RESEARCH PAPER

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Screening of anti-bacterial activity of C-phycocyanin and its minimum inhibitory concentration (MIC) determination by agar dilution method

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C-phycocyanin was extracted from laboratory grown *Spirulina platensis*, and the identity was confirmed using standard C-phycocyanin, by HPTLC and SDS-PAGE. It was then partially purified and screened for antibacterial activity. The results were compared with standard C-phycocyanin (Sigma-Aldrich) and standard antibiotics. There were 6 bacterial isolates (Clinical isolates) assayed for antimicrobial activity. The study proved that the C-phycocyanin possessed antibacterial activity which was more potent than standard C-phycocyanin

Key words : C-phycocyanin, Antimicrobial activity, Partial purification, Screening, Minimum inhibitory concentrations

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INTRODUCTION

The blue green algae are the oldest oxygenic photosynthetic organisms. They are classified under the prokaryotic kingdom and are the source of novel bioactive compounds (Patterson *et al.,* 1994). Microalgae has recently received considerable attention, because of it's a source of novel antimicrobial substances, in addition to enzyme inhibiting, immune-stimulant, cytotoxic and anti-plasmodial activities The micro alga *Spirulina platensis*, is used as a source of food since ancient times due to its high nutritional value(Dillon *et al.,*1995). *Spirulina platensis* was also reported to present antimicrobial activity (Demule *et al.,* 1996; Ozdemir *et al.,* 2004) as well as to inhibit the replication of several viruses, such as herpes simplex and HIV-1(Ayehunie *et al.,* 1998; Hernandez-corona *et al.,*2002).

The main objective of the present study was to find out the antibacterial activity of C-phycocyanin obtained from laboratory grown *Spirulina platensis* and to compare the efficacy with standard C-phycocyanin and the available chemotherapeutic agents.

RESEARCH METHODOLOGY

Cultivation and maintenance:

The mother culture of *S.platensis* was obtained from Centre for advanced studies, Department of Botany, University of Madras, Chennai, Tamilnadu, India. The culture was maintained in the laboratory on Zarrouk's medium (Modified-1966) in a 1000 ml Erlenmeyer flask. The pH of the medium was 9.2. A white fluorescent lamp served as light source(7000 lux) and an aquarium aerator was used to provide aeration. The sub culturing was performed once in a month.

Harvesting:

The test culture was grown in an Erlenmeyer flask at optimum condition for a period of 20 days. After incubation the culture was centrifuged at 5000rpm for 10 minutes. The supernatant was discarded and the pellets were washed three times with sterile distilled water and used for phycocyanin extraction.

Extraction of phycocyanin:

The C- phycocyanin was extracted from fresh biomass as per procedure described by Sarada *et al.* 1999. Fresh biomass was homogenized with 50mM sodium phosphate buffer, the homogenate was subjected to alternate freezing and thawing (3 to 4 cycles) and centrifuged at 5000rpm for 10 minutes. The phycocyanin content was estimated by the method of Sigelman and Kycia (1978).

Confirmation of C-phycocyanin:

The identity of extracted C-phycocyanin was confirmed by high performance thin layer chromatography (HPTLC) and the molecular weight determination by SDS-PAGE.

HPTLC:

About 8µl of the extract was spotted on a 10x10cm size pre-coated silica gel 60F254 TLC plate using CAMAG linomat IV automatic sampler spotter. The plate was developed in the solvent system (40% ethyl acetate, 60% acetone) and dried at room temperature. The plate was scanned using CAMAG: TLC scanner II at UV254 and the denstiogram was obtained which recorded the Rf value spectra and peak area of the resolved band. The Rf value and the absorbance maxima of different compounds separated out in the crude phycocyanin were compared.

Molecular weight comparison:

The molecular weight analysis was carried out as per the procedure of Kao *et al.* (1975). To identify the proteins(C-phycocyanin) based on their molecular weight, standard C-phycocyanin P-2172(Sigma Aldrich, USA) was used. Electrophoresis was carried out in vertical slab gel apparatus. The stacking and separating gel was prepared in the ratio of 4% and 16% and allowed to polymerise. Samples were pre-incubated with 4%(w/v)SDS, 12%(v/v) glycerol and 2%(v/v) mercapto ethanol0.025%(w/v) bromophenol blue and 50mM tris pH-6.8 for about 5 minutes at 95^{0}C. The gel was run at room temperature and stained with 0.1% (w/v) coomasie brilliant blue for 30 min. destained with diluted acetic acid to visualize the bands.

Partial purification of c-phycocyanin (Crag and Carr, 1968):

The crude phycocyanin was centrifuged at 10,000rpm for 10 minutes to obtain cell free extract and precipitated with 50% saturated ammonium sulphate and incubated at 4^{0}C for over night. The precipitate was obtained by centrifugation at 12,000 rpm and dialyzed with phosphate buffer (pH 6.8).The dialyzed sample was re-suspended in phosphate buffer and subjected to 35% ammonium sulphate precipitation for overnight incubation. After centrifugation a pellet rich in C-Phycocyanin and supernatant rich in allophycocyanin was obtained. The pellet was re-suspended in phosphate buffer (pH 6.8) solution and stored at 4^{0}C for further use and the purity was determined (Abalde *et al.*, 1998)

Antimicrobial activity:

Screening:

Preparation of inoculum:

Colonies were picked up from pure cultures, made an emulsion to give a standard density of inoculum of 10^4 colony-forming units (CFU) per spot on the agar. A 0.5 McFarland standard was used for visual comparison of the suspension to a density equivalent to approximately 10^8 CFU/ml.

Preliminary test (Bauer *et al.*, 1966):

The phycocyanin was tested against the pathogens by disk diffusion method. Sterile 6 mm disk was obtained from Hi Media, impregnated with 50µl of partially purified C-phycocyanin and air dried under laminar airflow at room temperature for 8 hours. The bacterial cultures *Escherichia coli* and *Bacillus subtilis* were adjusted to 0.5 McFarland standards and swab inoculated on MHA plates. The inoculated plates were kept at room temperature for 30 minutes. The standard partially purified C-phycocyanin impregnated disks were placed on MHA plates along with positive control, and incubated at 37^{o}C for 18 hours. The incubated plates were observed for anti-bacterial activity. A standard antibiotic disc-cephalosporin 30µg for *B.subtilis* and kanamycin 30µg for *E.coli* served as positive control.

MIC determination by agar dilution method: (EUCAST, 2000):

Preparation of stock solution:

10 mg of standard C-phycocyanin was (Sigma-Aldrich) dissolved in 1 liter of sterile phosphate buffer to obtain the initial concentration of 10,000 µg/l. It was diluted as per the following table to obtain final working concentrations on agar plates. The potency of the antimicrobial agent could be calculated by using the following formula:

$$\text{Weight of powder (mg)} = \frac{\text{Volume of solvent (ml) x concentration (mg/1)}}{\text{Potency of powder (mg/g)}}$$

Preparation of working solution:

A twofold dilution series were used to determine MIC of phycocyanin. Twenty milliliters volumes of agar in standard Petri-plates were used to determine MIC by agar dilution method. (Table 1).

Test organisms:

The standard C-phycocyanin (P2172 - SIGMA-

ALDRICH) and partially purified C-phycocyanin were tested against the following clinical isolates obtained from SRM Hospital Chennai. 1. *Staphylococcus aureus*, 2. *E. Coli*, 3. *Bacillus subtilis*, 4. *Streptococcus pyogens,* 5. *Proteus valgaris* and 6. *Vibrio cholerae.* MHA medium was sterilized and allowed to cool at 50ºC. Different concentrations of phycocyanin were prepared in series in 25ml container. 19 ml of molten agar was added to each container, mixed thoroughly, poured to the pre-labeled Petriplates, and allowed to settle. Phycocyanin free plates were maintained as control. 1µl of diluted bacterial suspension was spotted on the marked area of the plates and allowed to dry at room temperature. Plates were incubated at 35-37ºC for 18 hrs. The MIC for all the test organisms were read by naked eye and tabulated. The MIC is the lowest concentration of phycocyanin that completely inhibited the visible growth as observed by naked eye.

RESULTS AND ANALYSIS

The amount of crude C-phycocyanin obtained from laboratory grown *Spirulina platensis* was estimated spectrophotometrically and the value was 0.8 mg/ml. With purity of 1.17 .Upon partial purification the concentration and purity was improved to 0.9mg/ml and 2.68 respectively. The identity of the obtained C-phycocyanin was confirmed by the HPTLC analysis and SDS PAGE and compared with standard C-phycocyanin (Fig. 1).

Fig. 2 comparison of molecular bands in SDS PAGE, obtained from both standard and partially purified C-phycocyanin for the confirmation. Two bands were observed for the test sample and also in standard C-phycocyanin. Moreover, the position of the bands was found to be almost same (17,000 and 19,500), which showed that the molecular weight of the partially purified

High performance thin layer chromatography: Phycocyanin-dark blue-acetone extraction

Fig. 1: In this five peaks were formed. In that, peak no. 1 (highest absorption peak at 620nm) shows highest range of Rf value and represented C-phycocyanin

C-phycocyanin was similar to standard C-phycocyanin. The molecular weight of the 2 subunits of C-phycocyanin were approximately 16 and 17kda, respectively (Madhyastha *et al.,* 2006) on SDS PAGE separation. Treatment with SDS caused C-PC to dissociate into two subunits and were comparable with parallelly run Sigma standard which were 17,000 and 19,500).

The preliminary disk diffusion method showed sensitive zone against *Escherichia coli* and *Bacillus subtilis.* Standard antibiotic discs such as Kanamycin-30µg/disc for *E.coli* and cephalosporin-30µg/disc for *B.subtilis* were used for comparison. Screening of antibacterial activity of partially purified C-phycocyanin was compared with standard C-phycocyanin (Sigma) and standard antibiotics.

Six clinical isolates obtained from SRM medical College Hospital and Research Centre, kattankulathur,

Table 1: Preparation of dilutions of C-phycocyanin for agar dilution susceptibility tests

C-PC stock Soln. (µg/l)	Vol.of stock soln.(ml)	Vol.phosphate buffer.(ml)	Final CPC conc.(µg/l)	Resultant conc. in agar(µg/20ml)
10,000	1.024	0.000	10240	512
10,000	0.512	0.488	5120	256
10,000	0.256	0.744	2560	128
10,000	0.128	0.872	1280	64
1000	0.64	0.36	640	32
1000	0.32	0.68	320	16
1000	0.16	0.84	160	8
100	0.8	0.2	80	4
100	0.4	0.6	40	2
100	0.2	0.8	20	1

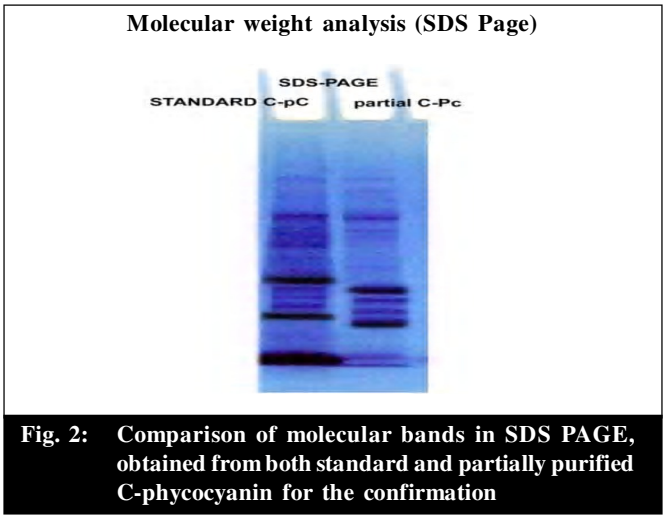

Fig. 2: Comparison of molecular bands in SDS PAGE, obtained from both standard and partially purified C-phycocyanin for the confirmation

Chennai, India, were used for screening the antimicrobial activity of both partially purified and standard C-phycocyanin. The strains were *B.subtilis*, *S.aureus*, *S.pyogens*, *E.coli*, *P.vulgaris* and *V.cholerae*. 15µg/ml of partially purified C-phycocyanin obtained from laboratory grown *S.platensis* was able to inhibit the growth of the *B.subtilis*, *E.coli* and *V.cholerae,* 30µg/ml for *S.aureus* and *P.vulgaris*, 60µg/ml for *S.pyogenes*. Whereas the amount of standard C-phycocyanin required to inhibit the growth of the test organisms were, 30µg/ml 60µg and 120µg, respectively (Fig. 3).

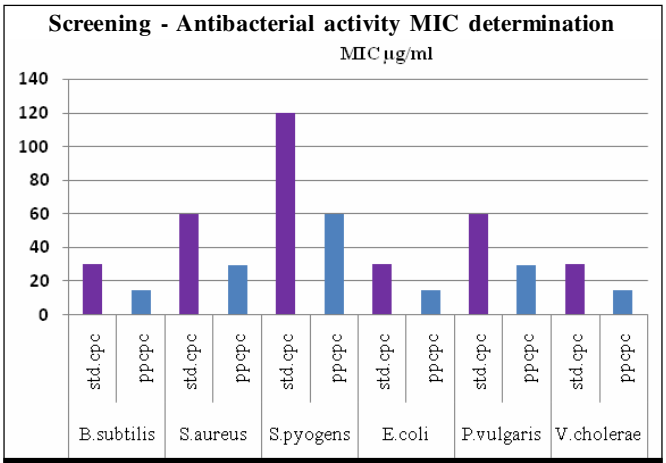

Fig. 3: Compare antimicrobial activity of Standard C-phycocyanin(std.cpc) and partially purified C-phycocyanin (ppcpc) to the test organisms

Conclusion:

C-phycocyanin extracted from *Spirulina platensis* was comparable to Sigma standard C-phycocyanin with respect to its molecular weight and absorbance values by HPTLC. Partially purified C-phycocyanin proved to be more potent than the standard phycocyanin with respect to its antibacterial activities as assessed by minimum inhibitory concentration against 6 bacterial species tested.

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