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Research Article

Antibacterial Activity of Desiccated Cyanobacterium *Anabaena* sp. Isolated from Terracotta Monuments of Bishnupur, West Bengal

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ABSTRACT

Anabaena sp. are the dominant cyanobacterial species on terracotta monuments of Bishnupur, which exposed to high solar radiation, ultraviolet, and in a desiccated condition in most parts of the year. In the present study, three *Anabaena* species were isolated from crust samples, and its antibacterial activities were evaluated against pathogenic bacteria *Bacillus subtilis*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella typhimurium*, and *Escherichia coli*. We observed good antibacterial activity in ethyl acetate and ethanol extract of *Anabaena* sp. (VBCCA 052002) which has the highest antibacterial activity against *Staphylococcus aureus*, *Salmonella typhimurium*, *Staphylococcus aureus*, and *E. coli* respectively. We have validated the antibacterial assay by using resazurin based anti-microbial assay in microtiter plates and calculated the minimum inhibitory concentration (MIC) value of ethyl acetate extract of *Anabaena* sp. (VBCCA 052002) which is found 100 µg/mL against *Staphylococcus aureus* and 150 µg/mL against *Salmonella typhimurium*.

INTRODUCTION

Multidrug-resistance to the pathogenic bacteria is a great concern for public health throughout the world. The routinely used drugs against many clinical pathogens such as *Mycobacterium tuberculosis*, *Enterococcus*, *Pseudomonas* sp., *Streptococcus pneumoniae*, and *Staphylococcus aureus* are not working. To overcome this alarming situation novel antibacterial product development from unknown sources is the need of the hour. Cyanobacteria, photosynthetic prokaryotes are a prolific source of natural products with a great choice for new drug developments in biotechnology and pharmaceutical industries. Cyanobacteria being able to tolerate many stresses and found in extreme climatic conditions, is a very potential candidate for novel drug discovery.^[1-3] Many cyanobacteria occurred

in desiccated state on sub-aerial surfaces like ancient monuments and building facades. *Anabaena* sp. is one of the subaerial cyanobacterium, which can survive in desiccated conditions. Many of the subaerial cyanobacteria are having polysaccharide covering in the form of slime, capsule or sheath, which acts as a protective boundary between the cells and its surrounding environment.^[4-5] Among cyanobacteria, marine cyanobacteria are studied well for their bioactive metabolites and till now 569 natural products have been reported in MarinLit^[6] from them. However, bioactive metabolites of sub-aerial cyanobacteria are not well known. Since in subaerial environment cyanobacteria have to adapt a wide variation of stresses, they might have produced some novel antimicrobial compounds. Therefore we have initiated this study with objectives of isolation and culture of subaerial

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cyanobacteria and evaluate its anti-microbial properties against some pathogenic bacteria.

MATERIALS AND METHODS

Collection Site, Isolation, Growth Conditions and Identification

Bishnupur temples are unique for its excellent terracotta works and located between 23°4'48" N latitude and 87° 19'12" E longitude in Bankura districts of West Bengal. In these terracotta monuments cyanobacteria colonises and can withstand high temperature and desiccation conditions in the form of crusts. Cyanobacterial crust samples were collected from the Bishnupur terracotta using non-destructive double-sided tapes, and a pinch of the collected crust was soaked with 12–48 hours. When some bluish-green color appeared on the crust, it was transferred to BG-11 medium with 1.2% w/v agar with or without nitrogen sources and incubated with fluorescent light (7.5 W/m²) at 25 ± 1°C.^[7] After repeated sub-culturing, pure cultures of the cyanobacteria were established. The isolated cyanobacteria were observed using a trinocular research microscope (Leica DM 750) and microphotographs were taken using Leica EC3 scientific digital camera and further analyzed with LAS EZ software. Three *Anabaena* sp. [*Anabaena* sp. (VBCCA 052002), *Anabaena* sp. (VBCCA 052 009), and *Anabaena sphaerica* (VBCCA 052 010)], were isolated, assigned with a strain number, and the pure cultures were deposited in the Visva-Bharati Culture Collection of Algae (VBCCA) which is affiliated to the World Federation of Culture Collection (WDCM 931). The isolated cyanobacteria were identified using standard monographs.^[8-11]

Extraction and Fractionation of the Metabolites

The isolated cyanobacteria were cultured in the batch culture system using the BG-11(-N) medium for 30 days and centrifuged at 10,000 rpm for 10 minutes to get the cell biomass. The algal pellet was washed in distilled water to remove the salts and dried in a lyophilizer. Five grams of the respected dried cyanobacteria samples were extracted with 50 mL of ethyl acetate, ethyl alcohol, and water using a sonicated water bath for 30 minutes. The extracts were filtered and dried in a rotary vacuum evaporator under reduced pressure and treated as crude extracts. The crude extracts were dissolved in 1 mL Dimethyl sulfoxide (DMSO) and stored in 4°C for further anti-microbial studies. Crude extracts (10 mg) of the metabolites are further fractionated using LH₂₀ and silica column chromatography using standard National Institute of Health (NIH), USA fractionation scheme. In brief, the fractionations of crude extract were carried out using silica column chromatography and eluted with non-polar to polar solvent systems such as hexane (100%), hexane: ethyl acetate (50:50), ethyl acetate (100%), ethyl acetate: methanol (50:50), methanol (100%), and water (100%).

Further purification of the metabolites was carried out using LH₂₀ column chromatography and eluted with mobile phase chloroform (100%), chloroform:methanol (50:50), methanol (100%), and methanol:water (50:50). All the fractions were dried using a rotary vacuum evaporator, and antibacterial assay were carried out using crude as well as silica and LH₂₀ fractions.

Antibacterial Assay

Antibacterial activities of crude and all the fractions of three *Anabaena* sp. were carried out using the agar well diffusion method.^[12] The five test pathogenic bacteria [*Bacillus subtilis* (MTCC 121), *Listeria monocytogenes* (MTCC 657), *Staphylococcus aureus* (MTCC 96), *Salmonella typhimurium* (MTCC 98), and *Escherichia coli* (MTCC 1667)] were procured from microbial type culture collection (MTCC), IMTech, Chandigarh. The bacterial test strains used were picked up from the agar slants and inoculated in freshly prepared slants and incubated inside an incubator with 28 or 37°C temperature. Further, the bacterial suspensions were evenly inoculated in nutrient agar (NA) plates and holes of 5 mm in diameter was punched aseptically with a sterile cork borer. In each well, 50 µL of crude and the column fractions dissolved in DMSO were added and incubated at 28 or 37°C temperature. After 24 hour of incubation, the zone of inhibition was observed, and the diameter of zones of inhibition was measured in mm. Ciprofloxacin (100 µg/mL) and DMSO were used as positive and negative control, respectively.

Microtiter Plate-based Anti-microbial Assay

Purified natural compounds are generally found in a very low amount, which can create some challenges for the bioassay. Therefore, we have also used a resazurin microtiter plate-based assay following a modified method of Sarker *et al.* 2007.^[13] In brief sterile 96 well microtiter plate is opened under aseptic condition. Several working concentrations of the crude extract were made and added to the plate resulting in final concentrations (µg/mL) of 200, 150, 100, 50, 25, 12.5, 10, and 5. Both the crude extracts and the fractions were dissolved in sterile DMSO, and tested against the pathogenic bacterial strains. Each of the test metabolites solution was added a volume of 2.5 µL to the respective wells. Pathogenic bacterial cultures were grown over-night and diluted by sterile nutrient broth and measured to a specific OD of 0.00075 in a spectrophotometer. In each well, 97.5 µL of bacterial suspension was added, making the final test volume in each well to 100 µL. Ciprofloxacin was used as a positive control. For sterility control, all the test materials were added, and sterile nutrient broth was added instead of bacterial suspension to check whether the test materials is sterile. For negative control, only bacterial suspensions were added to the wells to check whether the bacteria were growing or not. To check the solvents (water and DMSO) were not inhibiting growth of bacteria, another control

were used with water and DMSO were added in respective wells and bacterial suspension to them. Resazurin solution was prepared at 0.01% concentration with sterile water. A volume of 4 μ L was added to each well (except the blank) and mixed well.

Plates were incubated at 37°C overnight and any color change was detected visually and measured in an ELISA microplate reader.

Minimum Inhibitory Concentration (MIC)

We have calculated the MIC of those test metabolites, which gave positive results in the antibacterial assay. Different concentration of the extract e.g. 10, 15, 20, 25, 50, 100, 150, and 200 μ g/mL were prepared in DMSO. These different concentrations were added to nutrient broth containing respective test tubes and a fixed volume of bacterial cultures. The culture tubes were then incubated at 37°C overnight. After proper incubation, 100 μ L of cultures were picked up from each test tube and spread on Nutrient Agar (NA) plates and incubated at 37°C for overnight. MIC value was calculated using the colony-forming unit (CFU) counting method. To validate the data, MIC was also calculated using a microtiter plate-based anti-microbial assay.

RESULTS

Bishnupur monuments are famous terracotta temples, and the nature of substratum in these temples varies from bare rocks to terracotta tiles and sculptures. In summer, the temperature on the surface of the monument goes above 60°C and along with high light intensity, UV and extreme dryness make it an extreme environment: three *Anabaena* sp. [*Anabaena* sp. (VBCCA 052002), *Anabaena* sp. (VBCCA 052 009), and *Anabaena sphaerica* (VBCCA 052 010)] are the predominant cyanobacteria in these terracotta moments forming black brownish crust (Fig. 1). Since these cyanobacteria were isolated from the extreme habitat of terracotta monuments with exposure to high light, UV, and desiccation, we carried out the

anti-microbial activity of these cyanobacterial extracts against some gram-positive and gram-negative bacteria. Fig. 2 showing the zone of inhibition (in mm) of different solvent extract of three *Anabaena* sp. on the test pathogenic bacteria *Staphylococcus aureus*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Listeria monocytogens*, and *E. coli*.

The results indicated that ethyl acetate extract and ethanol extract of *Anabaena* sp. (VBCCA 052002) have the highest antibacterial activity against *Staphylococcus aureus*, *Salmonella typhimurium*, *Staphylococcus aureus* and *E. coli* respectively. In the water extract no antibacterial activity observed against the tested organisms. In ethyl acetate extract of *Anabaena* sp. (VBCCA 052002) maximum zone of inhibition 19.07 mm was found against *Staphylococcus aureus* and 13.33 mm against *Salmonella typhimurium*, followed by the ethanol extract of *Anabaena* sp. (VBCCA 052002) was detected



Fig. 1: Figure showing the growth of *Anabaena* sp. on the terracotta monuments of Bishnupur

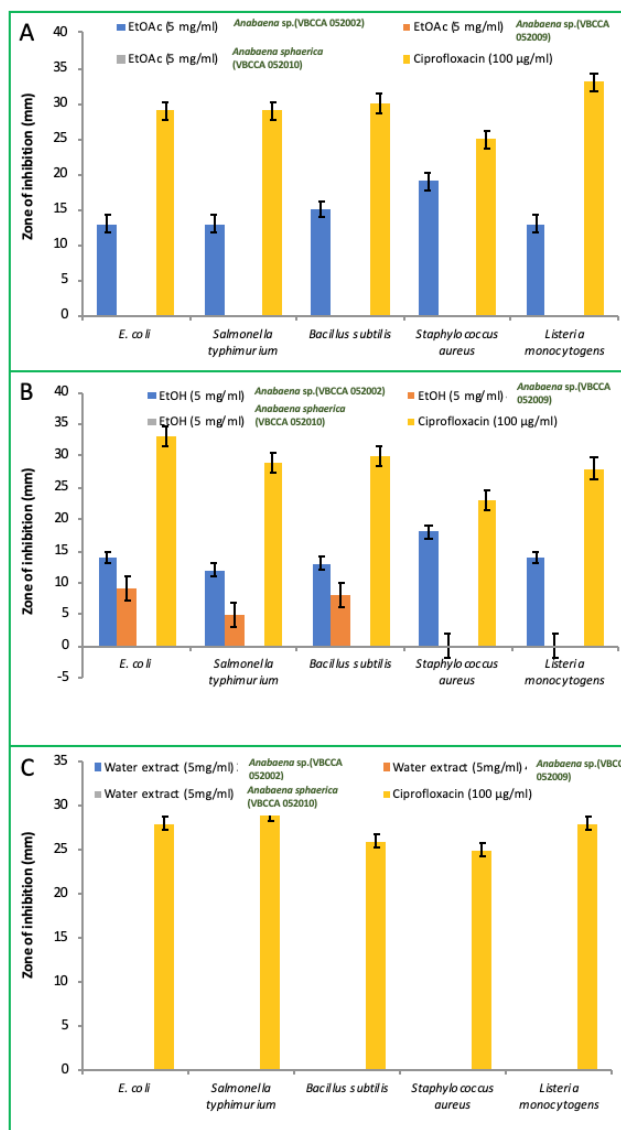


Fig. 2: Anti-microbial activity of *Anabaena* sp. isolated from terracotta monuments of Bishnupur.



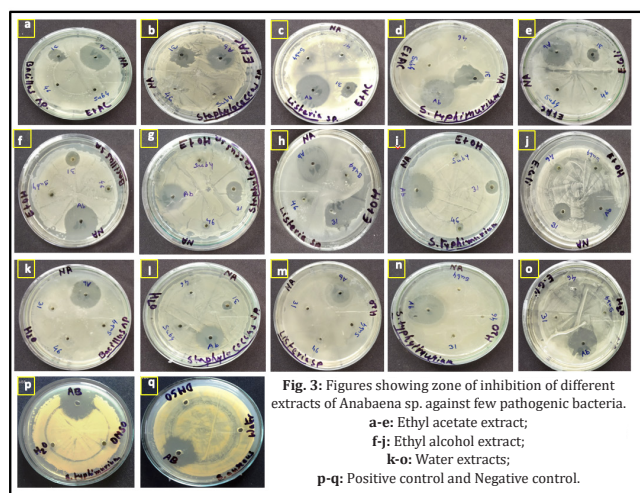


Fig. 3: Figures showing zone of inhibition of different extracts of *Anabaena* sp. against few pathogenic bacteria. a-e: Ethyl acetate extract; f-j: Ethyl alcohol extract; k-o: Water extracts; p-q: Positive control and Negative control.

Table 1. Data were showing a growth of *S. aureus* and *S. typhimurium* in different test concentrations for calculation of MIC.

Concentration ($\mu\text{g/mL}$)	CFU/mL	
	<i>Staphylococcus aureus</i>	<i>Salmonella typhimurium</i>
Control	7.8×10^{10}	1.7×10^{10}
10	8.6×10^9	1.3×10^{10}
15	1.9×10^9	2.8×10^9
20	1.9×10^9	6.2×10^8
25	2.0×10^8	5.1×10^7
50	1.9×10^4	1.1×10^6
100	0	1×10^2
150	0	0
200	0	0

18.22 mm against *Staphylococcus aureus* and 14.19 mm against *E. coli*. The ethanolic extract of *Anabaena* sp. (VBCCA 052009) showed minimum sensitivity in terms of inhibition zone against *Bacillus subtilis*, *E. coli*, and *Salmonella typhimurium* (Fig. 3). To confirm the results obtained in the anti-microbial test using agar diffusion method we further carried out the sensitive resazurin based anti-microbial assay in microtiter plates to calculate the MIC. Resazurin is an oxidation-reduction indicator and can indicate microbial growth in the plate at a very low concentration of the bioactive compounds in respect to color which was measured in a ELISA plate reader. Results obtained from agar diffusion method and the resazurin based microtiter plate-based method indicate MIC values of ethyl acetate extract of *Anabaena* sp. (VBCCA 052002) were 100 $\mu\text{g/mL}$ and 150 $\mu\text{g/mL}$ against *Staphylococcus aureus* and *Salmonella typhimurium* (Table 1, Fig. 4). The result indicates out of the three *Anabaena* sp. tested ethyl acetate extract of *Anabaena* sp. (VBCCA 052002) is having good anti-microbial activity against *S. aureus* and *S. typhimurium*.

DISCUSSION

In the microbial population, cyanobacteria considered being one of the potential organism for the development of novel antibiotics. *Anabaena* is a filamentous, heterocyst-

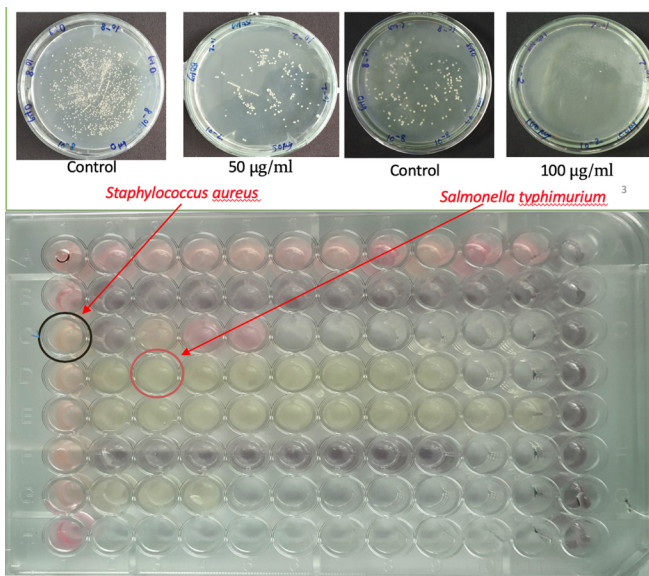


Fig. 4: Figure showing sensitive resazurin based anti-microbial assay for conformation of anti-microbial assay of *Anabaena* sp. extracts.

forming cyanobacterium having proven antibacterial activity. Heidari *et al.* (2012)^[14] reported that the ethanolic extract of *Anabena circinalis* have the antibacterial activity against *Serratia marcescens*, *E. coli*, *Klebsiella pneumonia* and the fungus *Aspergillus flavus*. Abdel-Raouf *et al.* 2011^[15] also reported antibacterial activity against *Sarcina maxima*, and *Micrococcus kristinae*, *Klebsiella pneumoniae*, as well as against the filamentous fungus *Aspergillus flavus*. Bhateja *et al.* 2006^[16] reported organic extracts of *Anabaena virabilis* showed activity against *Staphylococcus aureus*. Though few *Anabaena* sp. have been already tested for anti-microbial properties, subaerial cyanobacteria never been evaluated for its anti-microbial potential and our report of anti-microbial properties against two antibiotic-resistant bacteria *Staphylococcus aureus* and *Salmonella typhimurium* shows quite promising results. How the bioactive molecules help the cyanobacteria in surviving the stress condition is not well known, but since the cyanobacteria has wide biological adaptations and tolerance to environmental stress, some of these compounds might be produced in an attempt to confer advantages for their survival. The cyanobacterium *Nostoc commune* produces a novel antibacterial compound, called Noscomin, which is a diterpenoid.^[17] Though in the present study, we have not chemically characterized the antibacterial compound of the *Anabaena* sp. This is our first attempt to evaluate the antibacterial activity of subaerial cyanobacteria from ancient monuments, which is quite promising.

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