest phycocyanin yield of 47.856 ± 0.069 mg/100 mg with a purity of 1.402 ± 0.609 was obtained from the 30% $_{w/v}$ stressed *Spirulina sp*. culture, the yield was 1.9 folds greater than that of the control (i.e., unstressed culture). Recently in a study, Lauceri et al. (2022) used a two-step extraction process in which first the wet biomass of *Spirulina* was subjected to cell lysis by the addition of ammonium sulfate followed by ultrasonication. After the cell lysis, for extraction of the intracellular phycocyanin, the biomass was suspended in different extraction buffer systems, to obtain a yield of about 20–25% $_{w/w}$. Another study by Ebrahimi et al. (2023) focuses mainly on the extraction and purification of phycocyanin and showed that subjecting the *Spirulina* to a thermomixer along with the extraction buffer, followed by an aqueous two-phase purification system was able to achieve 90% phycocyanin recovery. However, phycocyanin purification using Pluronic copolymers and salts increases the overall production cost even though it increases purity. A study by Bachchhav et al. (2017) reportedly used different LEDs and enhanced the phycocyanin content within the *Spirulina platensis* with the highest yield of about 380 mg/g of dry biomass. Another study by Lee et al. (2016) has also experimented with the enhancement of phycocyanin content within Spirulina sp. using LEDs (a two-stage cultivation strategy was followed) and the final yield of phycocyanin was enhanced up to 353 mg/g of *Spirulina* biomass. These studies along with other research works involving enhancing phycocyanin yield using LEDs are cited in Hsieh-Lo et al. (2019). Apart from giving stress through light, other possibilities were also explored which include a change in cultivation strategy, Xie et al. (2015) used a fed-batch strategy for enhancing both phycocyanin production as well as enhancing cell growth of *Spirulina platensis*. It was observed that the productivity of phycocyanin was high at almost 94.8 mg/L/d and the phycocyanin content was measured to be 16.1%. One of the recently published articles used salt stress along with supplementation of glycine betaine and achieved 22.31% phycocyanin content with productivity of 57.63 mg/L/d (Yu et al. 2024). Only using salt stress another reported study has achieved almost 131.85 mg/g DW of phycocyanin (Prabhath et al. 2019). A significant increase in phycocyanin of about 180% of the control was observed in 200 mM NaCl-stressed *Spirulina* SP972 (Liu et al. 2016). Compared to these studies, the present study obtained a phycocyanin yield of about 25% $_{w/w}$ by subjecting the dried *Spirulina* biomass to the freeze–thaw method in acetate buffer which was further enhanced to 47.856 ± 0.069 mg/100 mg (about 47% $_{w/w}$) when the biomass was subjected to salt stress. Concerning purity, Lauceri et al. (2022) reported a purity ratio of > 3, and Ebrahimi et al. (2023) with a purity ratio of 5.9. Here it is worth mentioning that in food industry-based applications,

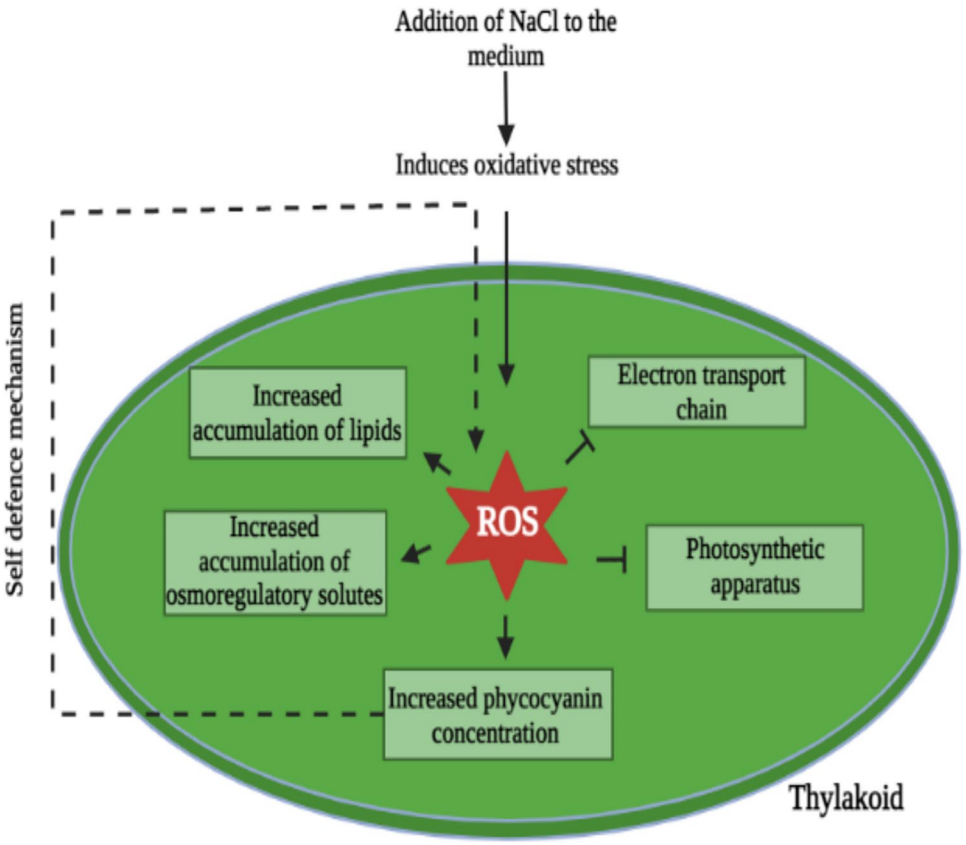

Fig. 6 Possible mechanism for enhancing the phycocyanin pigment by salt stress within the *Spirulina sp*

any purity ratio above 0.7 is considered acceptable. In the present study phycocyanin obtained after the purification with activated charcoal (which is a low-cost purification method), meets this criterion, making it suitable for food industrial applications.

Upon comparing the yield and purity of the extracted phycocyanin from the stressed culture with the control (Fig. 5), it was very evident that purity, as well as the yield, was higher in the stressed culture compared to the unstressed one. This yield enhancement can be explained using the concept of ROS (reactive oxygen species) generation. Salt stress produces ROS (reactive oxygen species) which inhibits the electron transport chain (ETC) process and arrests photosynthesis (Fig. 6). To cope with this problem, microalgal cells enhance the production of phycocyanin (an accessory photosynthetic pigment) which aids in photosynthesis, works as an antioxidant along with other osmoregulatory solutes, and scavenges ROS present inside the cell (Kaur et al. 2022; Yang et al. 2020).

There was a significant difference between the phycocyanin yield, purity, and concentration among *Spirulina sp.* cultivated under various salt stress concentrations, all the parameters increased as the concentration of the NaCl increased. There was a notable increase in the yield of phycocyanin which was about 34.808 ± 0.651 mg/100 mg from 10% $_{w/v}$ salt-stressed *Spirulina sp.*, followed by 39.973 ± 1.953 mg/100 mg from 20% $_{w/v}$ salt-stressed *Spirulina sp.* and the yield was about 47.856 ± 0.069 mg/100 mg at 30% $_{w/v}$ salt-stress *Spirulina sp.* The purity ratio of phycocyanin extracted from 10% $_{w/v}$ salt-stressed *Spirulina sp.* was 0.924 ± 0.159, 20% $_{w/v}$ salt-stressed *Spirulina sp.* was 1.158 ± 0.196 and 30% $_{w/v}$ salt-stressed *Spirulina sp.* was 1.402 ± 0.609. It can further be enhanced using a combination of activated charcoal and other polymers (such as chitosan, and polyethylene glycol) as a column in column chromatography. The enhancement in the purification strategy will enhance the yield of phycocyanin as well.

Physicochemical characterization of phycocyanin extracted from salt-stressed *Spirulina sp.*

The FTIR analysis was done for the phycocyanin extracted from salt-stressed and unstressed *Spirulina sp.* to compare the similarity and the difference in their functional groups for the given salt stress (Fig. 7). Most of the peaks obtained were similar for both the phycocyanin extracted salt-stressed and the unstressed *Spirulina sp.* like one major peak found at 3310 cm^{-1} corresponding to the alcohol group, 1654 cm^{-1} and 2498 cm^{-1} both corresponding to the carboxyl stretching and 543 cm^{-1} corresponding to nitrile group. Most of the major peaks were observed in

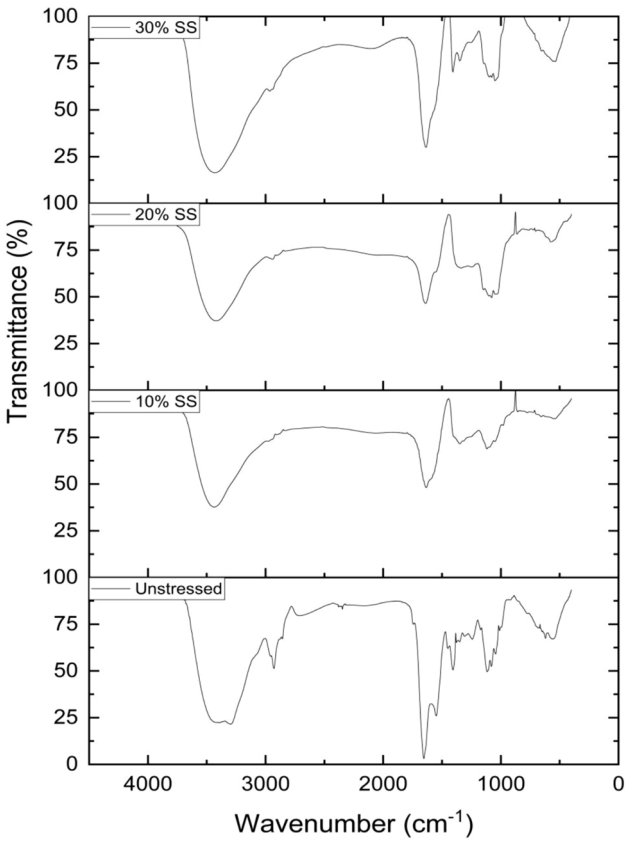

Fig. 7 FTIR spectra of phycocyanin extracted from the unstressed and the salt-stressed (SS) *Spirulina sp*

all the samples irrespective of the phycocyanin extracted from the salt-stressed or the unstressed *Spirulina sp.* However, the intensity of the peaks varied, as the salt-stress percentage increased, the transmittance was higher in the phycocyanin extracted from the 30% $_{w/v}$ salt-stress which shows the higher bond intensity in the sample.

Colorimetric analysis of phycocyanin extracted from unstressed and salt-stressed *Spirulina sp.*

The colorimetric analysis was done for the phycocyanin extracted from the unstressed and the salt-stressed *Spirulina sp.* (see Table 3). The L* of the phycocyanin extracted from unstressed *Spirulina sp.* was lower compared to the stressed ones. The higher negative a* and negative b* values of the phycocyanin suggest that the phycocyanin extracted from the unstressed *Spirulina sp.* has a lighter shade of green and blue. In contrast, the lowest negative a* and negative b* values of the phycocyanin extracted from the 30% $_{w/v}$ salt-stressed *Spirulina sp.* suggest that the samples possess a deeper shade of blue and green, indicating a higher concentration and better quality phycocyanin. The results obtained from colorimetric analysis of the extracted phycocyanin from different

Table 3 Colorimetric analysis of phycocyanin extracted from unstressed and salt-stressed *Spirulina sp*

Phycocyanin sample	L*	a*	b*	h°
Unstressed	20.47 ± 0.03	− 15.87 ± 0.05	− 29.40 ± 0.04	231.42 ± 0.13
10% salt-stressed	12.03 ± 0.02	− 10.22 ± 0.04	− 20.01 ± 0.06	214.80 ± 0.23
20% salt-stressed	10.28 ± 0.01	− 13.17 ± 0.12	− 23.15 ± 0.02	240.37 ± 0.19
30% salt-stressed	8.42 ± 0.02	− 2.42 ± 0.01	− 6.79 ± 0.11	266.44 ± 0.14

salt-stressed and unstressed cultures (see Table 3) align perfectly with the result obtained from the colorimetric analysis of the *Spirulina sp.* cultured different stressed conditions, indicating that the saline-stress have a positive effect on the phycocyanin production.

Conclusion

The present study aims to improve the phycocyanin yield from *Spirulina sp*. using salinity stress to amplify its production and further by optimizing the extraction strategy. The freeze–thaw method combined with acetate buffer (pH 5.1), was able to extract about 25% $_{w/w}$ phycocyanin from the dried *Spirulina sp*. biomass with a purity ratio of 0.806 ± 0.079. This yield was further enhanced by 1.9 folds with a purity ratio of 1.402 ± 0.609 when the culture was subjected to 30% $_{w/v}$ salt stress. Thus, subjecting *Spirulina sp*. to salt stress increases the production of phycocyanin within the species, by combining this with high-efficiency extraction techniques, it is possible to increase the efficiency and feasibility of the process industrially.

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Availability of data and materials All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Declarations

Conflict of interest The authors declare that they have no known competingfinancial interests or personal relationships that could have appearedto influence the work reported in this paper.

Ethical approval Not applicable.

Consent to participate Not applicable.

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