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# Isolation and Characterization of Two New Isolates of *Sprirulina Platensis* from the Rice Fields of Visakhapatnam

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**Abstract:** ***Spirulina belongs to the family Oscillatoriaceae which is characterized by spiral shaped chains of cells. Spirulina is one of the rich sources of proteins and is used as a protein supplement by humans. It is also used as a supplement is poultry industry and aquaculture units. It is also cultivated for its bluish green pigment, called Phycocyanin and of all the microbial sources Spirulina is the major source of phycocyanin. Purification and stability of the phycocyanin is often the limiting step in its use in the pharmaceutical applications. In the present study, two new cultures of Spirulina Platensis were isolated from the rice fields of Visakhapatnam and characterized. The two cultures were named SP2 and SP3. Phycocyanin was extracted from these two cultures using three different methods viz., Liquid Nitrogen method, freezing and thawing method and Lysozyme methods. Among these three methods, Liquid Nitrogen method was found to yield highest quantity of Phycocyanin. Absorption spectral scanning of phycocyanin was done using spectrophotometer at 500, 540, 580, 620, 660, 700 °A. The readings at these absorbances were 0.278, 0.59, 1.538, 2.157, 0.573, and 0.075 respectively. The highest absorbance values were obtained at 620 °A. The extracted phycocyanin was purified in a step wise manner using Ammonium sulphate precipitation, dialysis, Sephadex G25 and G100 column chromatography. The purity of the phycocyanin was checked by measuring the ratio of A620/A280 values. Maximum purity of 4.98 was obtained after the sephadex G100 step. With an aim of cloning the Phycocyanin gene from these new cultures, primers were designed using the sequence of phycocyanin gene from Synechococcus sp. in the NCBI nucleotide database. A partial sequence of apo α phycocyanin was amplified and cloned consisting of 271 bp of β subunit and 363 bp of α subunit. The α subunit and β subunit were separated by 103 bp intergenic sequence.***

**Keywords:** Micro algae, Spirulina platensis, Phycocyanin, Purification, cloning

## 1. Introduction

Microalgae can be utilized as protein source because of their fast growth rates and high protein contents. Microalgae represent all photosynthetic prokaryotic and eukaryotic microorganisms and most of them live in aquatic environments [7]. During the last two decades the utilization of microalgae and other forms of microorganisms as source of Single Cell Protein (SCP) has gained increasing interest. Among the microbial protein sources *Spirulina* occupies the foremost position. Commercial mass production of *SpirulinaPlatensis* as a food supplement has existed since the late 1970s [5]. At first glance *Spirulina* seemed to be a good protein source, however further research revealed that it is a rich source of Vitamins, Minerals and β - carotene. With these revelations *Spirulina* was declared as the best food for tomorrow by United Nations at the World Food Conference in 1974 due to its unmatched composition [6] Recently, *Spirulina* has been commercially cultivated for its bluish green pigment called Phycocyanin. Of allthe microbial source *Spirulina* is the major source of Phycocyanin. The phycocyanin from *Spirulina* has antioxidant [8], anti - inflamatory and hepatoprotective properties [9]. The Phycocyanin is used as colourant in food and cosmetic industries. The recovery of C - Phycocyanin has been attempted previously. In light of its many commercial applications in food and pharmaceutical industry, purity of the pigment plays a major role. Especially pharmaceutical industry demands a highly pure phycocyanin with a A620/A280 ratio of 4.0 and food industry a ratio of 2.0. Herrera et al., 1980 reported a protocol that involved stages of harvesting, drying and milling, extraction with salts (NaNO3 and CaCl2), adsorption, ultrafiltration, precipitation, dialysis, gel filtration and ion exchange chromatography. This procedure resulted in C - Phycocyanin of two types: Food grade with purity of 0.74 and reactive grade with a purity of 3.9. The main disadvantage with this procedure is that it contains ten number of unit operations. But Polmares et al., 2001 has developed a prototype which involves only five stages with a purity of 3.8. A simple and cost effective purification protocol with minimum number of steps is worth attempting.

The precursor for the biosynthesis of phycobilisomes is Heme. Heme is present in abundance in the cytoplasm of many prokaryotes and unicellular eukaryotes including microalgae. The heme is converted into biliverdin by the enzyme Heme Oxygenase (HOI). Biliverdin is converted into phycocyanobilin by an enzyme bilin reductase (PcyA). This phycocyanobilin combines with apo - α - phycocyanin to give holo - α - phycocyanin [17]. With an aim to reconstitute this entire pathway in E. Coli an attempt was made to clone apo - α - phycocyanin. As the multiplication rate of bacteria is faster than microalgae and can be done in fermenters this procedure can be utilized for commercial extraction of phycocyanin.

## 2. Materials and Methods

**Isolation of Local strains of *Spirulina Platensis*:** The water samples were collected from the rice fields of Visakhapatnam and are used for the isolation of *Spirulina Platensis*. The samples were inoculated into 100ml of sterile Zarrouk's medium (ZM) in Erlen Mayer flasks. The samples were incubated in the growth chamber having a facility to maintain a light intensity of 3000 lux with 16/8 h alternate

light and dark cycles at 28°C. The flasks were examined for algal growth periodically. They were isolated and microscopically identified as *Spirulina Platensis*. They were maintained on ZM agar slants.

**Purification of *Spirulina Platensis* culture:** The cultures were repeatedly transferred to Zarrouk's medium and observed for the purity under the microscope and further purified using agar streak plate method. The bacterial contamination was eliminated using triple antibiotic solution (10µg Penicillin+ 10µg Streptomycin+ 1µg Chloramphenicol in 1ml of water).

**Extraction and estimation of total proteins from *Spirulina platensis* cultures:** The cultures were ground in a pestle and mortar using ice cold 100mM Phosphate buffer (pH 7.2). The contents were centrifuged at 10, 000 rpm for 10 min. The aqueous phase was used for further experiments. The total protein content of the *Spirulina platensis* cultures were estimated by Lowry's method [12].

**Extraction of Phycocyanin from *Spirulina platensis* cultures:** Phycocyanin extraction was taken up on the 10th, 20th and 30th day after inoculation. Three different methods were followed for extraction of phycocyanin from *Spirulina platensis*.

**Liquid Nitrogen Method:** 10 ml of uniformly grown culture was centrifuged and the pellet was washed with distilled water. Liquid Nitrogen was added and the cells were macerated to a fine powder using pestle and motor. The powder was transferred to a tube containing 6 ml of sodium phosphate buffer (pH 7.0) and incubated for 2 h at 4°C. After the incubation, the contents were centrifuged at 4000 rpm for 30 min.

**Freezing and Thawing Method:** 10 ml of uniformly grown culture was centrifuged and the pellet was ground in 6 ml of sodium phosphate buffer pH 7.0 and kept for freezing at -70°C. Care was taken to keep the solution below 5°C while thawing the cells. The solution was centrifuged at 4000 rpm for 30 min. **Lysis buffer and Lysozyme method:** 10 ml of uniformly grown culture was centrifuged and the pellet was washed with distilled water. To the pellet 100µl of lysis buffer (1M Tris, 0.5M EDTA, 25% sucrose) was added. Finally Lysozyme was added to a final concentration of 5 µg/ml and the cells were suspended thoroughly and incubated at 37°C for 2 - 4 h. The contents were centrifuged at 4000 rpm for 30 min and bluish supernatant was collected.

**Purification of C - Phycocyanin:** C - Phycocyanin was extracted from Spirulina Platensis using liquid nitrogen procedure as described above. The blue supernatant was taken as crude phycocyanin and further purification were carried out as follows.

**Ammonium sulphate precipitation:** The protein precipitate from the 30% (w/v) ammonium sulphate was discarded. The precipitate from the 50% (w/v) ammonium sulphate solution was taken and dissolved in a small volume of 0.05M Na - phosphate buffer (pH 7.0). **Dialysis:** A dialysis tube of length 10 cm having a diameter of 6 mm was taken and in this tub the ammonium sulphate fraction was subjected to dialysis for 7 h against 0.05M Na - phosphate buffer (pH 7.4) which was diluted 1000 times. **Gel exclusion chromatography:** After incubation of sephadex G 25 and G 100 for 3 days in sterile water the columns (1.5x20cm length) were packed by running 0.005M Na - phosphate buffer (pH 7.4) continuously. The dialyzed fraction was subjected to the column with 0.005M Na - phosphate buffer at 1 ml $min^{-1}$. Fractions were collected every 5 min and the fractions were subjected to absorption scanning to identify the fraction which was showing absorption maximum at 620 nm. **Analysis of C - Phycocyanin for purity:** Purity of C - Phycocyanin was calculated by the ration A620/A280.

**Cloning of a fragment of C - Phycocyanin gene:** Genomic DNA from Spirulina platensis was isolated using a method using CTAB described by Melody (1997) with a slight modification. The DNA was used as a template to amplify a fragment of C - Phycocyanin gene using the primers sense – 5' AAGCAGCACCGAAATCCAAG 3' and antisense – 5' TTCAGGGCTTCAATGTACCA 3'. The primers were designed taking the gene sequence from *Synechocystis sp.* The amplified fragment was cloned into pBluescript vector and sent for sequencing to know the sequence.

## 3. Results

**Isolation of Spirulina cultures from the water samples:** Two cultures of Spirulina were isolated from the water samples of the Visakhapatnam district of Andhra Pradesh. These were identified as Spirulina Platensis and were designated as SP2 and SP3 respectively (Table 1) (Figure 1). The morphological characters of these cultures are similar to that of the reference strain SP4 with less spiralling and blunt ends with florescence green appearance.

**Table 1:** Table showing cultures used in the study and code given to the newly isolated cultures

| S No | Culture | Source | Code | Remarks |
|---|---|---|---|---|
| 1 | CS1 (Plate 1) | CFTRI, Mysore | SP1 | Reference strain |
| 2 | SP (Plate 2) | MRCC, Chennai | SP4 | Reference strain |
| 3 | Vizag (Plate 3) | Visakhapatnam | SP2 | New strain |
| 4 | Vizag (Plate 4) | Visakhapatnam | SP3 | New strain |

**Figure 1:** The four Spirulina cultures used in the study SP1 and SP4 are used as reference strains and SP2 and SP3 are the new isolates. The pictures were taken at 40 X magnification

**Total soluble protein content of Spirulina platensis cultures:** The total protein content of the different Spirulina platensis cultures was estimated at 10, 20 and 30 days after inoculation (DAI) (Table 2). There was a linear increase in the protein content with respect to increase in the incubation period. Among the cultures, the reference strain, SP1 registered masimum protein content in all the three sampling periods. Not much variation was observed between the SP2 and SP3 cultures. On the 30$^{th}$ day SP1 recorded the highest protein content (642.12 µg/mg dry weight) while SP3 registered the lowest (612.58µg/mg dry wt.) protein content. The total proteins of the four cultures were checked on SDS PAGE. The banding pattern was similar in all the cultures without much variation in the expression levels of the proteins among different cultures (Figure 2). The molecular weight of the proteins varied from 15kD to 80kD.

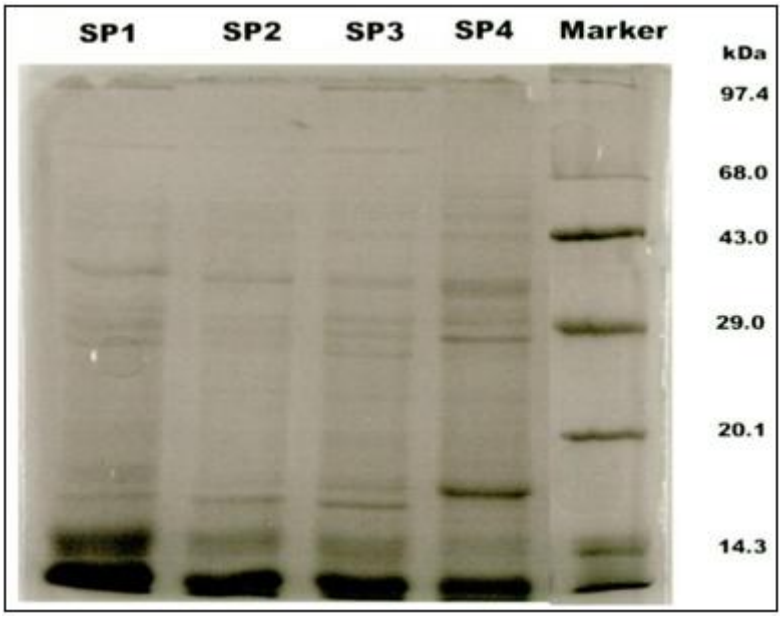



**Figure 2:** An SDS PAGE gel of total proteins from the four *Spirulina* cultures SP1, SP2, SP3 and SP4. Total proteins were extracted after 30 DAI.

**Table 2:** Total protein content of Spirulina platensis cultures after different days of inoculation

| Protein Content (µg mg $^{-1}$ dry wt) | | | |
|---|---|---|---|
| Cultures | 10 DAI | 20 DAI | 30 DAI |
| SP1 | 214.04 ± 1.6 | 418.12± 1.1 | 642.12± 1.4 |
| SP2 | 187.65 ± 0.9 | 405.90± 0.6 | 623.70± 1.6 |
| SP3 | 204.20± 1.8 | 409.69± 1.2 | 612.58± 1.7 |
| SP4 | 208.05± 1.7 | 414.98± 0.8 | 624.15± 1.9 |

**Phycocyanin pigment content of Spirulina platensis cultures:** The phycocyanin pigment extracted from Spirulina platensis culture was estimated at 10, 20 and 30 days after inoculation (DAI) using three different methods viz. Liquid nitrogen method, freezing and thawing method and lysozyme method and the data are given in Table 3. Among the three extraction methods used in this study the highest amount of phycocyanin was extracted using Liquid Nitrogen method. There was no significant difference between the amount of phycocyanin extracted by freezing/Thawing method and Lysozyme method. Among all the four culture used in this study, SP1 culture showed the highest phycocyanin content (110.2 µg/mg) at 30 DAI by liquid Nitrogen method followed by SP4. The least phycocyanin content (58.47 µg/mg) was extracted from SP3 culture (Table 3).

**Purification of Phycocyanin:** The phycocyanin pigment obtained by liquid nitrogen extraction method from the SP1 culture was crude by visible observation. The crude phycocyanin was purified in a step wise manner by ammonium sulphate precipitation, dialysis, followed by sephadex G25 column purification and sephadex G100 column purification. The purity of the phycocyanin was checked by A620/A280 ratio at each step of purification. The ratios were 0.97, 1.43, 3.73 and 4.98 for crude extract, ammonium sulphate precipitation, purification with dialysis, Sephadex G25 column purified phycocynin and Sephadex G100 column purified phycocyanin respectively (Table 4). Visible observation of phycocyanin after each purification step clearly showed the development of blue colouration (Figure 3).

**Table 3:** Phycocyanin content in four Spirulina cultures extracted by three different extraction methods at various stages after inoculationaverage of three replications with standard error. (µg mg $^{-1}$ dry weight)

| Spirulina culture | Liquid Nitrogen Method | | | Freezing and Thawing Method | | | Lysozyme Method | | |
|---|---|---|---|---|---|---|---|---|---|
| | **10 DAI** | **20 DAI** | **30 DAI** | **10 DAI** | **20 DAI** | **30 DAI** | **10 DAI** | **20 DAI** | **30 DAI** |
| SP1 | 36.73 ±0.78 | 71.45 ±0.57 | 110.2 ±0.39 | 32.98 ±0.68 | 62.12 ±0.55 | 101.8 ±0.97 | 31.03 ±0.59 | 61.96 ±0.55 | 101.62 ±0.77 |
| SP2 | 34.81 ±0.53 | 64.72 ±0.49 | 89.14 ±0.68 | 29.02 ±0.88 | 52.28 ±0.78 | 78.42 ±0.63 | 28.57 ±0.39 | 54.14 ±0.59 | 88.21 ±0.88 |
| SP3 | 31.70 ±0.29 | 61.24 ±0.55 | 65.12 ±0.69 | 28.22 ±0.77 | 38.98 ±0.49 | 58.47 ±0.65 | 28.01 ±0.53 | 58.84 ±0.46 | 60.24 ±0.49 |
| SP4 | 33.08 ±0.39 | 66.19 ±0.71 | 99.24 ±0.73 | 29.12 ±0.61 | 59.48 ±0.22 | 89.22 ±0.51 | 28.81 ±0.30 | 89.41 ±0.52 | 89.12 ±0.73 |

**Table 4:** Purity of Phycocyanin extracted from SP1 culture at various stages of purification

| Methods | Absorbance ratio A620/A280 (C - Phycocyanin) |
|---|---|
| Cell extract | 0.97 |
| Precipitation with (NH4) 2SO4 (50% w/v) | 1.43 |
| Sephadex G - 25 | 3.73 |
| Sephadex G - 100 | 4.98 |

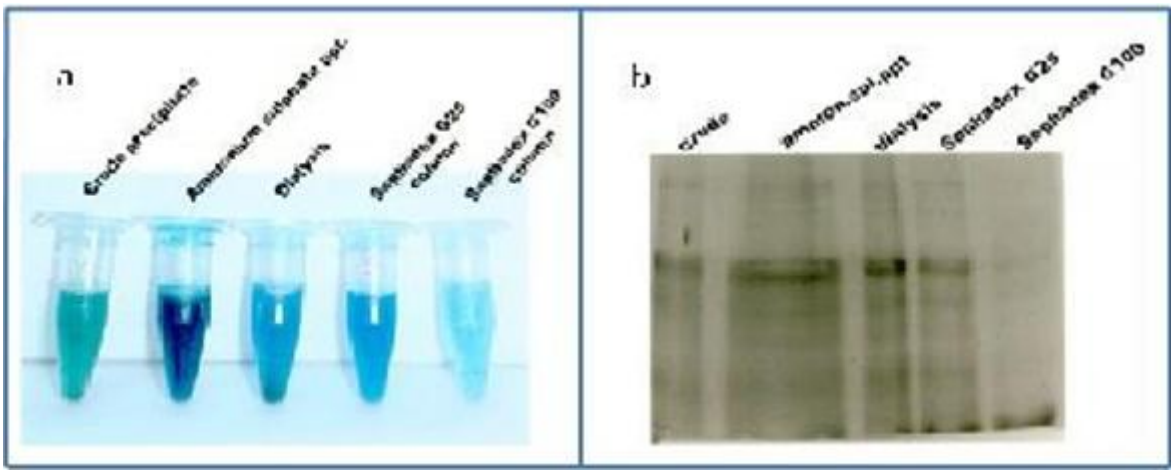
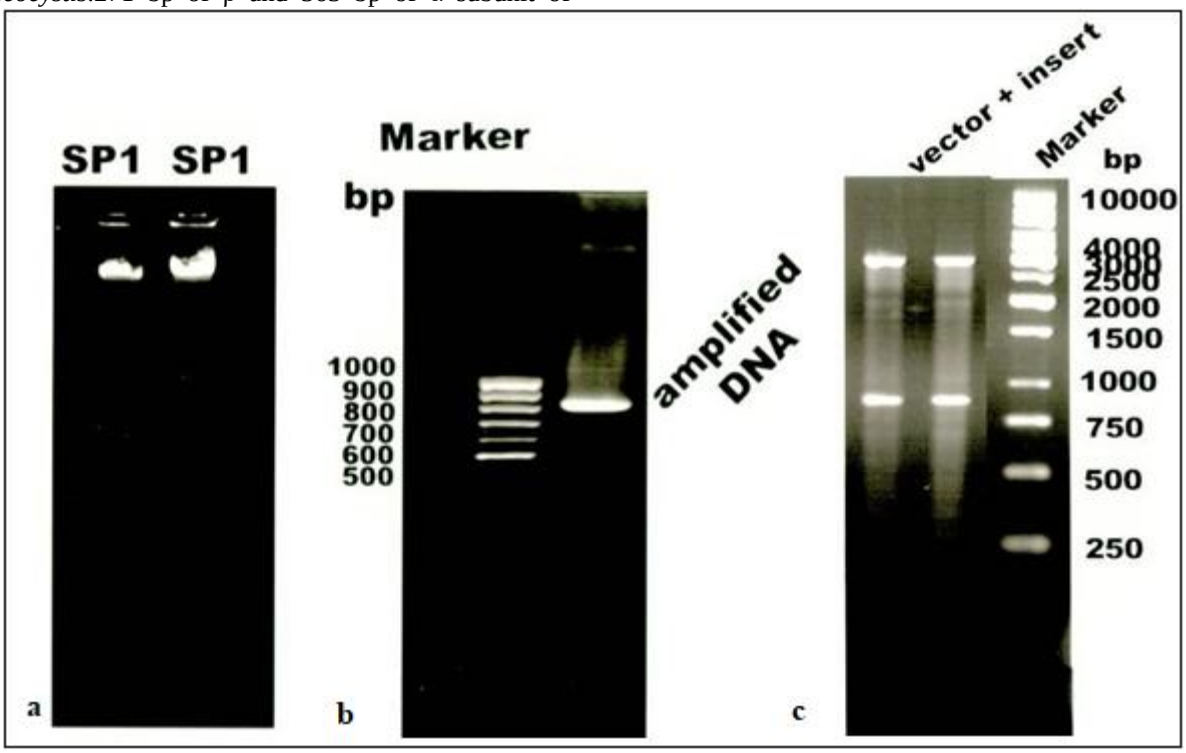



**Figure 3:** Purification of Phycocyanin pigment extracted from SP1 culture. a) Physical appearance of the pigment after various stages of purification. b) Corresponding SDS PAGE gel of the phycocyanin pigment after each step of purification.

**Cloning of apo α phycocyanin gene from the genomic DNA:** The PCR amplification was done using the DNA extracted from SP1 strain as the template and the primers mentioned previously. When the product was checked in 1.0% agarose gel, only a single band of around 750 bp was observed indicating there was no non - specific binding.

After cloning the amplified product into pTZ57R/T vector, it was purified and sent for sequencing (Figure4). The sequence showed 90% similarity with the reported sequence of *Synecocystis*.271 bp of β and 363 bp of α subunit of phycocyanin gene was obtained. A spacer region of 103 bp was obtained in between the β and α subunit of phycocyanin gene.




TTC CTG CGC TAT GTT AGC TAC GCT ACC TTA GCC GGC
GAT GCT AGC GTT CTC GAA GAT CGT TGT TTA AAC GTC
PGC GTG AAA CCT ACC AAG CTC TGG GTG TTC CTG GTG
CTT CCG TTG CGG CTG GCC TTG CCA AAA TGA AAG ACG
CTG CGG TTA AAA TTG CTA ACG ACC CCA ACG GCA TCA
CCC AAG GGG ATT GCA GCC AAT TAA TGT CTG AAG TTG
CTA GCT ACT TCG ATC GCG CTG CTG CTG CTG TTG CCT
AAA AAT AGC CTT GTC TAT CCG GTC TAG AGC AAT TAG
ACC TGC TCA ATT AGA CCT TCG GCA CAA ATT TTT TAA
TCA CAA ACA ATC CGG AAA CAG AGT TTA AGT TTA GGA
GAA TCC GAG AAC AAT GAA GAC CCC TAT CAC CGA AGC
AAT TTC TTC TGC TGA TTC CCA AGG ACG CTT TCT GAG
CAA CAG CGA ACT GCA ATC TGT ACG TGG CCG CTT CGA
GCG CGC TAC TGC CAG CAT GGA AGC GGC TCG CGC TTT
AAC TCA GAA TTC TAG CTC CTT AGT AAG CGG TGC GGC
TAA CGC CGT TTA CCA AAA ATT CCC TTA CAC CAC CCA
AAT GCA AGG GCC TAA CTA CGC TTA CGA CCA ACG CGG
TAA AGA CAA ATG CGC TCG TGA CAT CGG TCA CTA CCT
GCG CAT GGT GAC TTA CTG CCT GAT TTC TGG TGG TAC
CGG TCC TAT GGA TGA GTA CTT AAT TGC TGG TTT AGA
TGA AAT CAA CAG CAC CAA TGG GGG NGA AAA AA

**Figure 4:** a) Agarose electrophoresis showing the whole genome extracted from Spirulina sample. b) PCR amplified product of approximately 750 bp partial phococyanin gene was obtained using the gene specific primers. c) Agarose gel showing the

vector pTZ57R/T (3000bp) along with the insert (750bp) after the cloning. d) Sequenced PCR product of Partial phycocyanin gene, a 271 bp of β and 363 bp of α subunit of phycocyanin gene was obtained along with 103bp spacer DNA.

## 4. Discussion

The cyanobacterial pigments are gaining importance nowadays not only as a food colourant but also as a very effective medicine for curing various diseases in humans. An appealing colour of food and beverages is an important characteristic that goes a long way in enhancing the aesthetic value and consumer preference. Many synthetic dyes have entered the market in the middle of the last century but which have ill effect on the human health and can even cause cancer. Spirulina platensis is a promising cyanobacterium, has grown international demand for its high value chemical constituents. It has found application in the health food, therapeutics and specialized feeds [1]. In India during the last decade has seen a considerable interest both in the R&D front and in its commercialization [13]. Spirulina platensis is also cultured for its commercially important fluorescent bluish - green pigment, phycocyanin, which is used as a natural colouring agent for food, drug and cosmetic industries to replace the currently used synthetic pigments that are suspected to be carcinogenic. In the light of its many applications in the food and pharmaceutical industry, purity of the pigment plays an important role. Especially the pharmaceutical industry demands a very pure phycocyanin with a A620/A280 ratio of 4.0 and above and food industry a ratio of 2.0.

In the present study, the protein content of *Spirulinaplatensis* cultures was measured at 10, 20 and 30 days after inoculation and it was observed that the protein content has increased with increase in the incubation period. On an average the strains studied have a protein content of around 55%. These results are in accordance of with Bhattacharjee (1970) who stated that *Spirulina platensis* contain around 60% protein and about 50, 000 kg of protein per hectare could be produced annually. When the total proteins were extracted from the whole cells of *Spirulinaplatensis* and resolved on a 15% SDS PAGE gel, it showed a wide range of polypeptide subunits, the molecular weight of which ranged from 20 to 94 kDa. It is in corroboration with the finding of Chadgothia and Srivastava (1994) who stated that the total protein content of Spirulina platensis cultures varied widely when resolved on a SDS PAGE gel.

Among cyanobacteria, *Spirulina* is a rich and inexpensive source of the phycocyanin pigment [14]. The stability of the pigment at acidic pH of 4.5 is now being exploited in food products and soft drinks [13]. Pharmaceutical industry demands a highly pure phycocyanin with a A620/A280 ratio of 4.0 and above and food industry a ratio of 2.0. Herrera et al., (1989) reported a protocol that involves stages of harvesting, drying and milling, extraction with salts (NaNO3 and CaCl2), adsorption, ultrafiltration, precipitation, dialysis, gel filtration and ion exchange chromatography. This procedure resulted in C - phycocyanin with a maximum purity of 3.9. The main disadvantage of this method is the number of stages which increases the cost of production. In the present study three different extraction procedures were employed *viz*, liquid nitrogen method, freezing and thawing method and lysozyme method. Out of these three the simplest and most efficient method was found to be Liquid Nitrogen method of extraction (Table 3). The reason could be better breakage of the cell when exposed with - 196°C liquid nitrogen followed by mechanical shearing in a pistle which results in better release of the pigment. Similar results were observed by Chen and Zang (1997) who reported that freezing at - 20°C, followed by repeated thawing for 1 hr followed by sonication improved the phycocyanin extraction.

Lee et al., (2001) obtained pure phycocyanin from Spirulina platensis by combining ammonium sulphate precipitation with different chromatographic techniques. In the present study as well two step precipitation procedure followed by purification with dialysis and chromatographic techniques resulted in obtaining pure phycocyanin with a purity of > 4.0. When a concentration of 30% w/v ammonium sulphate was used it resulted in precipitation of unwanted proteins which were discarded. The supernatant was subjected to 50% w/v ammonium sulphate precipitation which resulted in the precipitation of the phycocyanin pigment which was verified by absorption spectral scanning. The precipitation was then subjected to dialysis and gel filtration using sephadex G25 column followed by sephadex G100 column which resulted in a purity of 4.98. It is in corroboration with the results obtained by Zhang and Chen (1999) who reported a ratio of 5.13.

The pathway for the biosynthesis of phycocyanin has been elucidated in *Synechocystis* sp. For the biosynthesis of phycobilisomes the precursor is heme. Heme is present in abundance in the cytoplasm of cyanobacteria. The heme is converted into biliverdin with the help of the enzyme Heme Oxygenase (HOI). Biliverdin is converted into phycocyanobilin by the enzyme bilin reductase (PcyA). The phycocyanobilin combines with apo - α - phycocyanin to give holo - α - phycocyanin with the help of lyase isomerase [17]. The entire pathway from heme to a particular holophycobiliprotein subunit has been reconstituted in *Escherichia Coli* [17]. In the present study a partial gene of apo α phycocyanin was amplified using gene specific primers and a product of about 850bp was obtained.271 bp of phycocyanin β subunit and 363 bp of α subunit was obtained in the amplified product. Another interesting finding is that a space sequence of 103 bp was observed in between the two subunits.

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