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Optimizing the conditions of phycocyanin extraction and purification from *Spirulina* sp.

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Abstract Phycocyanin (C-Pc) is a natural pigment with extensive use in the food, cosmetic, and pharmaceutical industries. *Spirulina* sp. is regarded as a natural source of C-Pc. The optimal growth conditions increased the pigment content. The extraction and purification process are crucial steps that shouldn't be disregarded to keep phycocyanin's quality at maximum level. Several buffers and concentrations of $(NH_4)_2SO_4$ were evaluated in this work to extract C-Pc. The optimal buffer was 100 mM KPB at pH 7 with a purity value of 0.809 and C-Pc concentration of 4.32 whereas, the optimal $(NH_4)_2SO_4$ concentration was 65% with purity ratio 0.91 and the C-Pc concentrations was 4.47. Ion exchange chromatography was used to purify C-Pc, yielding a purity of 1.73 using 50 mM KPB PH 7.

keywords: Phycocyanin, *Spirulina* sp., Potassium phosphate buffer, Ammonium sulfate, Pigment.

1.Introduction

Phycocyanin (C-Pc) is a pigment-protein complex belonging to the light-harvesting phycobiliprotein family, together with allophycocyanin and phycoerythrin [1]. Water-soluble phycocyanin (C-Pc) is present in up to 15% cyanobacteria [2]. The α and β subunits are the basic building blocks of phycocyanins which together form α and β-protomer subunits which can further form trimmers (α β)3 and hexamers (α β)6 [3,4]. An apparent the molecular mass is found to be 14–21 kDa [5-7]. The tetrapyrrole chromophore phycocyanobilin, which is linked to the apoprotein, causes C-Pc to appear dark Blue and upon that the maximum absorbance lies between 610-620 nm [8,9]. According to Safari *et al.* [10], C-Pc possesses anti-inflammatory, antioxidant, and anticancer activities. Certain algae species have polysaccharides and phycobiliproteins that can stop the proliferation of tumor cells, according to Cuellar-Bermudez *et al.* [11] and Ravi *et al.* [12]. The absorbance ratio of A_{620}/A_{280} is typically used to assess the purity of C-Pc; a purity of 0.7 is regarded as food grade, 3.9 as reactive grade, and more than 4.0 as analytical grade [13]. In 2004, Bhuyan *et al.* [14] found that it is necessary to look for renewable, alternative sources of natural dyes that don't interfere with the equilibrium of the ecosystem [15 ,16]. Algae may be the ideal source of natural dyes for industrial applications because of their rapid growth rate, wide range of colors, and widespread occurrence in various climatic circumstances [17, 18]. For phycobiliproteins to have the best yield and be commercialized, optimizing the extraction process is critical [19]. A number of techniques, including density gradient centrifugation, ammonium sulfate precipitation, chromatography methods, and aqueous two-phase extraction, have been developed for the separation and purification of C-Pc [20 - 24]. The cell rupture approach is employed in the extraction of phycobiliproteins in order to release these proteins with a good solvent that can be used in the food industry with lower cost at the same time [25, 26].

Inorganic solvent extraction has proven

to be an effective and different approach for recovering C-Pc in several investigations. A common method for extracting the contents of *Spirulina* is called Soxhlet extraction; however, Chemat *et al.* [27] state that this method is seemed to be less advantageous due to its high energy consumption and use of hazardous chemicals. Many techniques had been published for the purification of phycobiliproteins [30].

In this report, we are focusing on the evaluation of different buffers in the extraction step under exactly the same physical factors (time & temperature) followed by optimizing the steps of $(NH_4)_2SO_4$ precipitation and ion exchange chromatography for C-Pc purification.

2. Materials and methods

Extraction of phycocyanin

Spirulina sp., which was acquired from the Water Quality Lab and Algae Biotechnology Culture Collection Dept. of Botany, Faculty of Science, Mansoura University of Science in Egypt, was the source of C-Pc.

To determine the maximum C-Pc purity and concentration, the biomass of two-liter culture of *Spirulina* sp. was collected via centrifugation at 4000 rpm, and the pellet was combined with the tested extraction solvent buffers. About 10 mL of the different buffers were added to 0.1 g fresh weight biomass.

The buffers were: 100 mM phosphate buffer (KPB) pH 7, 100 mM acetate buffer pH 5, 100 mM ammonium buffer pH 9, and distilled water that served as a control. The cells were subjected to freezing at -20°C for 24 h then thawing at 4°C for 2 h. To get rid of cell debris, the extracts were centrifuged at 10,000 rpm using a cooling centrifuge (SIGMA Model 3-18K, Germany) at 4°C. After centrifugation, the purity was assessed. A JENWAY Model 7315 UV-Vis spectrophotometer (UK ST15 0SA) was used to measure the absorbance of the extracts at three different wavelengths: 280, 620, and 652 nm. The absorbance ratio A_{620}/A_{280} was used to assess the purity (equation 1) [29]. As reported by Boussiba and Richmond [30], the equation (2) was used to calculate the concentration of C-Pc (mg ml^{-1}). The samples were measured in triplicate.

$$Purity = \frac{A620}{A280} \qquad \text{eq (1)}$$

$$\mathrm{C-Pc(mg/ml)} = \frac{(\mathrm{A620 - 0.474\ A652})}{5.34} \ \text{eq (2)}$$

Purification of phycocyanin

Ammonium sulfate precipitation of extracted phycocyanin

Upon C-Pc calculations, the most favorable solvent buffer was used for further step after centrifugation. The supernatant, containing C-Pc, was subjected separately to 25, 45, 50, 55, 60, 65, and 70 % ammonium sulfate precipitation. Each $(NH_4)_2SO_4$ concentration was added gradually and the precipitation process involved constant stirring in an ice bath for 30 minutes, followed -by centrifugation at 10,000 rpm for 10 minutes at 4 °C. Pellets re suspended in an optimal extraction buffer of a specified volume. For each $(NH_4)_2SO_4$ concentration, the C-PC purity and concentration were measured.

Dialysis of phycocyanin

The optimal extraction buffer and the greatest purity ratio of ammonium sulfate obtained in this study were employed first. To get rid of salts, C-Pc dissolved pellets were dialyzed against 1 L of 10 mM KPB pH 7 using cellulose membrane dialysis tubing (SERVAPOR, dialysis tubing, MWCO 12000–14000 RC, diameter 21 mm) overnight at 4 °C with continuous stirring.

Purification of dialyzed phycocyanin

Sulphopropyl-sepharose "fast flow" anion exchange chromatography was used to purify the dialyzed C-Pc. Ion exchange chromatography was performed with "fast flow" sulphopropyl-sepharose resin. The first calibration of the column was performed in a 10 mM potassium phosphate buffer (PH 7). The dialyzed C-Pc was carefully loaded on the column at a volume of 5 ml. After that the same buffer of calibration was used for removing unbound fractions in three bed volumes. For C-Pc elution, 10 mM potassium phosphate buffer pH 7 containing 0.25 mM NaCl was used in and gradually the buffer concentration increased to 20 mM, 30 mM, 40 mM, 50 mM, 60 mM, and 70 mM. The collected fractions in blue color were collected and kept to evaluate conc. and purity of C-Pc.

Statistical analysis

All analyses were tested in triplicate and values were averaged. The standard errors (SE) were computed as well.

3. Results and Discussion

Extraction of phycocyanin

The right choice for the extraction and purification processes is crucial to getting C-Pc from *Spirulina* sp. Figure 1 displays the properties of the C-Pc extract based on different extraction buffers, in which the fixed volumes and physical factors were taking in consideration. The concentration and purity ratio of the crude C-Pc extracts were calculated in order to assess the extraction methods' efficacy. The A_{620}/A_{280} absorbance ratio, which shows the contamination of aromatic amino acid-rich proteins (A_{280}) and the maximum peak height for C-Pc (A_{620}), is commonly used to assess the purity of C-Pc, which is important in commercial applications. Food grade purity is defined as 0.7, reagent grade as 3.9, and analytical grade as greater than 4.0 [31]. Cyanobacteria cells are frequently ruptured in laboratories by freezing and thawing cycles [32 ,33]. Cell volume increases as a result of the formation of ice crystals during the freezing of intracellular fluid, and cell contraction occurs when the fluid thaws. Additionally, because of differences in the concentration of electrolytes in particular places, freezing causes adjustments to the pressure conditions of the cell membrane and osmotic chocks, which exacerbates the damage to the membrane [34,35]. The choice of solvent is an important parameter to consider in this technique. Of the solvents evaluated, sodium and potassium phosphate buffers (pH 7.0) [33, 36] proved to be the most suitable, leading to a higher extraction yield (217.18 mg/g) [38] and purity (0.87) [33]. In our study and regarding the findings shown in Figure 1, 100 mM potassium phosphate buffer (KPB) pH 7 was the most effective one for achieving the maximum level of purity, both in terms of the extracted C-Pc concentration and purity.

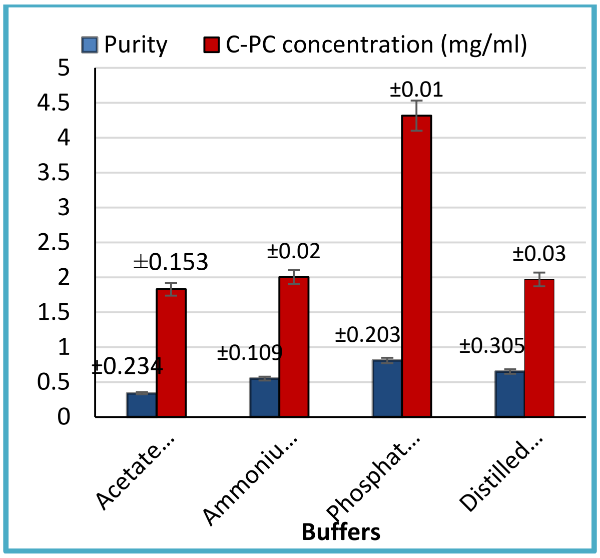

Figure (1): purity and concentration of C-Pc extract from different extraction buffers. 100mM, pH 5, 9 and 7.

Ammonium sulfate precipitation of extracted phycocyanin

Ammonium sulfate precipitation was the initial stage in the purification of C-Pc. One can employ a variety of precipitating agents, including polyethylene glycol (PEG), ethanol, and acetone. Ammonium sulfate was chosen above others because of its Ammonium sulfate precipitation was the initial stage in the purification of C-Pc. Another study could be employed a variety of precipitating agents, such as polyethylene glycol (PEG), ethanol, and acetone. However, ammonium sulfate has a superiority compared to others ones, owing to its many benefits, including its easy precipitation ability to prevent protein denaturation and bacteriostatic action [30]. There were seven distinct ammonium sulfate concentrations used. Based on the purity ratio, the optimal ammonium sulfate concentration for C-Pc purification was selected. As can be shown in Table (1), Figure (2), the precipitation step with 65 % in this investigation produced the highest purity ratio (0.91±0.045) with a C-Pc concentration of 4.47 ± 0.212 mg/ml. At 70% saturation, the purity ratio started to drop. Thus, 65% of the precipitation's ammonium sulfate saturation was chosen. After precipitating a 65% $(NH_4)_2SO_4$ solution, Kumar *et al.* [28] produced C-Pc with a purity ratio of 1.5. Even though their data show a larger yield, it's crucial to remember that their experiment involved multiple extraction stages in order to acquire more than 80% content.

Table (2): The quantity and purity of Phycocyanin extracted by different ammonium sulfate concentrations.

Ammonium sulfate conc.	Purity of C- Pc	Conc. of C-Pc (mg/ml)
25%	0.279±0.016	0.152 ± 0. 17
45%	0.533±0.017	1.895 ±0.106
50%	0.63±0.097	1.045 ± 1.03
55%	0.61±0.027	1.64 ± 0.385
60%	0.74±0.02	2.44 ± 1.3
65%	0.91±0.045	4.47 ± 0.212
70%	0.82±0.008	1.48±0.098

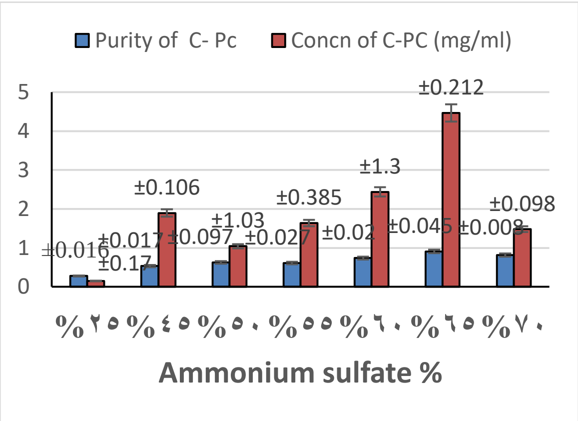

Figure (2): Purity and of C-Pc concentrations extracted by different ammonium sulfate concentrations.

Purification of phycocyanin

After precipitating C-Pc, 10 ml of extraction was dialyzed against 10 mM KPB. To prevent saturation in the solution buffer, the solution buffer was changed three times throughout dialysis. The goal of dialysis was to lessen the amount of salt that remained after the precipitation process. purity was 1.08 following dialysis. Fractions were separated after the dialyzed C-Pc was purified using sulphopropyl-sepharose "fast flow" and eluted with several concentrations of KPB (10 mM, 20 mM, 30 mM, 40 mM, 50 mM, 60 mM, and 70 mM). The C-Pc containing fractions, 4 to fraction 8, were designated by blue color. As shown in Table 2 and Figure 3,4 the highest purity, 1.738, was recorded for fraction 7. Using gel filtration, Julianti *et al.* [39] produced a pure C-Pc with a purity of 1.729. Phycocyanin from *Calothrix* sp. was refined by Santiago-Santos *et al.* [21] using ion exchange chromatography with Q-sepharose rapid flow, yielding a purity of 2.2. The following list (Table 2) includes the C-Pc purity, concentration, and separation factor.

Table 3: Purity, concentration and separation factor of C-Pc containing extracts and fraction eluted from sulphopropyl-sepharose column.

Fractions	Purity	C-Pc conc. (mg/ml)	Separation factor
Crude	0.8	0.304	0.144
Precipitated	0.907	0.240	1.62
Dialyzed	1.08	0.283	1. 81
F1	0.14	0.02	1.155
F2	0.19	0. 32	0.12
F3	0.37	0.43	0.168
F4	1.2	3.28	1.72
F5	0.99	3.491	1. 22
F6	1.01	3.49	1.75
F7	1.07	2.61	1.97
F8	1.73	2.3	2.17
F9	1.4	2.08	2.19

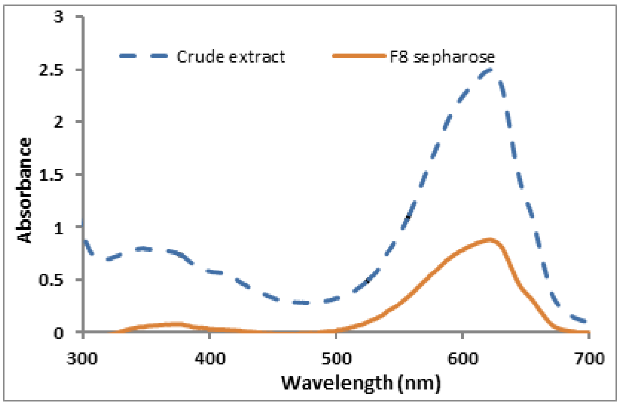

Figure 3: UV-Vis spectrum of crude and ion exchange chromatography fraction.

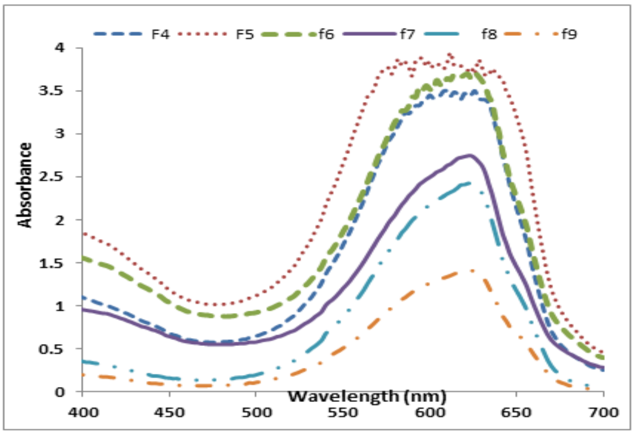

Figure 4: UV-Vis spectrum of ion exchange chromatography fractions.

Conclusion

One cyanobacterial species, *Spirulina* sp., has been studied for the commercial production of C-Pc. First, different buffers were examined to achieve the maximum extraction (based on purity ratio). The optimal operating parameters, including potassium phosphate buffer (KPB) (pH 7), which produced a purity of 0.809. To ascertain the proper $(NH_4)_2SO_4$ concentration for phycocyanin preciptation, $(NH_4)_2SO_4$ with 65% saturation percentages was found to give the highest purity C-Pc (0.907). Finally, sulphopropyl-sepharose led to a final purity being 1.739 which eluted with 50 mM KPB.

References

1 Glazer AN. (1989) Light guides: directional energy transfer in a photosynthetic antenna. *Journal of Biological Chemistry*. Jan 5;**264(1)**:1-4.

2 Patel A, Mishra S, Pawar R, Ghosh PK. (2005) Purification and characterization of C-Phycocyanin from cyanobacterial species of marine and freshwater habitat. Protein expression and purification. Apr **1;40(2)**:248-55.

3 Freindenreich P, Apell GS, Glazer AN. (1978) Structural studies on phycobiliproteins (II). *J Biol Chem*.; **253**:212-9.

4 Bryant DA, Hixson CS, Glazer AN. (1978) Structural studies on phycobiliproteins III. Comparison of bilin-containing peptides from the beta subunits of C-phycocyanin, R-phycocyanin, and phycoerythrocyanin. *The Journal of biological chemistry*. Jan 10;**253(1)**:220-5.

5 MacColl R. (1983) Stability of allophycocyanin's quaternary structure. Archives of Biochemistry and Biophysics. May 1;**223(1):**24-32.

6 MacColl R, Berns DS, Koven NL. (1971) Effect of salts on C-phycocyanin. Archives of biochemistry and biophysics. Oct 1;**146(2):**477-82.

7 Maccoll R, Csatorday K, Berns DS, Traeger E. (1981) The relationship of the quaternary structure of allophycocyanin to its spectrum. Archives of Biochemistry and Biophysics. Apr 15;**208(1)**:42-8.

8 Sidler WA. (1994) Phycobilisome and phycobiliprotein structures. In The molecular biology of cyanobacteria (pp. 139-216). Dordrecht: Springer Netherlands.

9 Hirata T, Tanaka M, Ooike M, Tsunomura T, Sakaguchi M. (2000) Antioxidant activities of phycocyanobilin prepared from Spirulina platensis. *Journal of applied Phycology*. Oct; **12**:435-9.

10 Safari R, Raftani Amiri Z, Esmaeilzadeh Kenari R. (2020) Antioxidant and antibacterial activities of C-phycocyanin from common name Spirulina platensis. *Iranian journal of fisheries sciences.* Jul 1;**19(4)**:1911-27.

11 Cuellar-Bermudez, S.P., Aguilar-Hernandez, I., Cardenas-Chavez, D.L., Ornelas-Soto, N., Romero-Ogawa, M.A. and Parra-Saldivar, R. (2015) Extraction and purification of high-value metabolites from microalgae: essential lipids, astaxanthin and phycobiliproteins. Microbial biotechnology, , **8(2)**, pp.190-209.

12 Ravi M, Tentu S, Baskar G, Rohan Prasad S, Raghavan S, Jayaprakash P, Jeyakanthan J, Rayala SK, Venkatraman G. (2015) Molecular mechanism of anti-cancer activity of phycocyanin in triple-negative breast cancer cells. BMC cancer. Dec; **15**:1-3.

13 Patil G, Chethana S, Sridevi AS, Raghavarao KS. (2006) Method to obtain C-phycocyanin of high purity. *Journal of chromatography* A. Sep 15;**1127(1-2)**:76-81.

14 Bhuyan R, Saikia CN, Das KK. (2004) Extraction and identification of colour components from the barks of Mimusops elengi and Terminalia arjuna and evaluation of their dyeing characteristics on wool.

15 Yusuf M, Shahid M, Khan MI, Khan SA, Khan MA, Mohammad F. (2015) Dyeing studies with henna and madder: Research on effect of tin (II) chloride mordant. *Journal of Saudi Chemical Society.* Jan 1;**19(1)**:64-72.

16 Raza A, Iqbal N, Mahmood S, Parveen S, Azeem M, Nawaz M, Javed MT, Noman A. (2018) Harnessing natural colorants for sustainable textile dyeing an eco-friendly approach using sweet cane (Saccharum bengalense Retz.) inflorescence. Brazilian Archives of Biology and Technology. Nov 14;61: e18170802.

17 Batool F, Adeel S, Azeem M, Khan AA, Bhatti IA, Ghaffar A, Iqbal N. (2013) Gamma radiations induced improvement in dyeing properties and colorfastness of cotton fabrics dyed with chicken gizzard leaves extracts. Radiation Physics and Chemistry. Aug 1; **89**:33-7.

18 Mir RA, Adeel S, Azeem M, Batool F, Khan AA, Gul S, Iqbal N. (2019) Green algae, Cladophora glomerata *L*.–based natural colorants: dyeing optimization and mordanting for textile processing. *Journal of Applied Phycology.* Aug 1; **31**:2541-6.

19 Niu JF, Wang GC, Tseng CK. (2006) Method for large-scale isolation and purification of R-phycoerythrin from red alga Polysiphonia urceolata Grev. Protein Expression and Purification. Sep 1;**49(1):**23-31.

20 Viskari PJ, Colyer CL. (2003) Rapid extraction of phycobiliproteins from cultured cyanobacteria samples. Analytical biochemistry. Aug 15;**319(2)**:263-71.

21 Santiago-Santos MC, Ponce-Noyola T, Olvera-Ramırez R, Ortega-López J, Cañizares-Villanueva RO. (2004) Extraction and purification of phycocyanin from Calothrix sp. Process Biochemistry. Oct 29;**39(12)**:2047-52.

22 Reis A, Mendes A, Lobo-Fernandes H, Empis JA, Novais JM. (1998) Production, extraction and purification of phycobiliproteins from Nostoc sp. Bioresource technology. Dec 1;**66(3)**:181-7.

23 Galland-Irmouli AV, Pons L, Lucon M, Villaume C, Mrabet NT, Guéant JL, Fleurence J. (2000) One-step purification of R-phycoerythrin from the red macroalga Palmaria palmata using preparative polyacrylamide gel electrophoresis. *Journal of Chromatography B: Biomedical Sciences and Applications*. Feb 28;**739(1)**:117-23.

24 Wang G. (2002) Isolation and purification of phycoerythrin from red alga Gracilaria verrucosa by expanded-bed-adsorption and ion-exchange chromatography. Chromatographia. Oct; **56**:509-13.

25 Gacesa P, Hubble J. (1900)Tecnología de las enzimas. Editorial ACRIBIA. Zaragoza..

26 Kula MR, Schütte H. (1987) Purification of proteins and the disruption of microbial cells. Biotechnology Progress. Mar;**3(1)**:31-42.

27 Chemat F, Vian MA, Cravotto G. (2012) Green extraction of natural products: Concept and principles. *International journal of molecular sciences.* Jul 11;**13(7)**:8615-27.

28 Silva LA, Kuhn KR, Moraes CC, Burkert CA, Kalil SJ. (2009) Experimental design as a tool for optimization of C-phycocyanin purification by precipitation from Spirulina platensis. *Journal of the Brazilian Chemical Society*.; **20**:5-12.

29 Bennett A, Bogorad L. (1973) Complementary chromatic adaptation in a filamentous blue-green alga. *The Journal of cell biology*. Aug 1;**58(2)**:419-35.

30 Boussiba S, Richmond AE. (1979) Isolation and characterization of phycocyanins from the blue-green alga Spirulina platensis. Archives of Microbiology. Feb; **120**:155-9.

31 Rito-Palomares M, Nunez L, Amador D. (2001) Practical application of aqueous two-phase systems for the development of a prototype process for c-phycocyanin recovery from Spirulina maxima. *Journal of Chemical Technology & Biotechnology*. Dec;**76(12)**:1273-80.

32 Majdoub H, Mansour MB, Chaubet F, Roudesli MS, Maaroufi RM. (2009) Anticoagulant activity of a sulfated polysaccharide from the green alga Arthrospira platensis. Biochimica et Biophysica Acta (BBA)-General Subjects. Oct 1;1790(10):1377-81.

33 Moraes CC, Sala L, Cerveira GP, Kalil SJ. (2011) C-phycocyanin extraction from Spirulina platensis wet biomass. Brazilian

Journal of Chemical Engineering.; **28**:45-9.
34 Tavanandi HA, Mittal R, Chandrasekhar J, Raghavarao KS. (2018) Simple and efficient method for extraction of C-Phycocyanin from dry biomass of ASpirulina platensis. Algal research. Apr **1**; 31:239-
35 Acker JP, McGann LE. (2003) Protective effect of intracellular ice during freezing? Cryobiology. Apr 1;**46(2)**:197-202.
36 Roquebert MF, Bury E. (1993) Effect of freezing and thawing on cell membranes of *Lentinus edodes*, the shiitake mushroom. World *Journal of Microbiology and Biotechnology*. Nov; **9**:641-7.
37 Li Y, Zhang Z, Paciulli M, (2020) Abbaspourrad A. Extraction of phycocyanin—A natural blue colorant from dried *Spirulina* biomass: Influence of processing parameters and extraction techniques. *Journal of Food Science*. Mar;**85(3)**:727-35.
38 Silveira ST, Burkert JD, Costa JA, Burkert CA, Kalil SJ. (2007) Optimization of phycocyanin extraction from Spirulina platensis using factorial design. Bioresource technology. May 1;**98(8)**:1629-34.
39 Julianti E, Susanti S, Singgih M, Mulyani LN. (2019) Optimization of extraction method and characterization of phycocyanin pigment from Spirulina platensis. *J. Math. Fundam. Sci*. May 1; **51**:168-76.