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Extraction, partial purification, and antibacterial activity of phycocyanin from *Spirulina* isolated from fresh water body against various human pathogens

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ABSTRACT

The phycobilin pigments are intensively fluorescence and water soluble. They are categorized into three types: pigments containing high energies (phycoerythrins), intermediate energies (phycocyanins), and low energies (allophycocyanins). Among them, crude-phycocyanin (c-pc) has been considered to be the most preferred one. The present study was undertaken to evaluate the antibacterial activity of c-pc which was isolated from the blue green algae *Spirulina platensis*. C-pc was extracted by sonication, followed by centrifugation and filtration. The crude form of phycocyanin was purified by ammonium sulphate precipitation, membrane filtration, and dialysis method. Antibacterial activities were obtained from various human pathogens. The results showed that all *Spirulina maxima* extracts exhibited great potential antibacterial activities against five bacterial strains with inhibition zones of range 7–13 mm.

Key words: *Spirulina maxima*, c-phycocyanin, antibacterial activity

INTRODUCTION

Microalgae are a very diverse group of organisms that consist of both prokaryotic and eukaryotic forms. Although most microalgae are phototropic some species are also capable of showing heterotrophic growth. Some species of micro algae can be induced to over produce many components through simple manipulation of the physical and chemical properties of the culture medium. Microalgae are photosynthetic microorganism that converts sunlight, water, and CO_2 to algal biomass. Many microalgae are rich in carbohydrate, protein, and other pigments (Gilman, 2007).

Microalgal biomass consists of rich source of some nutrients, such as proteins, minerals, carbohydrates, and other essential nutrients, which depend upon the environmental condition the algal culture was grown. In all microalgae, *Spirulina* is one of the most nutritive and concentrated microalgal food source available in the market. It is a predominant species, commercially cultivated in many countries.

Spirulina is a photosynthetic, filamentous, spiral shaped, multicellular, and blue green microalgae. The two most important species are *Spirulina maxima* and *Spirulina platensis*. This microorganism's cell division occurs by binary fission. It contains carotenoid, chlorophyll, and major phycocyanin pigment. It belongs to Cyanophyceae class, Oscillatoriaceae family; this cyanobacteria is characterized by spiral chains of the cells enclosed in a thin sheath. *Spirulina* has a very long history of being served as a source of food for human being.

Spirulina is a spiral filament generally found in fresh water. It consists of 60%–70% protein in dry weight. The protein elements consist of 18 types of amino acids, several vitamins, such as vitamins A, B, E, and K, minerals, and fatty acids necessary to the body. The blue green algae *S. maxima* were maintained in the Zarrouk medium, a standard synthetic medium containing 2.5 gl^{-1} sodium nitrate as the nitrogen source (Zarrouk, 1966).

Algal pigments are colorful and chemical compounds which only reflect light at certain wavelengths. This makes them appear colorful as flowers, corals, and animal skins which contain pigments that give them their color. More important than their reflection of light is the ability of pigment to absorb certain wavelengths. Because they interact with light to absorb only wavelengths, pigments are useful to plants and other autotrophic organisms, which make their own food using photosynthesis. Oilments are the means by which the energy of sunlight is captured for photosynthesis. However, since each pigment reacts with only a narrow range of the spectrum, there is usually a need to produce several kinds of the pigments for each color, to capture more of the sun's energy.

There are three basic classes of pigments carotenoids, chlorophyll, and phycobilin. Chlorophyll is the pigment which contains a porpyrin ring. This is a stable ring shaped molecule around which electrons move freely; the ring has the potential to gain or lose electrons easily and the potential to provide energized electrons to other molecules. This is the fundamental process by which chlorophyll captures the energy of sun light. There are several kinds of chlorophyll, namely, chlorophylls a, b, and c.

Chlorophyll a is the molecule which makes photosynthesis possible by passing its energized electrons on to molecules which will manufacture sugar. All plants, algae, and cyanobacteria which photosynthesize contain chlorophyll a. Chlorophyll b is found only in green algae and plants. Chlorophyll c is found only in the photosynthetic membranes of the chromista as well as the dinoflagellates.

Carotenoids are red, orange, or yellow pigments. They include the familiar compound carotene, which gives the color. Phycocyanin is the blue pigment protein located in the photosynthetic lamella in the cytoplasm membrane. When the envelope is broken, thylakoid membrane together with phycocyanin is released. The blue green color is due to presence of two pigments, phycocyanin and chlorophyll. Phycocyanin extracted from *Spirulina* was first marketed in 1980 by Dainippon Ink and Chemicals under the brand name "Lina Blue–A."

The purity of c-pc is generally evaluated using the absorbance ratio of A_{620}/A_{280}, and a purity of 0.7 is considered as food grade, 3.9 as reactive grade, and more than 4.0 as analytical grade. Purity is directly related to process costs, and, in general, the more purified a product is, the more expensive to obtain it.

Of the protein purification techniques used, ammonium sulfate precipitation is of great value, since it can be applied on a large-scale, requires simple equipment, and is cheap to carry out (Reis *et al*., 1998). Moreover, for recovering c-phycocyanin biological activity, this process is usually excellent after precipitation and its dissolution is easy. There is no standard procedure to purify c-phycocyanin using ammonium sulphate precipitation; therefore, it is interesting to study and establish this procedure to reach the maximum possible purity.

Phycocyanin is a potent antioxidant and possesses significant immune enhancing and antiviral properties. It's enhancing biological defense activity against infections disease reduces allergies inflammation by the suppression of antigen-specific IgE antibody. The phycocyanin is used as coloring agent in food item like jellies, chewing gums, ice sherbaths, and dairy products. In Japan, China, and Thailand, phycocyanin is used in cosmetics like lipstick and eyeliners. It is used in biomedical research and pharmaceutical industries. It is used in immune diagnostic applications.

Any organism or an agent that produces such a disease is a pathogen. Its ability to cause disease is called pathogenicity. Some of the natural proteins are used against infections diseases. In *Spirulina*, phycocyanin is a phycobiliproteins; it is used against many bacterial infections and has antiinflammatory, antioxidant and antiviral properties. It mainly enhances biological defense activity against infectious diseases. In small concentration, phycocyanin effectively acts against many pathogens. This work was conducted to study the extraction of phycocyanin from *Spirulina platenisis* and the antibacterial activity of phycocyanin.

MATERIALS AND METHODS

ALGAL SOURCE

The blue green alga, *S. maxima*, was obtained from the water sample collected from the Bear Shola falls, Kodaikanal. The strain was maintained in the Zarrouk's medium.

ISOLATION AND MASS CULTIVATION OF SPIRULINA

The water sample was inoculated into the Zarrouk medium for isolation of *Spirulina* and incubated in a growth chamber with light flux of 16:8 h. The purity of the culture was ensured by repeated inoculation, and identification was accomplished by determining cellular morphology observed by using light microscope.

Modified Zarrouk medium was prepared and the purified form of *Spirulina* was inoculated and mass cultivated. After 2 days, fresh sterile Zarrouk's medium was added to the flask placed for mass cultivation at room temperature in a growth chamber with light flux of 16:8 hours.

EXTRACTION OF PHYCOCYANIN

Phycocyanin was isolated by the following procedure. First, approximately 3–7 g of algae was suspended in 30 ml of 0.1M Na-phosphate buffer, pH 7.0, containing 100 µg/ml lyzozyme and 10 mm EDTA. The enzymatic disintegration of the cell-wall was brought about by placing the algae in a shaking bath at 30 °C for 24 h. The slurry was then

centrifuged for 1 h at 10,000 rpm to remove cell debris, yielding a clear blue supernatant.

The concentration of total blue pigment phycocyanin was spectrophotometrically determined at A_{280}, A_{615}, and A_{652} nm as reported by Silverira *et al.* (2007). Phycocyanin concentration (PC) and extraction purity (EP) were calculated by the following equation:

(PC) = OD_{615} − 0.474 (OD_{652})/5.34 mg ml^{-1} and (EP) = OD_{615}/O_{D280}, respectively.

The crude phycocyanin was subjected to further purification.

PURIFICATION OF PHYCOCYANIN

C-pc crude extract was used for purification; for experiment comparison, the same crude extract was used to obtain the same initial conditions.

AMMONIUM SULPHATE PRECIPITATION

The blue supernatant was precipitated with 30%–50% saturation with ammonium sulphate at 4 °C for 12 h and centrifuged at 14,000 rpm for 30 min. The precipitate was dissolved in a minimal volume of 0.5m sodium phosphate buffer solution and was absorbed at A_{620}/A_{280} in UV spectrophotometer.

MEMBRANE FILTRATION

After ammonium sulphate precipitation, the phycocyanin was filtered through the membrane filter under vacuum pressure. The filtered sample was absorbed through UV spectrophotometer.

DIALYSIS

The membrane filtrated phycocyanin was dialyzed with dialysis bag for further purification.

PRE-TREATMENT OF DIALYSIS MEMBRANE

The dialyzed membrane was cut to 5 cm length and pre-treated for removal of chemical and other components from the membrane.

Glycerol was removed by soaking and washing the membrane in running water for 3–4 h followed by distilled water and the membrane was always immersed in water at 4 °C. The heavy metals were removed by boiling solution 1 (10 mM EDTA); sulphide was removed by boiling in solution 2 (2% $NaHCO_3$) for 10 min. The remaining heavy metals, glycerol, and sulphur (which may inactive the enzyme) was removed from the membrane by placing it inside a beaker containing 500 ml of solution 3 (2% $NaHCO_3$ with 10 mm EDTA) and boiled for 20 min (caution was taken to prevent the membrane from touching the walls of the beaker).

After 20 min the inside and outside of the membrane was washed with distilled water using squeeze bottle. Again the membrane was boiled for 10 min in solution 4 (1 mM EDTA) to remove excess $NaHCO_3$. Then the membrane was washed inside and outside with solution 5 (10% ethanol) at 4 °C.

The pre-treated dialysis membrane was taken (gloves was used to handle the membrane) and rinsed with distilled water both inside and outside. One end of the membrane was tied securely with the thread. The membrane was filled with water and gently squeezed to check the leakage and assumed there was no leakage. Then the water was removed and the phycocyanin sample solution was filled using a pipette. Then the open end was also closed securely by a thread. The bag was placed in 0.5m sodium phosphate buffer solution. The sample was dialyzed for 24 h at room temperature. Now partially purified phycocyanin was obtained. The sample was absorbed through UV-spectrophotometer.

ANTIBACTERIAL ACTIVITY OF PHYCOCYANIN

Bacterial strains were used (*Escherichia coli*, *Streptococcus* sp., *Pseudomonas* sp., *Bacillus* sp., *Staphylococcus aureus*) for antibacterial evaluation.

ANTIBACTERIAL TESTING

The bacterial strains were inoculated in the nutrient broth and incubated at 37 °C for 24 h. Muller Hinton agar plates were prepared. The given culture was spread with sterilized swabs to form a lawn of culture. Phycocyanin disc was prepared, filter paper disc impregnated with 100-400 ml of phycocyanin. Then the discs were placed gently on the pathogen incubated Muller Hinton agar plates spaciously. The plates were incubated at 37 °C for 24-48 h. After incubation the results were observed.

RESULTS AND DISCUSSION

The water samples were collected from Bear Shola falls at Kodaikanal, Tamilnadu and *Spirulina* sp. was isolated from it and cultured in the Zarrouk medium. The samples of green colonial unicellular microalgae which can grow in fresh salt water as well as in brackish bodies of water were isolated. It grows best in a highly alkali environment of pH 10–12. Such condition currently exists in certain lakes and rivers (Capelli, 2010). The isolated *Spirulina* samples were mass cultivated in larger amount of Zarrouk medium. *Spirulina* is highly nutritive and shows great diversity and higher concentration of nutrients compared to other food sources. Infact, it is among the most nutritious, concentrated whole food sources found in nature, contributing to its being known as a super food.

The crude pigment, phycocyanin was extracted by sonication, followed by centrifugation and filtration. Here, lysozyme was used for cell disruption and to maximize the phycocyanin yield. Lysis buffer contains Tris, EDTA, and sucrose which disrupts the cell and releases phycocyanin pigment into the medium. The concentration of total crude pigment phycocyanin was spectrophotometrically determined at 615, 652, and 280 nm.

The crude form of phycocyanin was purified by ammonium sulphate precipitation, membrane filtration, and dialysis method. The purity of phycocyanin increased after every stage of fraction (Table 1). The first step in the fractional precipitation started with 30% ammonium sulphate saturation, which mainly salted out proteins with little improvement in the purity ratio. The c-pc fraction was then salted out with 50% ammonium sulphate concentration and dissolved in phosphate buffer, eliminating other basic proteins to a remarkable degree with a purity, which results by fractionation better than those obtained by direct precipitation at 50% saturation.

Table 1. Purification of phycocyanin

S. No.	Purification process	Extraction purity at 615/280 nm	Phycocyanin concentration (mg/ml)
1	Crude phycocyanin	0.61	1.94
2	Ammonium sulphate precipitation	0.73	2.39
3	Membrane filtration	0.89	2.64
4	Dialysis	0.99	2.90

Table 1 shows the purity of c-pc after various stages of purification which can be implied for further analysis of grading the phycocyanin. The table signifies that the purity can be increased and a food grade phycocyanin was isolated.

The purification technique, ammonium sulphate precipitation is of great value, since it can be applied on a large-scale and requires simple equipment and is simple and cheap. Moreover, for recovering C-Phycocyanin biological activity, it is usually excellent after precipitation and its dissolution in easy (Reis *et al*., 1998). The partial purification of phycocyanin concentration was observed in dialysis method in range of 2.90 mg ml^{-1}.

The antibacterial activity of *S. mamima* 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 dependant. This phenomenon was in agreement with that found by Ozdemir *et al*. (2004). The data in Table 2 showed that the most susceptible bacteria were *Streptococcus* sp. and *B. subtilis* to *S. maxima* with highest inhibition zones ranged 6–13 mm at concentrations 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*.

Table 2. Antibacterial activity of phycocyanin

S. No.	Name of the microorganism	Zone of inhibition at various concentrations of c-pc (mm)			
		100 µl	200 µl	300 µl	400 µl
1	*Streptococcus* sp.	9	11	12	13
2	*Pseudomonas* sp.	8	10	11	12
3	*Bacillus* sp.	8	10	11	13
4	*Staphylococcus* sp.	6	7	8	9
5	*E. coli*	7	9	10	11

The antibacterial activity of c-pc was determined by the zone of inhibition against common pathogens. They showed a considerable zone of inhibition which implies that this phycocyanin can be used as supplements or as coloring agents or additives in food products.

CONCLUSION

Many microalgae are involved in the pharmaceutical product formation, particularly *Spirulina* sp. produced high amount of protein. It is gaining more and more attention not only for the food aspects but also for the development of pharmaceutical industries.

The present work describes an efficient method of extraction and purification of food grade phycocyanins from *Spirulina* sp. The purity ratio of phycocyanins at the end of the process is 0.99 mg ml^{-1}. Mainly when applied to food, since low cost and high recovery are necessary, ammonium sulphate precipitation is an economically feasible alternative with no toxic reagents, allowing this precipitation technique in food use.

The sonication method is very effective in cell wall disruption of *Spirulina* sp. The extraction process needs pH and temperature control to obtain a good yield with more stable form of phycocyanins. C-Phycocyanins extracted from *Spirulina* can have a preventive effect of drug-induced cardiac side effects as well as a protective effect during heart attacks.

The *Spirulina* extracts of phycocyanins could inhibit human pathogens and cancer in human and animals. Phycocyanin is a natural blue pigmented protein with linear tetrapyrrole prosthetic groups. They are water soluble and highly fluorescent. It is effectively active against human pathogens such as *Streptococcus* sp., *Staphylococcus* sp., *E. coli*, *Bacillus* sp., and *Pseudomonas* sp.

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