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EXTRACTION AND PURIFICATION OF PHYCOCYANIN FROM *Spirulina platensis* BY USING DIFFERENT METHODS: A REVIEW

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ABSTRACT

Spirulina is a cyanobacteria known as blue green algae and consist of large amount of protein. This algae has been used as source of protein and vitamins. *Spirulina* mainly known for high content of Phycocyanin protein. Phycocyanin belong to the phycobiliprotein family, which is characterized as blue colorpigment, light harvesting, soluble in water which has high nutritional value, and antioxidant, anticancer and anti-inflammatory properties. It has application in a food, cosmetic as well as pharmaceutical industry. This review article describes the extraction of phycocyanin from *spirulina platensis* by using different extraction methods, and purification of phycocyanin by different purification methods. Extraction of phycocyanin carried out by different method includes physical, chemical, and enzymatic method. Physical methods include homogenization and freeze thawing, chemical method include inorganic acid and organic acid treatment and enzymatic method includes lysozyme treatment. Purification of phycocyanin is carried out by different method steps ammonium sulfate precipitation, dialysis, gel electrophoresis and ion exchange chromatography. Present review will highlight the methods involved in extraction and purification of phycocyanin.

Keywords: Phycocyanin, *Spirulina*, Extraction, Purification, Application

1. INTRODUCTION

Spirulina is belonging to the family Oscillatoriaceae **[18]**. *Spirulina* is a cyanobacteria, known as a blue green algae, class of gram negative bacteria

found in lakes, fresh water. It is a unicellular algae **[16]**. It is a natural source of nutritions compositions including protein, vitamin, lipid, minerals, carbohydrates and also consist phenolic acids, tocopherols, and gamalinolenic acid **[23]**. It has been used as a healthy food **[17]**, drugs and dyestuff **[24]**. *Spirulina* mainly used for source of Protein and vitamin in human **[17]**. These algae characterized as blue green algae due to the presence of both chlorophyll and phycocyanin protein *Spirulina* measure 250 µm in length **[19]**. Now cultivation of *spirulina* take place in many countries **[14]**. It consist highest protein source than meat and fish or soybean **[19]**. It has antiviral, anticancer, and anti-inflammatory properties **[18]**. It boosts immune system. It has application in a treating several diseases **[25]**. *Spirulina is* also known for its free radical scavenging and antioxidant activity **[26]**.

1.1. *Spirulina* as human food

*Sprulina i*s a beneficial food has numerous application. *Spirulina* are a cyanobacteria living in a subtropical and tropical areas **[27]**. *Spirulina* consist high protein, vitamin, nutritions and mineral which make it excellent dietary source. *Spirulina* contains pigments large amount of betacarotene as well as vitamin B12 **[19]**. Since 1980, the cultivation of *spirulina* occur in many countries including India, China, United states, Frances **[14]**. It is used by NASA as dietary supplement for astronaut on space mission **[18]**. It is also used for preparing baby food **[19]**. *Spirulina* not consist cellulose in their cell membrane therefore easily digested by human **[19]**.

Table 1: Composition of *spirulina*

Nutrient	Composition	References
Protein	55-70%	19
Carbohydrates	15%	28
Essential fatty acid	18%	29
Lipid	5-7%	30
Vitamins	0.75%	19
Minerals	8%	30

Spirulina has been promoted as the food of the future. It increase the growth of lactobacillus in the intestine, has cholesterol lowering effect **[18]**. *Spirulina* consist all essential and nonessential amino acids. *Spirulina* considered as natural source of vitamin B12 and betacarotene **[19]**.

1.*2 Spirulina* consist Phycocyanin

Spirulina can produce large amount of Phycobiliproteins **[32]**. Phycobilisomes are molecular complex located on the surface of thylokoid membrane in the

cyanobacteria **[33]**. On the basis of size and shape "Phycobilisome" termed first described by the Gant and Conti **[1]**. Phycobilisomes are composed of phycobiliprotein molecule **[8]**. Phycobiliprotein are colored light harvesting, water soluble protein known as phycobilins **[10]**. These protein present in algae that has application in clinical and immunological assay **[1]**. Phycobiliprotein has application in flow cytometry, histochemistry, immunoassay and natural dyes **[10]**. Phycobiliprotein classified into four types;- 1 phycoerythrins, 2 Phycoerythrocyanin 3 Phycocyanin 4 Allophycocyanin. Phycobiliprotein composed of these 4 classes in order of Allophycocyanin in core, Phycocyanin in middle and phycoerythrocyanin and phycoerythrins at the tip **[11]**. Phycobiliprotein are easily isolated as colored pigment protein complexes **[14]**. Phycobiliprotein also has application in the gel electrophoresis, isoelectric focusing, and gel exclusion chromatography as convenient markers **[32]**. It consist of subunit of polypeptide that form monomer .These consist trimers and hexamers **[12]**. It is stable protein consist chromophore group which is responsible for its fluorescent properties **[34]**. Spirulina consist only two Phycobiliproteins, allophycocyanin and C phycocyanin. Phycocyanin is present as major protein, whereas allophycocyanin is present as minor protein in the *spirulina*. *Sprulina* mainly known for its high content of phycocyanin protein. Blue green appear blue intense color due to content of phycocyanin **[19]**. Phycocyanin extracted from spirulina which may contain 14% of this pigment within total cell protein **[17]**. Phycocyanin is a deep blue colored pigment, light harvesting, water soluble protein that has numerous functions. Phycocyanin is used in food industry as food colorant, natural dye, also used as potential therapeutic agent and fluorescent markers in research **[8]**. It is used in cosmetic industry because of its non toxic and non carcinogenic properties **[8]**. Phycocyanin stability depend upon on its pH, temperature, light **[20]**. It may serve as nitrogen source during nitrogen starvation in *Spiruluna platensis* **[5]**.

Figure 1: Phycocyanin

1.3 Phycocyanin structure

Phycocyanin is main phycobiliprotein present in cyanobacteria. Phycocyanin belongs to the phycobiliproteins **[22]**. Phycobiliprotein are light harvesting and water soluble protein **[7]**. Phycocyanin is composed of an apoprotein and non protein component known as phycocyanobilin **[6]**. It has natural blue deep color, less sensitive than chlorophyll to phto-destruction **[35]**. Crystal structure of phycocyanin has been determined as 22 angstorng. Phycocyanin has intense blue color due to presence of open chain tetrapyrrole **[36]**. Phycocyanin composed of α and β subunits. The α chain with one phycocyanobilin which attached at cystein 84, and β chain with two phycobilins attached at cystein 84 and 155 **[14]**. The α and β subunit composed of 162 and 172 amino acids and role in a energy transfer cascade **[37]**. Both chains are rich in conserved α helices **[6]**. The αβ monomers which is turn into aggregate into trimers(α3β3) and hexamers(α6β6) **[6]**. It has molecular weight between 44 and 260 kDa **[14]**. Phycocyanin has properties such as odourless, light harvesting, water soluble, and deep blue color **[14]**. Phycocyanin has 615 nm absorption peak and 647 nm fluorescence emission peak **[9]**.

EXTRACTION OF PHYCOCYANIN

Freezing and Thawing

Freezing and thawing is a cycle in which biomass is freezing and melting. It is a simplest method, and does not cause any biological function of protein.

Spirulina wet biomass was to suspended to freezing and thawing cycle for 24 or 48 hours intervals **[3]**.

The dry *Spirulina* biomass was suspended into independent solvents and repeated freezing and thawing cycle for freezing at -22 ˚C and thawing at 20 ˚C, then phycoyanin collected by centrifugation at 3140 × g for 5 min **[38]**.

Dock *et al.*, 2005, reported that*spirulina* wet biomass repeated freezing and thawing at respectively -21 ˚C and 4 ˚C for 4 hours. Phosphate buffer at pH 7 used to carried out the procedure. Extract was centrifuged at 6000 rpm for 10 min, Phycocyanin estimation by spectrophotometric method **[39]**.

Spirulina biomass repeated to freezing and thawing for 1, 2, and 3 hours cycle. After cell disruption phycocyanin was carried out in shaker for at 4 hour **[33]**.

Minkova *et al.*, 2003 reported that extraction of phycocyanin was carried out by *spirulina* biomass with phosphate buffer frozen at -15 ˚C, thawed and heated under stirring. Extract was centrifuged at

18000 g for 30 min, after centrifugation phycocyanin was collected **[4]**.

Sonication

Sonication is the process of applying ultrasound to the biomass.

The wet biomass suspended to the ultrasound bath at 50 kHz in the proportion of 1:1.1 (g biomass: g glass pearls) for 40 minutes, after 40 minutes phycocyanin was collected **[3]**.

The biomass sonication at 10^4 keycles^{-1} for 5 min.and extract was centrifuged at 10000 g for 15 min, after centrifugation phycocyanin was collected **[10]**.

Shubham *et al*., 2018, concluded that biomass with distilled water sonicated at 40 kHz for 20 min, after centrifugation phycocyanin was collected **[23]**.

Homogenization

Moraes *et al*., 2011, reported that spirulina wet biomass homogenized in mortar pestle in the presence of diatomaceous earth, after centrifugation phycocyanin was collected **[3]**.

Sivasankari *et al*., 2014 concluded that homogenization of biomass in the presence of phosphate buffer, after centrifugation phycocyanin extract was examined under UV- Vis spectrophotometer **[41]**.

The *spirulina* biomass homogenized in a homogenizer in presence of phosphate bufferphycocyanin extract was collected after centrifugation **[39]**.

Chemical Method

In chemical method inorganic acid and organic acid are used. Inorganic acid involve the hydrochloric acid and organic acid involve the acetic acid **[3]**.

The *spirulina* biomass treated with 12M HCL in the proportion 1:2 and incubate at room temperature for 24 hrs. The wet biomass treated with 1M acetic acid in the proportion 1:2 and incubate at room temperature. After centrifugation phycocyanin extract was collected by centrifugation **[41]**.

Moraes *et al*., 2011, reported that wet biomass treated with different concentration of HCL such as 2,4,6,8 and 12M HCL in the proportion of 5:1 and incubated at room temperature. The wet biomass treated with 1m acetic acid as a same proportion and incubated at room temperature for 24 hours, phycocyanin extract was collected after centrifugation **[3]**.

Enzymatic Method

The biomass suspended into Na-Phosphate buffer containing lysozyme enzyme and EDTA then placing the biomass in a shaking bath for 24 hrs, then extract centrifuged to remove cell debris **[42]**.

The biomass suspended into HEPES buffer consist HEPES and EDTA,and containing lysozyme and incubated in shaking bath for 16 hrs **[43]**.

Vernes *et al.*, 2015, reported that lysozyme hydrolyse the glycosidic bond linkage between peptidoglycan from spirulina wall and release and protein from the *spirulina* **[14]**.

Table 2: Extraction methods of Phycocyanin

Treatment	Condition	References
1.Freeze and Thaw	FT (24 &48 hours)	[3]
	Biomass + Independent solution, FT (-22 ˚C to 22˚C)	[38]
	Biomass + phosphate buffer, FT (-28˚C to 4 ˚C)	[39]
	Biomass + phosphate buffer FT(15 ˚C , heat stirring)	[4]
2. Sonication	1:1.1(g biomass:g glass pearl) (50 kHz)	[3]
	10^4 keycles^{-1} (15 min.)	[10]
	40 KHZ (20 min)	[40]
	Biomass + Diatomaceous earth	[3]
	Biomass + Phosphate buffer	41
	Biomass + Phosphate buffer (Homogenizer)	[39]
3. Chemical	12M HCL (1:2) 1 M Acetic Acid (1:2)	[41]
	2,4,6,8 , 12 M HCL (5:1) 1 M Acetic Acid (5:1)	[3]
4. Enzymatic	Biomass + Phosphate buffer, Lysozyme , EDTA	[42]
	Biomass + HEPES Buffer, EDTA	[43]

PURIFICATION OF PHYCOCYANIN

Ammonium sulphate precipitation

Ammonium sulphate precipitation is a commonly used for protein purification. It used to seprate the several proteins based on their tendancy to precipitated **[14]**.

Khazi *et al.*, 2018, reported that put the beaker containing phycocyanin extract in another ice containing beaker and strir by magnetic stirrer,while stirring ammonium sulphate was added. After stirring ammonium sulphate precipitation solution is centrifuged for 15 minutes, then pellet was collected **[22]**.

The phycocyanin extract was suspended to single step precipitation using 65% ammonium sulphate and kept for overnight, then pellet was collected after centrifugation for 15 minutes **[7]**.

Zheng *et al.*, 2019, concluded that 50% ammonium sulphate extract was added to the phycocynin extract and kept 4 ˚C for 2 hrs then solution is centrifuged for 13 min, pellet was collected after centrifugation **[16]**.

Dialysis

The phycocyanin extract was dialyzed against Na-phosphate buffer for overnight at 4 ˚C, then phycocyanin fraction was collected **[44]**.

The crude phycocyanin was dissolved in a Na-Phosphate buffer and subjected into dialysis membrane to dialysis over night against same buffer, fraction was collected after dialyzed **[45]**.

Gel filtration

Gel filtration used to separate protein based on their size.

Khazi *et al*., 2018, concluded that dialyzed sample was suspended into sephadex G – 25 column. The column was eluted with Na -phosphate buffer, than blue colour fraction was collected **[22]**.

The crude phycocyanin was dissolved in Na – phosphate buffer was suspended to seralosegel filtration column,. The column was eluted with same buffer, then pphycocyanin fraction was collected **[46]**.

Kamble *et al*., 2013 reported that dialyzed sample was further passing through a sephadex G -25 column. The column which eluted with Na – phosphate buffer. Phycocyanin fraction was collected at 0.5ml/min flow rate **[44]**.

Ion exchange chromatography

Ion exchange chromatography is used to purify protein separate based on their affinity towards Ions. DEAE – sephadex was suspended into Na – phodphate buffer, pour these slurry into column. Load the phycocyanin fraction was obtained from the gel filtration on DEAE column. The column eluted witj same buffer, then fraction was collected **[22]**.

Dialyzed sample was suspended to anion exchange column containing Q – sepharose, then column was washed with Na -phosphate buffer and NaCl to remove bound or unbound proteins. Blue color fraction was collected **[47]**.

Ion exchange chromatography carried out by dialyzed sample was subjected to ion exchange column containing Q – sepharose, the column was eluted with acetate buffer, after sample was loaded the column was rinsed with same buffer and fraction was collected **[10]**.

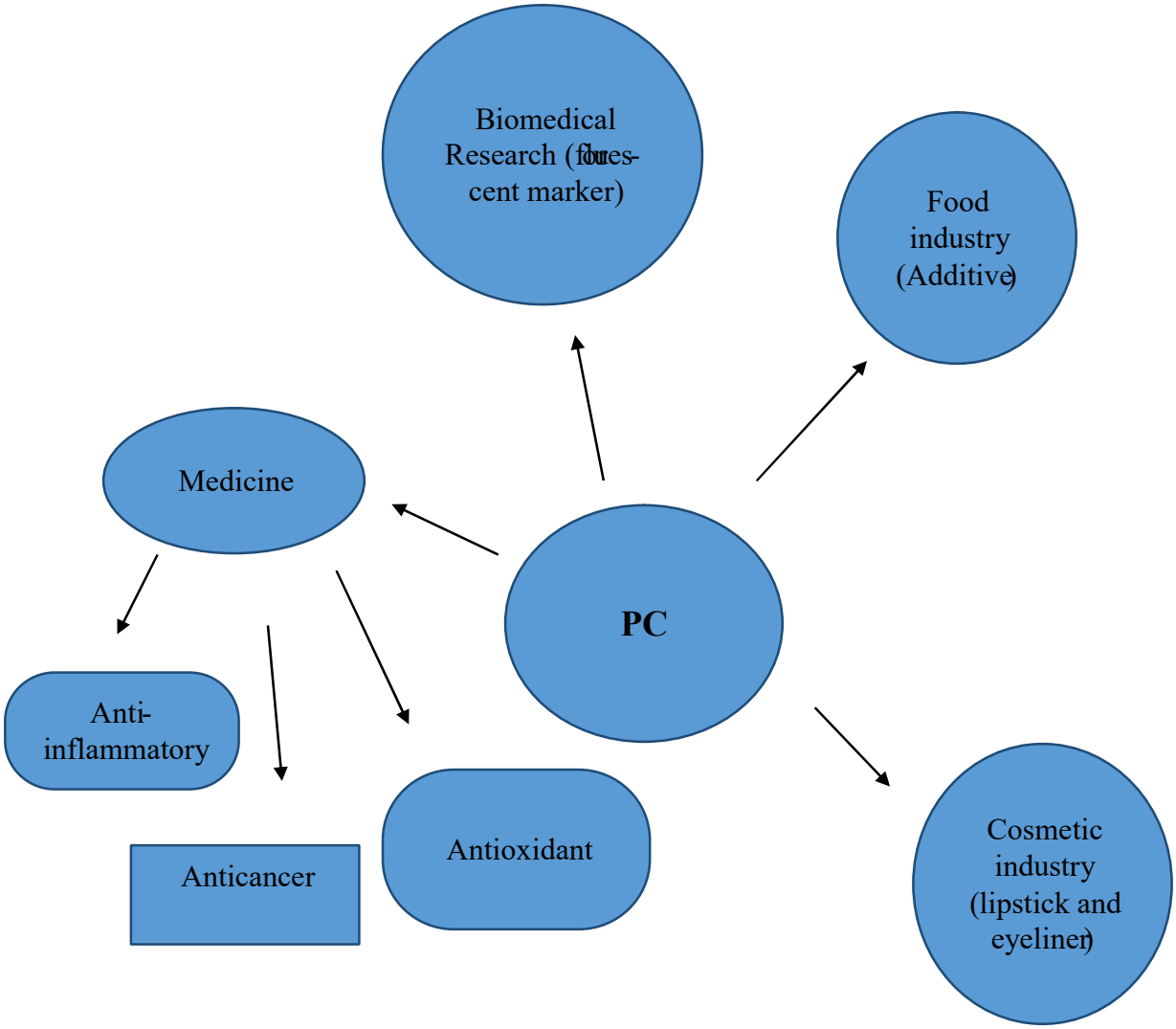

Figure 2: Application of Phycocyanin

APPLICATIONS OF PHYCOCYANIN

Phycocyanin gives blue color to some cyanobacteria. Phycocyanin is a warer soluble, fluorescent protein **[8]** and non toxic and non carcinogenic. It is used as fluorescent label for cell sorting **[9]**. Phycocyanin may be nitrogen storage compound in the cell **[5]**. Phycocyanin has numerous application in the food, cosmetic and pharmaceutical industry **[48]**. Phycocyanin has beneficial properties such as antioxidant **[40]**, anti-inflammatory **[7]**, also has properties such as radical scavenging properties **[20]**. Phycocyanin is used to diagnosis of several diseases, and in biomedical research as fluorescent markers **[8]**. The combination of Phycocyanin with other drugs that treat a particular disease **[9]**.It is used in cosmetic industry such as lipstick and eyeliners **[7]**.

Phycocyanin in food industry

Phycocyanin has been used as naturally occurring colorant for food additive purpose **[1]**. Phycocyanin has been used for healthy foods. It is a source of nutraceuticals **[8]**. Phycocyanin boost immune system, has properties such as antioxidant, antiviral anticancer, and antinflammatory **[6]**. Phycocyanin is used in chewing, gums, dairy products, **[7]** sherbets, soft drink **[23]**. It is used as food colorant, nontoxic, coloring agent in Food Industry. Phycocyanin used in a artificial modification of food. It has application as natural colorant in food and cosmetics. Some study reported that phycocyanin is used in foods with regards to color stability and rheological properties **[49]**. In 2013 phycocyanin was the first food and drug administration authorized natural dye in the united states to be used by food industry, since then demand of phycocyanin has been increased in other countries **[58]**. Consumers have some issue related to the use of food colorants. FDA have restricted the use of food colorant **[50]**. Therefore food manufacturers focus towards the natural food colorant. Microencapsulate phycocyanin has application in jelly candy as natural colorant **[51]**.

Phycocyanin as a natural dye

It is used as natural dye due to its deep blue color **[1]**. It is used as natural dye because of its having a stable color and rheological properties **[14]**. It has maximum fluorescence emission at 640nm **[2]**. Phycocyanin used in biomedical research as fluorescent markers since they have broad excitation spectrum and fluroresense with high quantam yield **[1]**. It is used in flowcytometry, histochemistry, immunoassay **[52]**. It is used in a cell sorting as flourscence probe. It is used for analysis of cell and molecules.

Phycocyanin as Antioxidant

Phycocyanin is resistant to oxidation because of its antioxidant properties **[51]**. Phycocyanin has deep blue color due to open tetrapyrrole chromophore **[53]**. It has similar structure as bilirubin, which is known as scavenger **[54]**. It consist GLU, ASP ALA LEU,ARG, ILE, SERGLY and THR, Which reported to be related to higher antioxidant activity level **[20]**. Phycocyanin is major biliprotein has antioxidant properties **[18]**. The antioxidant activity of a phycocyanin due to its ability to scavenge free radicals and react with other oxidant of pathological relevance **[54]**. Antioxidant activity of phycocyanin determine by using radical – scavenging assay **[20]**, free oxygen radical

scavenging system and fenton reaction phycocyanin were shown transfer electrons **[55]**.

Phycocyanin in a pharmaceutical industry

Phycocyanin has application in pharmaceutical industry. It has also reported that phycocyanin can inhibit cell proliferation **[14]**, induce apoptosis in carcinogenic cell lines and affect gene regulation in mammalian cell **[1]**. Phycocyanin inhibit tumor cell proliferation by block the DNA synthesis **[56]**.

Piroxicam is a drug that is used for rheumatoid arthritis. The piroxicam combine with phycocyanin on rat colon that increase the tumor inhibition rate **[9]**. Phycocyanin with doxorubician drug which reduce better antitumor effect and less toxic effect than single drug treatment **[9]**. Phycocyanin is a also applicable for inhibition of growth of drug resistant bacter. Phycocyanin can inhibit other enzyme activity than NADH oxidase and affect the gene regulation **[6]**. Different chemical agent used for radio sensitizing purposes ,but researcher believe that agents has side effect on human body therefore researcher has focus towards the natural radio sensitizing for radiation therapy, which not has any side effect to the human body that inhibit the COX-2 activity **[57]**. Several study show that phyocyanin has anticancer effect in both vitro and vivo on different cancer like lung cancer, colon cancer,breast cancer.It has potential application in nanotechnology **[58]**. Phycocyanin is a natural anti-inflammatory agent inhibiting the effect of response mediated by the histamine release from mast cell **[19]**. It has also used in DNA staining **[1]**.

CONCLUSION

Spirulina is a gram negative cyanobacteria found in a tropical and subtropical area. *Spirulina* is a natural source for proteins, vitamins, nutrients, and minerals. *Spirulina* mainly known for its high content of phycocyanin. Phycocyanin is a blue deep color, light harvesting, and water soluble protein. Phycocyanin has numerous application as natural dye. It has fluorescent properties therefore used as flourescent tag in flow cytometry, histochemistry, immunoassay. Phycocyanin is used as natural colorant in food and cosmetic industry. In Recent trends of phycocyanin for their potent anticancer activity have attracted much attentions. Phycoyanin to be considered as promising chemotherapeutic agent in the future. Phycocyanin has been extracted from *spirulina* by using different extraction method, and purified by using different purification method. In these

different methods freeze and thaw considered as simplest, reproducible and does not change any biological function of protein. **Moraes *et al*., 2011, [3]** get the highest yield by using sonication with glass pearls and 12 M Hcl. **Sivasankari *et al*., 2014, [41]** get the highest yield using freeze and thaw method and using 12 M HCl get the poor yield. Using freeze and thaw at 21 ˚C and thawed at 4 ˚C. **Doke *et al*., 2005, [39]** got the highest yield and using homogenization show less yield compare to freeze and thaw. **Saran *et al*., 2016,** get the highest yield of phycocyanin using freezing at -20 ˚C and thawing at 4 ˚C. **Kumar *et al*., 2014, [7]** get the highest purity of phycocyanin using ion exchange chromatography compare to dialysis and concluded that purity increased nearly six time after purification with ion exchange chromatography. **Abalde *et al*., 1998 [10]** get the best result of purification by using ion exchange chromatography containing Q -sepharose and isocratic system with mobile phase. **Patil *et al*., 2013 [2]** reported that phycocyanin by aqueous two phase extraction yield 3.23 purity. **Thangam *et al*., 2013 [46]** get the pure phycocyanin by using precipitation technique combine with chromatographic method.

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