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Optimization of cost effective method for extraction and fractionation of Phycocyanin pigment from Spirulina

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Abstract
Phycocyanin, a dietary supplement is a major light harvesting accessory pigment of algae *Spirulina plantensis* due to its high protein content. The intense blue pigment has wide commercial applications in food and pharmaceutical industry. Due to its fluorescent property, phycocyanin is required in small quantities in immunoassay. In the current investigation different extraction and fractional purification methods were compared in order to optimize for the industrial production of phycocyanin pigment. Extraction was carried out by 3 different methods: (a) Solvent method using water and 0.1M Sodium Phosphate buffer pH-7.0, (b) Freeze- thaw method and (c) lysozyme mediated method. Various methods used for purification of C-Phycocyanin are heat denaturation method, methanol precipitation method, ace tone precipitation method and silica adsorption. Extraction using water (10%) as solvent showed highest yield of 53.82%, 0.5% purity and 5.83g/ml phycocyanin concentration extract. The Silica precipitate method showed a greater fractionization of pigment yielding 64.3% with 6.4 g/ml PC content, 0.14% purity and increased protein concentration of 150mcg/ml compared to other purification methods. Amongst all the methods, 10% water with 24 hours of extraction and purification by means of Silica adsorption method was very effective and efficient method optimized for C-PC by considering C-PC %purity, yield and pigment protein concentration.

Keywords: C-Phycocyanin (C-PC), pigment, extraction, acetone, spirulina

1. Introduction
Phycocyanin is a water-soluble pigment, potent antioxidant which possesses significant immune enhancing and anti-viral property. C-PC could be extracted from gram-negative cyanobacteria such as *Spirulina platensis* which is a primitive organism that has the ability to utilize carbon dioxide dissolved in sea water as a carbon source. After the process of photosynthesis, pigment phycocyanin will be synthesised in the inner member of thylakoid (Roman.*et.al.* 2002) [16]. Spirulina is a multi-layered cell wall which consists of 4 barriers; the outer layer is made up of lipopolysaccharides which consist of magnesium and calcium ions, the inner layers are made of protein and peptidoglycan layer. Extraction of phycocyanin is a tedious work due to the variations in stability and multi-layered cell wall. Several methods have been reported for extraction and purification of PC (chem. *et al.*, 2006; Patel *et al.*, 2006) but these methods comprised of multi-step process, high cost for production, time consumption, yield reduction, purity reduction which may affect the widespread applications.

Many methods had been reported for C-PC extraction from cyanobacteria like freeze-thaw method (Sarada *et.al.*, 1999; Bermejo *et.al.*, 2006) [6], sonication (Bermijo *et.al.*, 2006 abalde *et.al.*, 1998), homogenization (Sarada *et.al.*, 1999) [6], lysozyme mediated method (Stewart and Farmer *et.al.*, 1984) [17] and acid treatments by cold maceration (Suresh. P. kamble *et al.*, 2013) [4]. Purification of PC by means of gel filtration, dialysis, ammonium sulphate precipitation was also been well studied (Suresh. P. Kamble, 2013) [3].

Researchers concluded that PC enhances biological defence activity against infectious diseases through the mucosal immune system and also suppresses antigen specific IgE antibody. In vivo studies of nutraceutical science explain that the PC acts as neuroprotector, nephroprotection, eye protector. Phycocyanin had been widely used as nutritional ingredients, natural dyes, florescent markers (Glazer and Stryer 1984), pharmaceuticals such as antioxidants (Romay and Gonzalez 2000) and anti-inflammatory reagents (Qureshi *et al.* 1996). Phycocyanin is used as colorant in food (chewing gums, dairy products, gellies etc.) and cosmetics such as lipstick and eye liners in Japan, Thailand and China. It had been shown to have therapeutic value by showing increased immuno-modulating activity and anticancer activity (Lijima and Shimamatsu 1982).

Due to requirement of natural pigment phycocyanin in various fields, there are various methods implied to ease the production. Here we are trying to find the most efficient amongst the existing and novel methods of extraction and fractionization of PC pigment from spirulina dry powder.

2. Materials and Methods
2.1 Extraction methods
Spirulina is a good source of phycocyanin and hence spirulina dry powder was procured from Ramsun Rasayanics Pvt. Ltd, Bengaluru India. PC was extracted by 3 different methods such as Solvent method which includes water and 0.1M Sodium Phosphate Buffer extraction; Freeze-Thaw method and lysozyme mediated extraction.

2.1.1 Solvent method
Dry spirulina powder was crushed with varied percentage of 1, 5, 10, 20 % w/v of 0.1M phosphate buffer - pH 7 and kept in shaker incubator at 160 rpm at 30 degree Celsius with 24 to 48 hours of incubation. Same protocol was repeated with water as solvent.

2.1.2 Freeze-Thaw method
PC was extracted by repeated freezing and thawing of spirulina cells in 10% phosphate buffer and 10% water following the method of Sigelman and Kyeia, 1978 with slight modification of freezing time exposure increased to 2-4 hours with constant thawing time of 5 minutes for each cycle.

2.1.3 Lysozyme mediated method
Lysozyme was added to the biomass with different % (1, 5, 10, and 20% w/v) in 0.5mM Sodium Phosphate buffer pH-7 containing tris -EDTA lysis buffer. This biomass was then incubated at 30 degree Celsius for 2 hours according to the method of Boussiba and Richmond, 1980 [8].
After extraction of phycocyanin from each method, the samples were centrifuged and the supernatant was used to verify the analytical yield.
PC concentration was calculated using the equation given by Bernett and Bogoard 1973.

CPC (mg/ml) = (OD at 620nm - 0.474 OD at 652nm) / 5.34
PC Yield was calculated using the equation given by Silveria *et.al.* 2007

Yield (mg) = CPC × Volume of Solvent (ml) / Dry biomass (g)
PC Purity percentage was calculated using equation given by Bernett and Bogoard 1979

% pure CPC = Absorbance at 620nm x 10 x 100 / 7.3 x (mg. sample) x (% dry wt.)

2.2 Fractionization of PC
Purification was done by fractionation using 4 different methods – chilled methanol, chilled acetone method, heat denaturation method and silica adsorption method. Water extract of phycocyanin was done with 10% with incubation of 24 hours was selected as efficient and effective method of extraction for further purification process.

2.2.1 Methanol/Acetone precipitation method
One volume of sample extract was added with 4 volumes of chilled acetone: water (4:1, v/v) and incubated in -20 degree Celsius overnight and was cold centrifuged at 13,000rpm. Repeat washing of pellet with acetone mix solution and centrifuge each time for 10 minutes. Finally, pellet was dissolved in 25 Moles of ammonium acetate and purity, yield and concentration of PC was calculated. Same protocol was followed with methanol solvent also.

2.2.2 Heat denaturation method
Simple method of heat denaturation was performed by incubating the sample extract in hot water bath at 90 degree Celsius for 1 hour. Further, the extract was centrifuged and the supernatant was used to verify yield and purity.

2.2.3 Silica adsorption method
10g of dry silica (HPLC grade) powder was added to 20ml of sample and 30 ml of methanol at room temperature. Further, centrifuged and supernatant was kept for evaporation. The PC residue was verified for purity yield.
Crude spirulina was also taken for comparison with all fractionized sample methods to assess purity yield and concentration.

2.3 Protein estimation
Fractionized samples along with the crude were taken for protein estimation following the protocol of Lowry, 2013 *et.al,* method using bovine serum albumin as standard to interpolate the concentration of each sample protein.

2.4 SDS-PAGE
Fractionized sample extract along with standard protein ladder and crude spirulina was run on SDS-PAGE with 5% of stacking gel; 12% of resolving gel buffer was used to confirm the purity of phycocyanin. The bands were documented by Coomassie blue staining under UV transilluminator.

3. Results
3.1 Extraction of PC
Isolation of phycocyanin from spirulina was done using solvent method, freeze thaw method and lysozyme mediated method and was checked for % purity, yield and concentration.

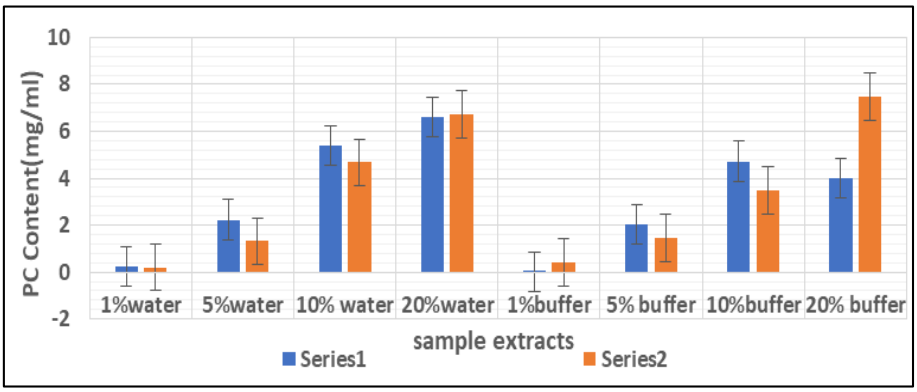


Fig 1: Solvent Extraction- PC Content.

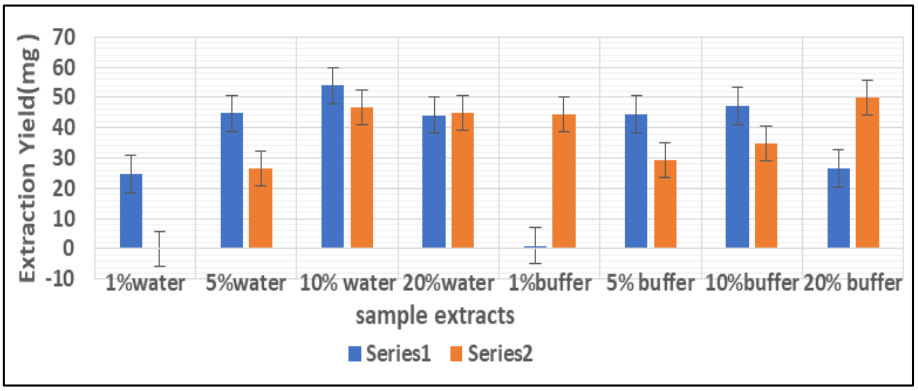


Fig 2: Solvent Extraction- Yield

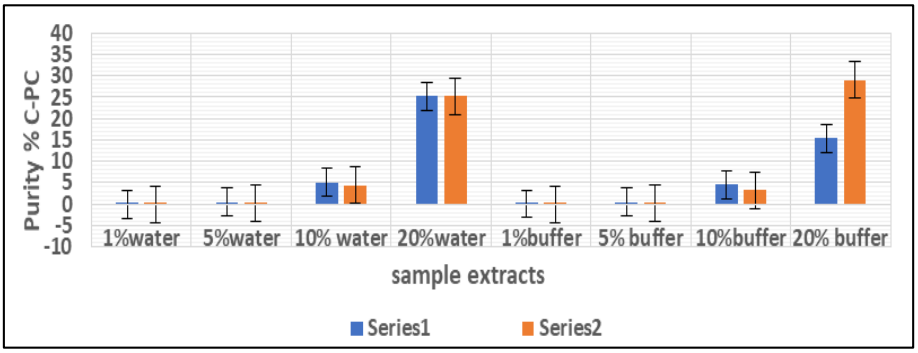


Fig 3: Solvent Extraction-Purity%

Fig 1, 2 and 3: Graphical depiction of the mean PC content, yield and purity percentage of PC extracted by solvent treatments of water and 0.1 M Sodium phosphate buffer pH-7 with different percentage (1, 5, 10, 20%) and 24hours to 48 hours of incubation. 24 hours of water extract gave high yield, purity and concentration compared to 48 hours incubated samples. Series 1- 24 hours incubated extracts;

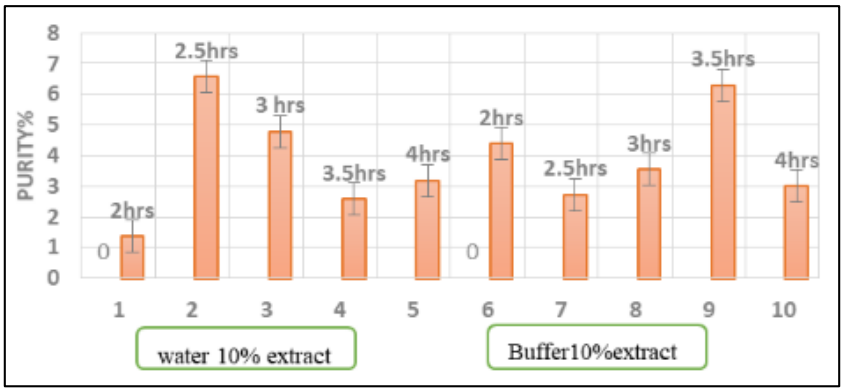


Fig 4: Freeze-Thraw extraction -Purity%

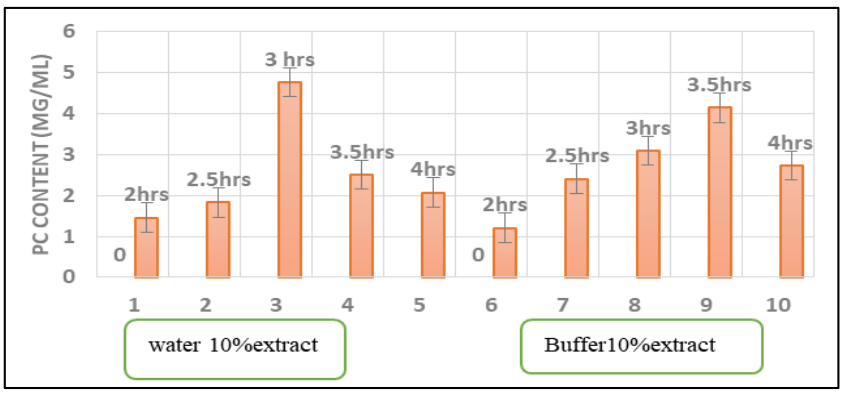


Fig 5: Freeze-Thraw extraction -PC content

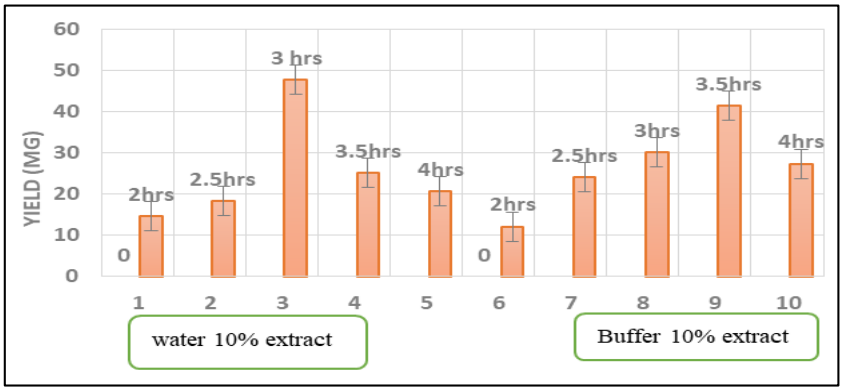


Fig 6: Freeze-Thraw extraction -Yield

Fig 4, 5 and 6: Represents the mean yield, purity percentage and PC concentration from spirulina cells using freeze-thaw method, 10% water and buffer extracts increasing freeze time from 2-4 hours with a constant thawing time of 5 minutes. Water extract of 2.5 hours of freeze time expressed high purity (~7%) with comparatively low yield (~20mg/g) and phycocyanin concentration (~1.8mg/ml) Series 2- 48 hours incubated extracts.

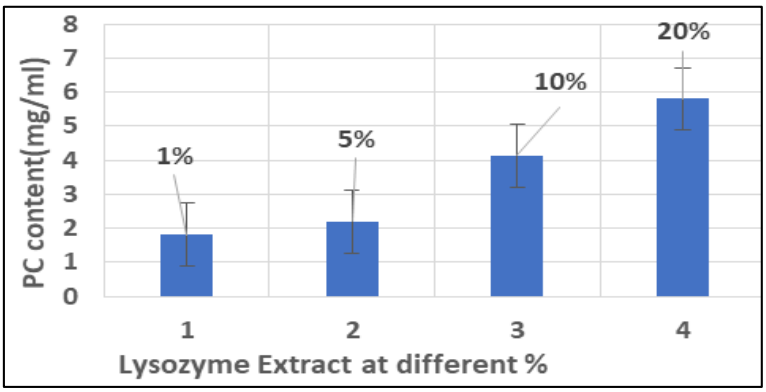


Fig 7: Lysozyme- mediated extraction PC content

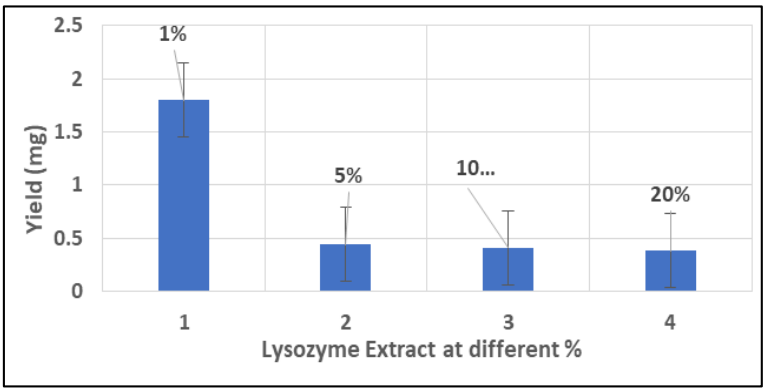


Fig 8: Lysozyme mediated extraction-Yield

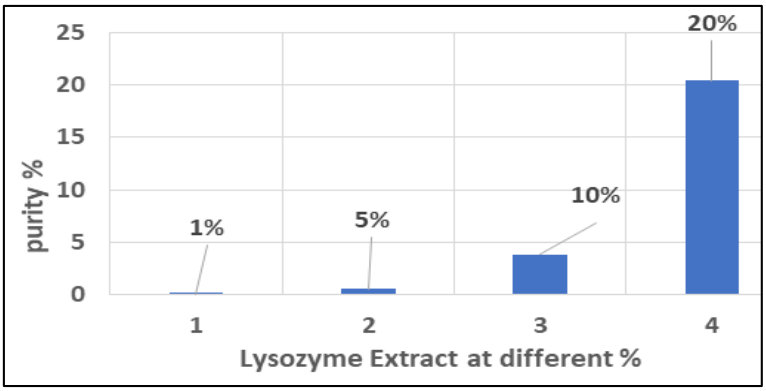


Fig 9: Lysozyme mediated extraction- Purity%

Fig 7, 8 and 9: Illustrates the yield purity and concentration of PC from spirulina cells by using lysozyme mediated extraction. Different percentages of 1%, 5%, 10% and 20% was represented in columns 1, 2, 3 and 4 respectively.

PC concentration was high in 20% solvent extracts and yield was reduced as the purity increased. Solvent method of extraction showed 5.38mg concentration, 5 % purity and 53.82mg of yield in water 10 % with 24 hrs of extraction; 6.62 mg and low yield of 44.14mg was observed in 20% extract; 1% and 5% buffer and water extracts showed less pigment concentration, low purity and yield. (Figure 1, 2 & 3) Freeze-thaw method of extraction done with phosphate buffer and water showed comparatively less yield of 14-27 mg and purity of 1-3% and concentration of 1-2mg/ml. Concentration may be low due to the denaturation effect of disruption of cells (Figure 4, 5 & 6). Enzymatic extraction involving lysozyme with different % (1, 5, 10, 20%) showed a range of 1-4mg PC concentration; 0-20% purity; 0-1mg yield. Lysozyme 20% extract showed highest purity but very low yield which could not be considered for industrial purpose (Figure 7, 8 & 9). Crude spirulina expressed a PC concentration of 4.32mg/ml, 0.16% purity and 43.2 mg of yield. Among all the phycocyanin extraction methods, concentration, purity and yield was optimal and expressed high when extracted with water 10 % 24 hours.

3.2 Purification of C-Phycocyanin

Solvent extraction with 10% water 24 hours which showed maximum concentration, yield and purity percentage was selected for purification. Purification was done by fractionation using chilled methanol/acetone, heat denaturation and silica adsorption methods and was graphically represented in figure 10, 11 and 12.

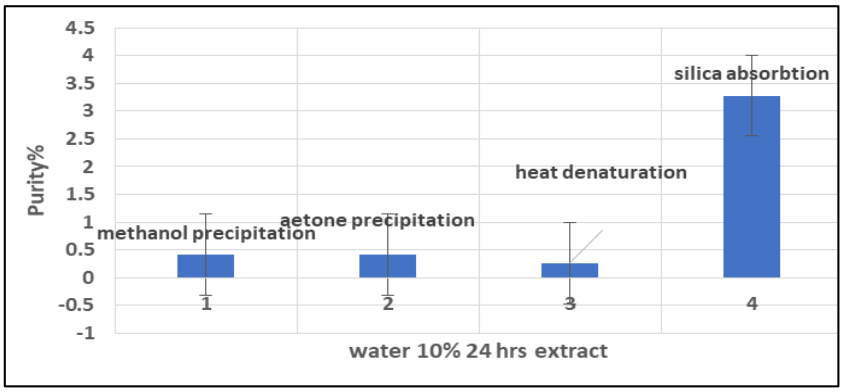


Fig 10: Fractionization of PC purity by different methods

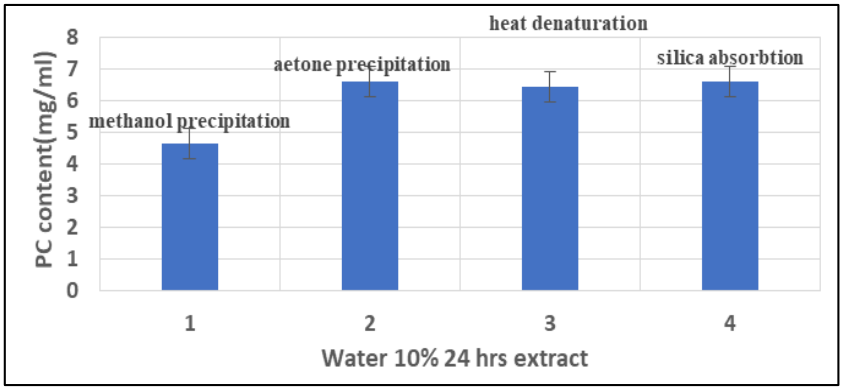


Fig 11: PC concentration by different methods

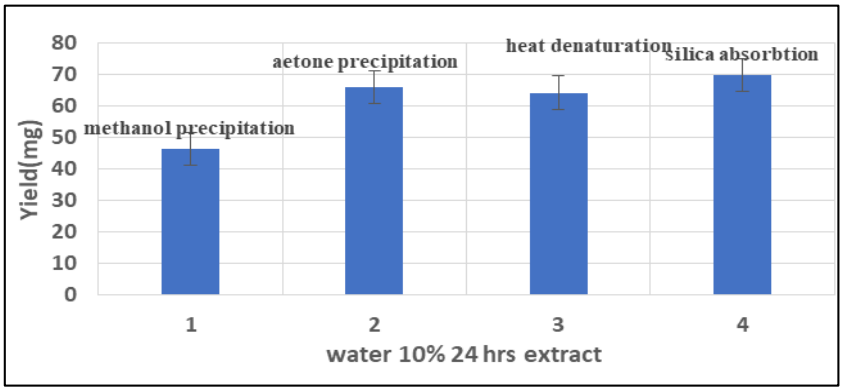


Fig 12: Fractionization of PC yield by different methods

Fig 10, 11 and 12: Illustrates mean yield, purity % and content of PC of different fractionization methods which includes chilled methanol, chilled acetone, heat denaturation and silica precipitation. Yield and PC concentration was high in all the fractioned water 24hours extracts but purity was expressed high (~3%) only in silica absorption method.

Purification of phycocyanin was done by the process of fractionation comparing water 10% 24 hours PC extract and crude spirulina by different methods such as methanol precipitation, acetone precipitation, heat denaturation and silica adsorption method. Among these silica adsorptions was very effective method due to regenerative property and ease to adsorb molecules, and can be feasible for industrial purification. PC content was expressed high in silica adsorption method (6.72mg/ml) with high purity of 5.2% and 70 mg of highest yield compared to other methods. Phycocyanin purification by acetone method showed high yield of 66.1mg with low purity (0.4%) and heat denaturation method had low protein concentration (110mcg/ml) compared to silica method (150mcg/ml) (Figure 10, 11 & 12). Therefore, silica adsorption of Phycocyanin was considered to be best effective and efficient method for purification of phycocyanin.

3.3 Protein estimation of C-phycocyanin

Total protein content in the spirulina sample was found out using a standard curve plotted from different concentrations of BSA (mcg/ml) according to Lowry's method. Protein concentration values of fractionized extracts and the crude were graphically represented in figure no 13 and 14.

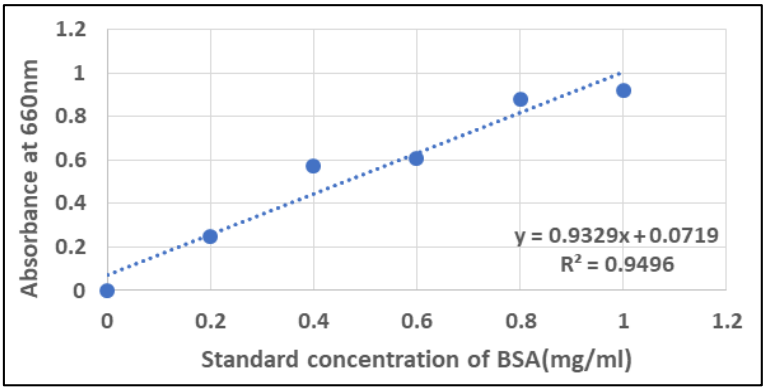


Fig 13: Standard graph of protein by lowrys method

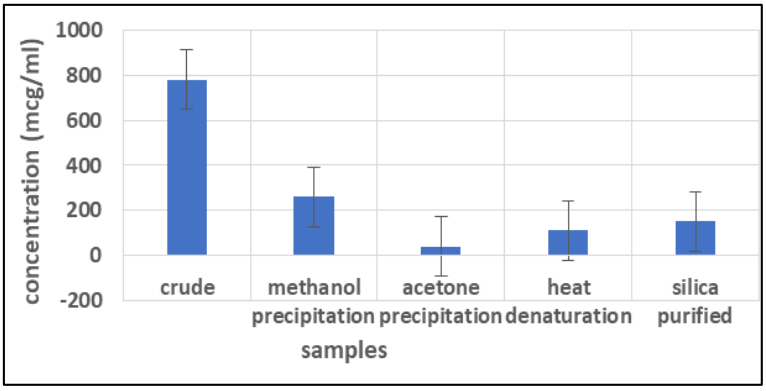


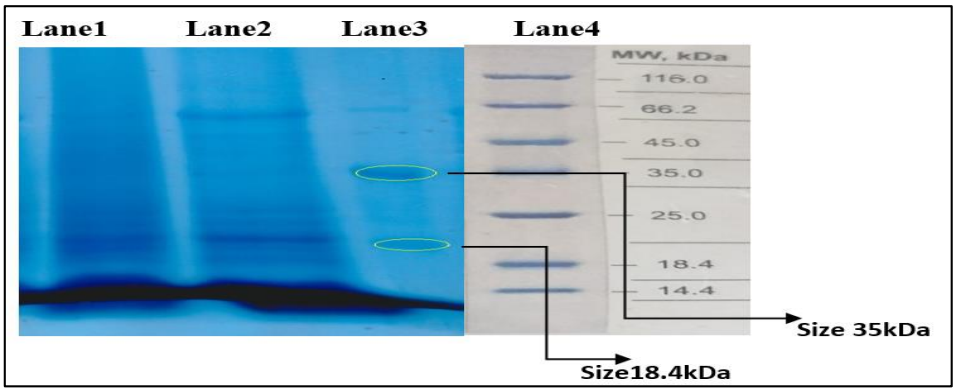
Fig 14: Protein Estimation

Fig 13: Protein standard curve; **Fig 14:** Protein concentration of spirulina crude and phycocyanin purified extracts with respect to standard protein.

Results interpret that crude extract had 780mcg/ml of protein concentration whereas silica method had 150 mcg/ml and methanol precipitation was 260 mcg/ml (Figure 13&14).

3.4 Determination of purity of C-Phycocyanin

The extracted and purified PC along with crude sample was run on SDS-PAGE following the protocol of Shigh. *et. al.* 2010 to determine the molecular weight and purity.



Lane1: Crude; **Lane2:** Water Extract; **Lane3:** Purified Phycocyanin; **Lane4:** Molecular weight marker

Fig 15: SDS- PAGE comparison of crude, extract and purified phycocyanin

SDS-PAGE was done to confirm the quality of Phycocyanin obtained through selected extraction method (water 10% 24 hours extract), silica absorption method extracts with comparison to crude (Figure15). The purification of C-PC was carried out by silica absorption expressed high purity through specific bands when compared to crude and water extract. SDS- PAGE analysis of the purified fraction showed 2 protein bands corresponding to the alpha and beta subunits of C-PC which migrated as approximately 18.4kDA and 35k Da bands.

4. Discussion

Phycocyanin is an industrially important pigment which can used in different fields such as cosmetics, food industry *etc.,* comparison was made among different procedures for extraction and the concentration of PC was represented in terms of mg/ml units. One of the most important requirements for obtaining C-PC from spirulina dry powder was selection of significant and efficient extraction and purification protocol. In the present study, solvents such as water and 0.1M sodium phosphate buffer pH 7 with different concentration and incubation time was checked along with freeze- thaw method with different freezing times and constant thawing times of water and phosphate buffer 10% and lysozyme enzymatic method with different percentage concentration of treatments was compared. Solvent method of extraction expressing 5.38mg PC content, 5 % purity and 53.82mg yield in water 10 % 24 hrs extract was highly efficient in terms of solvent, source requirement, utilization. PC content was high in silica adsorption method (6.72mg/ml); yield of (150mg) and 3% purity. Previous research showed extractions carried out through hexane extraction process combined with high pressure, cold maceration and sonication methods and purified through ammonium sulphate precipitation, dialysis and gel filtration and presented a final extraction yield of 3.27mg with a purity of 2.317% (Stewart *et.al.,* 2008) [17]. C-Phycocyanin extraction from wet biomass gave yield of 43.7mg/g but concentration of only 0.21mg/ml (Moreas *et al.,* 2010); Cold maceration with sodium phosphate buffer and distilled water gave 0.60 mg/ml, 0.16% purity and 0.57mg/ml, 0.14% purity respectively. Ammonium sulphate precipitation showed 4.176mg/ml concentration and 0.28% purity; 5.674mg/ml pigment concentration and 1.08% purity in dialysis method and 3.27 mg/ml concentration, 2.317% purity in sephadex G25 purification (Kamble *et.al.,* 2013) [3]. Even though the purity obtained was high, the method of extraction and purification done was not realistic for large scale production. For purifying C-PC various other precipitating methods using PEG, ethanol, acetone, TCA and ammonium sulphate, column chromatography *etc.,* were used and achieving purification for large scale production among all these methods is not cost effective and a non-reliable process due to the source and precipitating agents requirement, process-time consumption.

5. Conclusion

Amongst all the methods analysed, we concluded that C-PC from *Spirulina platensis* powder showed significant yield, purity and concentration in water extraction and purification through silica absorption method was more effective. In this process we also had the advantage of reutilizing silica due to its regenerative property and cost-effective method when compared to gel filtration matrix.

6. Acknowledgements

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7. References

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