

Antibacterial activity of extracted phycocyanin from *Oscillatoria* sp.

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ABSTRACT

The intention of the proposed work is to study on extraction of phycocyanin pigment from blue green algae and its antibacterial activity. The water sample was collected from Mettur dam and brought to the laboratory for isolation and microscopic analysis. The isolated colony was found to be *Oscillatoria* sp. and it was grown in BG 11 medium. The *Oscillatoria* sp. was grown in 1000 mL and centrifuged for biomass. Then the centrifuged biomass was weighed and used for extraction of phycocyanin. On analysis of the phycocyanin from *Oscillatoria* sp. it was found that the phycocyanin concentration was 2.39 mg/mL and Extraction yield was obtained to be 4.735 mg/g. The protein estimation of the phycocyanin pigment using Lowry's method was found to be 273.8 µg/ mL. Phycocyanin pigment was used to identify the antimicrobial activity against three pathogenic bacteria. Finally the antimicrobial activity was carried out on Muller hintonQ agar plates with different concentrations of phycocyanin such as 20 µL (4.48 µg), 40 µL (13.44 µg) and 60 µL (17.92 µg). The obtained results shows the ability of phycocyanin as antimicrobial agent against human pathogens.

INTRODUCTION

Microalgae and cyanobacteria are photosynthetic microorganisms that can be used in a wide variety of applications. These microorganisms can be effectively used in CO₂ capture from the atmosphere or from flue gas emissions and can remove nutrients from wastewaters, playing an important role in wastewater treatment processes (Razzak *et al.*, 2013). Nitrogen removal efficiencies between 80 and 90% and phosphorus removal efficiencies close to 100% have already been reported (Zhu *et al.*, 2013). Cyanobacteria, also known as blue green algae (BGA) are considered to be the oldest form of life on the earth. They possess a wide range of coloured components, including carotenoids, chlorophyll and phycobiliproteins. Phycobiliproteins (PBPs) are large water

soluble supra molecular protein aggregates involved in light harvesting in these organisms and may comprise as much as 40–60 % of the total soluble protein in these cells (Bogorad, 1975). Cyanobacteria with high levels of specific phycobiliproteins are of commercial interest. One common pigment is phycocyanin, which is a blue color. 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 has also shown to have therapeutic value (immuno-modulating activity and anticancer activity) (Lijima *et al.*, 1982).

The extraction solution most frequently used for extraction is a phosphate buffer in a relatively large concentration (0.001 M to 0.05 M) (Furuki *et al.*, 2003), but in some case 90% acetone (Zimba, 2012) or lysozyme (Boussiba and Richmond, 1979) is also suitable for cell wall rupturing. In these studies, the extraction protocols were followed by spectrophotometric (Sarada *et al.*, 1999), liquid chromatographic, capillary (Viskari and Colyer, 2003) and gel electrophoresis (Soni *et al.*, 2006) or single step chromatographic analyses.

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Most studies have dealt with species originating from tropical/subtropical freshwater alkaline lakes (*Spirulina platensis*, Silveira *et al.*, 2007, Furuki *et al.*, 2003), salt water bodies (*Oscillatoria quadripunctulata*, Soni *et al.*, 2006) or marine environments (*Synechococcus* sp., 6301 Yamanaka and Glazer, 1980; *Lyngbya* sp., Patel *et al.*, 2005). The marine cyanobacterial group is well documented for their bioactive compounds possessing antimicrobial properties, while there are only few reports available with fresh water cyanobacteria. In this work, we report the antibacterial activity of the C-phycoyanin extracted from *Oscillatoria* sp. isolated from fresh water.

MATERIALS AND METHODS

Sample collection and processing

The water sample was collected from the Mettur dam in 15 mL centrifuge tubes and brought to laboratory. The water sample was serially diluted and the single colonies were picked and inoculated in 1 mL of BG11 Medium.

BG 11 media For Growth

The isolated colonies of Microalgae grown in 1 mL of media were then transferred to 4 mL media followed by scaling up of media to 1000 mL medium. After the growth was completely observed it was taken for further study.

Morphology and Identification

The morphology of the microalgae was observed using Microscope at different magnification i.e. 10 X and 40 X . The culture was observed for its morphology and its structure.

Extraction of phycocyanin

1 M Tris-HCl Stock Solution Preparation

Phycocyanin was extracted from the fresh cyanobacterial strain biomass using the modified methods of Sarada *et al.* (1999). Thirty-four day grown cyanobacterial cells were harvested by centrifugation at 3000x g for 5 min at 20°C. Cell pellets were washed with 1M Tris-HCl buffer (pH 8.1). One volume of washed cell mass was re-suspended in five volumes of the same buffer and treated for the extraction of phycocyanin by physically (freezing-thawing method).

Physical extraction

The experiment was carried out to examine the effect of physical (freezing-thawing) method of phycocyanin produced by the cyanobacterial strain. In the physical approach, the wet biomass was frozen at -20°C (for at least 4 hours) then thawed at each 25°C in four cycles. In all the treatments, the cell debris were removed by centrifugation at 10,000 X g and the supernatant was pooled and labeled as crude extract.

Partial purification

The partial purification of the extracted phycocyanin pigment was carried out using ammonium sulphate precipitation

for overnight at 4°C on magnetic stirrer. After the Ammonium sulphate precipitation the pigment is centrifuged and taken for further studies.

Phycocyanin yield

The phycocyanin yield and extraction yield of phycocyanin is calculated by the following formulae (Bennett and Bogorad, 1973):

$$\text{CPC} = \frac{(\text{OD}_{620} - 0.474 \times \text{OD}_{650})}{5.34}$$

$$\text{Extraction Yield} = \frac{\text{CPC (V)}}{\text{DB}}$$

where, V is the solvent volume (mL) and DB is dry biomass (g).

Protein Estimation

Protein was estimated by the method of Lowry *et al.* (1951) using Bovine serum albumin as standard.

Collection of clinical specimens

The three clinical pathogens were obtained from Fermentation Technology Laboratory. Bacterial pathogens obtained were *Pseudomonas* sp., *Klebsiella* sp. and *Staphylococcus aureus*.

Maintenance of clinical pathogens

The samples were streaked on Blood agar, Nutrient agar and Mac-conkey agar and incubated at 37° C for 24 hours. The plates were observed after 24 hours of incubation to identify the types and number of colonies.

Examination of clinical pathogens

The examination of the pathogens was done using Microscopic examination and Biochemical tests to confirm the bacterial isolates.

Antimicrobial activity

The antimicrobial activity of the partially purified phycocyanin was carried out using Disc diffusion method against the three clinical pathogens and the results were observed and tabulated.

RESULTS

Sample Collection and Identification

The water sample was collected from Mettur dam in 15 mL centrifuge tubes and brought to the laboratory. The water sample was serially diluted and the single colonies were picked and inoculated in 1 mL of BG11 Agar. The identification were made using microscopic observation under light microscope. They show long filaments of cells which can break into fragments called hormogonia when were seen under 40 X observation (Fig. 1).

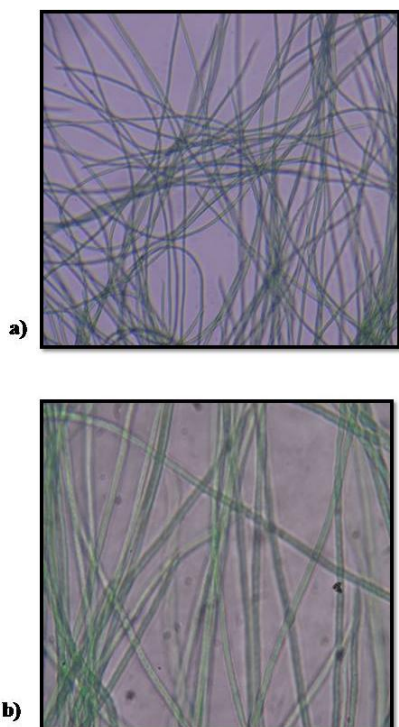


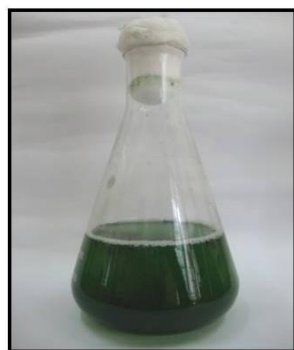
Fig. 1: Microscopic identification of *Oscillatoria sp.* In light microscope a) 10 X image, b)40 X image

Cultivation of Microalgae

The obtained strain was cultivated in 1000 mL of Erlenmeyer's Flask and it was kept in 16 hrs light and 8 hrs dark cycle. The growth was observed for three weeks and the OD value was checked using UV Spectrophotometer. The Maximum growth is attained when the OD value is more than 1 (Fig. 2).



a) *Oscillatoria sp.* In BG 11 medium (250 mL)



b) *Oscillatoria sp.* In BG 11 medium (1000 mL)

Fig. 2: Cultivation of cyanobacteria in BG 11 medium.

Biomass production

The *Oscillatoria sp.* grown in 1000 mL was centrifuged for biomass. Finally the centrifuged biomass was weight and used for extraction of phycocyanin (Table 1). The Fresh weight and dry weight of the Biomass was calculated (Fig.3a).

Table 1: Amount of Biomass recovered after 1 month

S.NO	Name of the organism	Weight of Wet biomass / mL	Weight of the dry Biomass
1.	<i>Oscillatoria sp.</i>	3.55g	1.2 gms

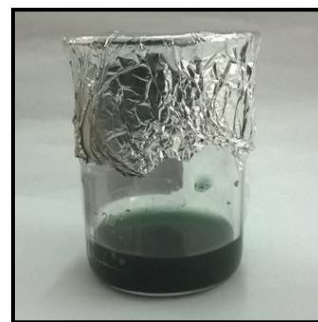


Fig. 3: a) Wet biomass of the *Oscillatoria sp.*



Fig. 3: b) Extracted phycocyanin pigment by freeze-thaw method.

Extraction of Phycocyanin Pigment

The Freeze thawed Biomass was centrifuged at 6000 rpm for 10 mins and the supernatant was taken for further studies (Fig.3.b). The C- phycocyanin concentration (CPC) in mg.mL^{-1} was calculated from the optical densities at 650 and 620 nm. On analysis of the phycocyanin from *Oscillatoria sp.* it was found that the Phycocyanin concentration was found to be 2.39 mg/mL (Table.2)

Table 2 : Phycocyanin yield obtained from *Oscillatoria sp.*

S.No	Processed Phycocyanin	C-phycocyanin concentration (mg.mL^{-1})	Extraction Yield (mg.g^{-1})
1.	Crude phycocyanin	1.21	3.51
2.	Ammonium sulphate precipitation	2.39	4.37

Partial purification

Partially purified pigment is centrifuged and its supernatant is taken as partially purified pigment. This phycocyanin is then processed for further studies. (Figure.7)

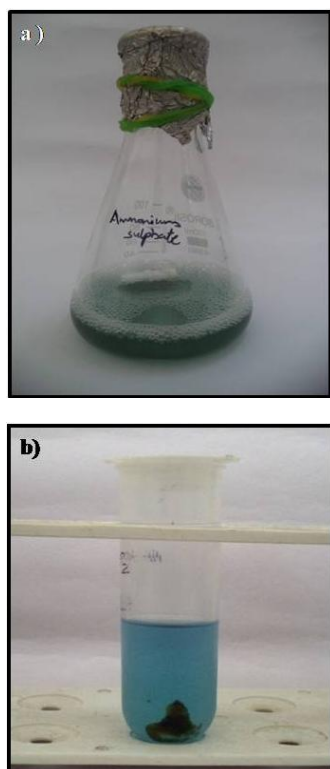


Fig. 7: Partial purification of phycocyanin pigment using ammonium per sulphate precipitation
a) Before centrifugation b) After centrifugation

Protein Estimation and Determination

Using lowry's method the protein was extracted and determined. The colour changes absorbance was read at 660 nm. Bovine serum albumin (BSA) was used as a standard and the R^2 value obtained was 0.995. The values were plotted and the sample value was identified (Table 3).

Table 3: Estimation of protein in *Oscillatoria sp.*

S.NO	OD value at 660nm	Amount of protein present in sample
1.	0.64	278.3 $\mu\text{g/mL}$

Collection of Bacterial Colonies

Pathogenic bacteria were obtained from Fermentation Technology Laboratory, Department of Microbiology, Periyar University, Salem.

Examination of Bacterial Isolates

Only three predominant individual colonies were selected and identified on the basis of colony morphological characteristics, Gram staining and biochemical characteristics (Table 4.)

Table 4: Morphological characteristics of the Bacterial Isolates.

Bacterial isolates	Microscopic examination			
	Gram staining	Spore staining	Motility test	Capsule Staining
S1	Gram Negative, Rod	Non spore forming	Motile	Non capsulated
S2	Gram negative, Rod	Non-spore forming	Non-motile	Capsulated
S3	Gram positive, Cocci	Non spore forming	Non-motile	-

Microscopic Examination

All the three selected bacterial colonies were examined under microscope after Gram staining, capsule staining, spore staining and motility test. The results were observed and recorded (Fig.4).

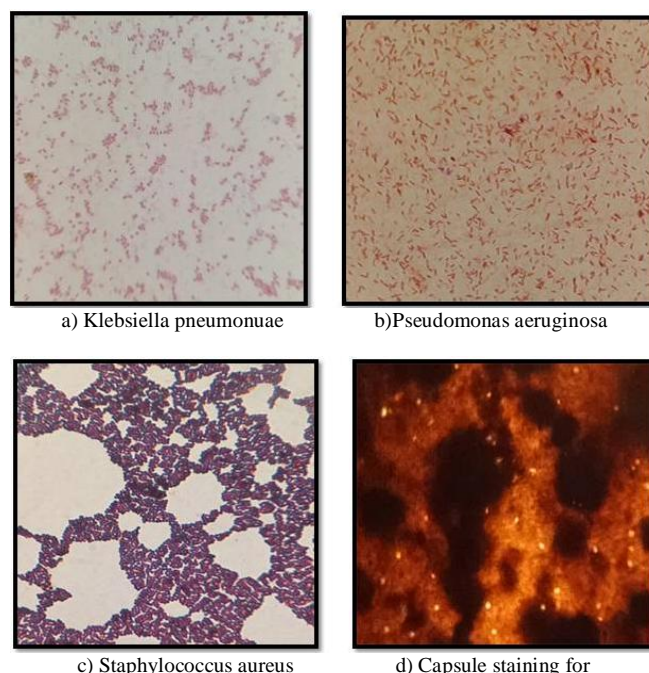


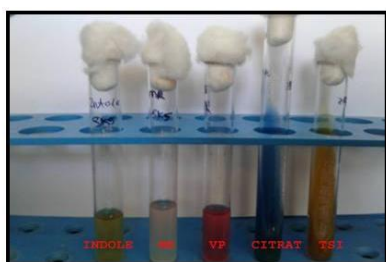
Fig. 4: Microscopic identification of clinical isolates gram staining (a, b, c,) Capsule staining (d).

Biochemical analysis of isolated bacterial strains

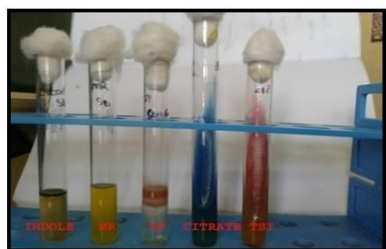
All the selected three bacterial isolates were characterized by the following biochemical analysis: Indole test, Methyl red test, Voges proskauer test, Citrate test, catalase test, oxidase test, Triple sugar iron test, Urease test, Carbohydrate fermentation test. Biochemical analysis results were observed and recorded (Table.5) (Fig.5).

Table 5: Biochemical characteristics of the Isolated Bacterial colonies.

Strains No	Indole	MR	VP	Citrate	TSI	Oxidase	Catalase	Urease	Carbohydrate fermentation test
S1	-	-	+	+	G+, H ₂ S-	+	+	-	-
S2	-	-	+	+	A+, H ₂ S-	-	+	+	+
S3	-	+	-	+	G+, H ₂ S-	+	+	-	+



Klebsiella pneumoniae



Pseudomonas aeruginosa

Fig. 5: Biochemical characteristics of clinical isolates.

Antimicrobial activity

Crude extract of *Oscillatoria sp.* showed maximum antibacterial activity against gram positive bacterial pathogens compared with Streptomycin. The phycocyanin pigment showed high activity against *Klebsiella sp.* compared to Streptomycin. The phycocyanin pigment showed high activity against *Klebsiella sp.* compared to the other two pathogens (Table 6) (Figure 8).

Table 6: Antibacterial activity of extracted phycocyanin by Disc diffusion method

Pathogen	Zone of inhibition			
	20 μ L	40 μ L	60 μ L	Streptomycin
<i>Pseudomonas aeruginosa</i>	5 mm	8 mm	9 mm	-
<i>Klebsiella sp.</i>	10 mm	12 mm	13 mm	6 mm
<i>Staphylococcus aureus</i>	-	-	1mm	11 mm

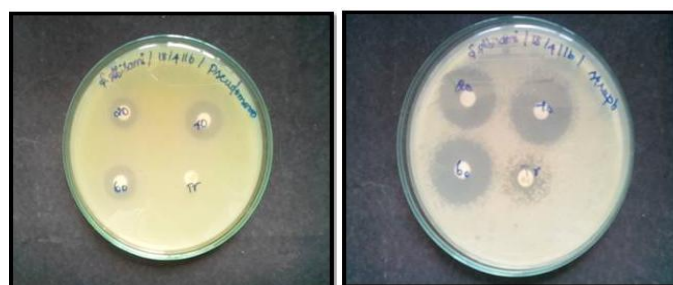
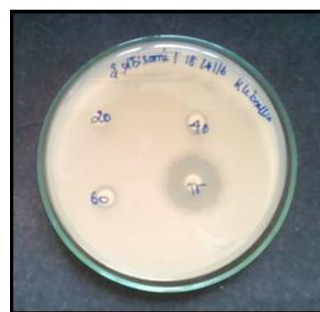
a) crude phycocyanin extracted from *Oscillatoria sp.*Fig. 6: Extracted phycocyanin from *Oscillatoria sp.*a) *Pseudomonas sp.*b) *Klebsiella sp.*c) *Staphylococcus aureus.*

Fig. 8: Antibacterial activity of the extracted phycocyanin against three human pathogens.

DISCUSSION

Rippika *et al.*, (1979) isolated *Anabaena constricta* and *Oscillatoria curviceps* from paddy fields of Cuddalore District. The filamentous cyanobacterium *Anabaena constricta* and *Oscillatoria curviceps* were grown in culture tube at $30^{\circ}\text{C} \pm 2^{\circ}\text{C}$ in the nitrogen-free BG-11 liquid and nitrogen medium respectively and pH was adjusted to 7.9. *Anabaena sp.* was grown in BG-11 medium without nitrogen source and *Oscillatoria sp.* was cultured in BG-11 medium with nitrogen source. These cultures were maintained in laboratory conditions in daylight fluorescent tubes for 16 hrs a day for 10 days. The cultured cyanobacterial species were used for further study.

Similarly, Bennett and Bogorod., (1973) took one volume of biomass and then resuspended in five times the volume of the same buffer followed by repeated freezing (-20°C) and thawing (4°C) shocks for the release of phycobiliproteins. The above procedure was continued till coloured supernatant was obtained from the biomass. The cell debris was removed by centrifugation at 12,000 rpm for 10 min. The absorbance of phycobiliprotein containing cell-free supernatants obtained by the centrifugation was measured at 562 nm, 615 nm and 652 nm using phosphate buffer as a blank. These wavelengths correspond to the absorption maxima of phycoerythrin (PE), phycocyanin (PC) and allophycocyanin (APC) respectively and was determined in spectrophotometer (Shimadzu, UV-1800, Japan). Showed that crude extract was subjected to a single step precipitation using 65 % $(\text{NH}_4)_2\text{SO}_4$ (Bio Xtra, [99 %; Sigma-Aldrich) and kept overnight at 4°C . The pellet was recovered by centrifugation at 27,000 rpm for 15 min at 4°C and dissolved in 10 mL of the same extraction buffer and termed as ammonium sulfate extract (ASE).

10 mL of ASE was dialyzed against the extraction buffer using dialyses membrane (Dialyses membrane-70, MWCO; 12–14 kD) procured from Hi-Media. Dialyses was performed twice against 1,000 mL extraction buffer, first at room temperature and again dialysed against 1,000 mL of extraction buffer at overnight. The resultant extract was recovered from the dialyses membrane and filtered through 0.45mL filter.

The antibacterial activity of *S. Maxima* extracts were assayed against five bacterial strains (*P. aeruginosa*, *B. subtilis*, *S. aureus*, *E. coli* and *Streptococcus* sp.) by evaluation of the inhibition of zones. Generally, all *S. maxima* extracts were found to be effective with antibacterial activity and were dose dependent. This phenomenon was in agreement with that found in this study. The most susceptible bacteria were *Streptococcus* sp. and *B. subtilis* to *S. maxima* with highest inhibition zones ranged 6–13 mm at concentrations of 100–400 µl/disk. It is of interest to note that all *S. maxima* extracts manifested similar degrees of susceptible towards both Gram-positive and Gram-negative bacteria. The lower inhibition zone ranged from 6 to 9 mm of extracts obtained for *S. maxima*.

CONCLUSION

The present work describes an efficient method for extraction and antibacterial activity of phycocyanin obtained from *Oscillatoria* sp. The partially purified extracts of phycocyanins could inhibit some Bacterial pathogens. The efficacy of phycocyanin on cancer therapy and antimicrobial activity against other bacterial pathogens will be tested in future. Phycocyanin is a natural blue pigmented protein is a water soluble and highly fluorescent. It is effectively active against human pathogens such as *Klebsiella* sp., *Pseudomonas* sp., and *Staphylococcus aureus*.

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Conflict of Interests: There are no conflicts of interest.

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