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Antimicrobial Evaluation of some Freshwater Cyanobacteria Collected from Local Rice Fields of Orissa, India

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Freshwater cyanobacteria from local rice- fields of Mayurbhanj district of Orissa, India were studied for their antimicrobial potentials. Crude metabolites were extracted using three organic solvents of different polarity viz. chloroform, methanol and ethyl acetate. The metabolites were tested against some clinically significant microorganisms. The results showed that nine freshwater cyanobacteria (*Oscillatoria* sp., *Oscillatoria curviceps*, *Phormidium* sp., *Aphanothece microscopica*, *Nostoc piscinale*, *Anabaena variabilis*, *Anabaena spiroides*, *Cylindrospermum muscicola* and *Microcystis* sp.) were active in inhibiting the test pathogens with chloroform extracts displaying considerable antimicrobial activity. Among the strains, three freshwater cyanobacterial species (*Phormidium* sp., *Aphanothece microscopica*, *Cylindrospermum muscicola*) effectively inhibits at least one of the test pathogens indicating that these cyanobacteria might have active antimicrobial metabolites. Minimum inhibitory concentration (MIC) was carried out in selected cyanobacterial strains that showed good zone of inhibition against the test pathogens during agar cup diffusion assay. MIC ranged from 500 μg/ml to 250 μg/ml with highest values recorded against *E. coli* and *S. aureus* and lowest value against *B. subtilis*. The findings revealed that the crude extract showed higher antimicrobial activity against Gram positive and Gram negative bacteria than pathogenic fungi. This study illustrates that cyanobacteria occurring in rice fields could be a potent source of antimicrobial agents.

Key words: Crude metabolites, Antimicrobial activity, Rice-fields, Cyanobacteria.

Microbial resistance to present available antibiotics is increasingly becoming a global public health concern. Currently used antibiotics are failing to bring an end to many infections due to super resistance strains. For this reason, search for new and effective antimicrobial agents are underway. Cyanobacteria also known as blue-green algae are found to be rich sources for various products of commercial, pharmaceutical and toxicological interest (Borowitzka, 1995). These organisms have drawn much attention as prospective and rich sources of biologically active constituents and have been identified as one of the most promising group of organisms capable of producing novel and important bioactive compounds. Cyanobacteria are known to produce metabolites with diverse biological activities such as antibacterial, antifungal, antiviral, anticancer, algicide and immunosuppressive activities (Barja *et al*., 2001). The importance of cyanobacteria as a potential drug resource is evident by the launching of the "cyanomyces" project in Europe, anticipated to generate novel therapeutic substances by combining genes from

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actinomycetes and cyanobacteria (Asthana *et al*., 2006).

Most of the important bioactive compounds discovered so far, have been isolated from marine cyanobacteria. However, freshwater cyanobacteria remain poorly investigated for their bioactive potentials. Preliminary investigations on freshwater cyanobacteria for their antimicrobial properties have drawn much attention as rich source of biologically active constituents. (Harada, 2004); has identified freshwater cyanobacteria as rich sources of lethal toxins such as microcystins and anatoxins. Recently, heterocystous cyanobacteria from locally occurring rice fields have also been reported to produce antifungal and antibacterial metabolites and have been identified as one of the promising group of organism to produce bioactive compounds (Skulberg, 2000). Considering specific and intraspecific diversity of cynobacteria, the rice fields of different areas and ecological niches can play considerable role in metabolic diversity of this organism in acquiring novel metabolites. Therefore the objective of this study was to evaluate the antimicrobial potentials of selected cyanobacterial species obtained from local rice fields against some clinically significant microorganisms.

MATERIAL AND METHODS

Collection of samples

Samples were collected from local rice fields of Mayurbhanj district of Orissa, India situated between 21°16'-23°34'N latitude and 85°40'-87°91'E longitude in different seasons during July, 2006- 2007. Collections were carried out following method adopted by (Rippka *et al*., 1979). The samples were collected from both soil and water using clean forceps, sampling bottles, polythene bags and planktonic net. All the samples were deposited and maintained at Department of Botany, North Orissa University, India.

Isolation and Identification of the samples

The samples were brought to the laboratory and processed immediately. Pure culture was obtained by serial dilution and agar plate method (Kaushik, 1987). The organisms were identified following the monograph of (Desikachary,1959); and Anand (1989). The morphological identifications were done measuring the cell size and shape of the organism. The systematic enumerations were carried out following the above mentioned monograph.

Cultivation and extraction of metabolites

Pure culture of cyanobacteria were cultivated in freshly prepared BG-11 ± N broth medium (Rippka *et al*., 1979); and incubated at 28 ± 2^0C and illumination at 3000 Lux with a white continuous light for 30-45 days. After incubation period the cultural broth were homogenized and separated into two fractions. The cell mass were filtered out by muslin cloth and culture filtrates were extracted thrice using equal volume of ethyl acetate in a separating funnel by vigorously shaking for 10 min. Cell mass were shade dried and grounded into powdered and then extracted using two different organic solvent (i.e. chloroform and methanol). All the organic solvents were evaporated in a rotary vacuum evaporator and concentrated to yield the crude extracts. The crude extracts were then dissolved in dimethyl sulphoxide (DMSO) for antimicrobial bioassay.

Screening for antimicrobial activity

The crude extracts were screened for their antimicrobial activity against some clinically significant microorganisms using agar cup diffusion method (Grammer, 1976). The test organisms include three gram positive organisms namely *Bacillus subtilis* (Bs), *Staphylococcus aureus* (Sa), *Staphylococcus epidermidis* (Se); five gram negative organisms namely *Escherichia coli(Ec)*, *Klebsiella pneumoniae* (Kp), *Shigella dysenteriae* (Sd), *Shigella flexneri* (Sf), *Vibrio cholerae* (Vc) and three pathogenic fungi namely *Candida albicans* (Ca), *Candida tropicalis* (Ct) and *Candida krusei* (Ck). All the test pathogens were procured from Institute of Microbial Technology (IMTECH), Chandigarh, India and maintained on freshly prepared nutrient agar medium. For antimicrobial assay, bacterial pathogens were cultured in nutrient broth and fungal pathogens were cultured in Sabouraud's broth. Nutrient agar plates were then inoculated with the overnight culture suspension of each test bacterial pathogen and sabouraud's agar plates were inoculated with the overnight culture suspension of each test fungi pathogen. The plates with inoculated organisms were then evenly spread out with sterile cotton swabs. Agar cups

were prepared by scooping out the media with a sterile cork borer (6mm in diameter). The cups were then filled with 100ìl of the crude extract that was already dissolved in DMSO. The plates were then incubated at 36±1°C for 24 h and the zone of inhibition was recorded and compared with the control (i.e a cup filled with DMSO solution only). Three replicates were maintained in each case.

Determination of minimum inhibitory concentration (MIC)

Minimum inhibitory concentration was determined for those extracts which showed considerable zone of inhibition during the antimicrobial bioassay. Broth micro-dilution method using 96 wells micro-titer plates and tetrazolium salt, 2, 3, 5, Triphenyl tetrazolium chloride (TTC) as indicator to microbial growth was used for determination of minimum inhibitory concentration (Eloff, 1998). 100ìl overnight culture of the test organisms were seeded into the wells serially and at the same time two fold dilution of the crude extracts to be tested were loaded into each wells. Wells containing DMSO and streptomycin sulphate (100ìg ml^{-1}) were used as negative and positive control respectively. Then 5ìl of 0.5% TTC was loaded into each well. The final volume of all the wells was 205ìl. The microplate was sealed and incubated at 37°C for overnight. Each assay was repeated thrice. The result was read by observing the growth of microorganisms in the wells. MIC was determined as the least concentration of extracts that inhibited the growth of test pathogen.

RESULTS AND DISCUSSION

Altogether 212 samples were collected from three agricultural zone of Mayurbhanj district of Orissa, India (Fig 1). A total of 58 taxa belonging to 20 genera were characterized. Out of these 19 forms were heterocystous and 39 were non-heterocystous. They belonged to 2 orders namely Chroococcales and Nostocales of Cyanophyta. Among the taxa 11 morphologically different cyanobacterial species were selected and evaluated for antimicrobial activity. Out of which 9 strains (81%) showed antimicrobial activity. The antimicrobial activities of the crude extracts were evaluated against some clinically significant microorganisms that include three gram positive bacteria, five gram negative bacteria and three pathogenic fungi by agar cup diffusion method. Crude metabolites were extracted using three different solvents namely chloroform, methanol and ethyl acetate. All the crude extracts showed antimicrobial activity inhibiting at least one of the test pathogens. However, crude extracts of two cyanobacterial species namely *Nostoc calcicola* and *Microcoleus* sp did not showed antimicrobial activity against the test pathogens. The methanolic extract of all cyanobacterial species showed antibacterial activity and 63% of the species showed antifungal activity inhibiting at least one the test pathogens. Among the bacterial pathogens *Shigella dysentriae* was found to be resistant and *Bacillus subtilis* was recorded to be susceptible to the crude extract. The methanol extract of all cyanobacterial species were resistant to the fungal pathogen, *Candida krusei* (Table 1). Considerable antimicrobial activity was detected in the chloroform extract. Among the cyanobacteria, *Phormidium* sp. showed antimicrobial activity against all the tested microbial strains. The extract was active in inhibiting both gram positive and gram negative bacterial pathogens (Table 2). Among gram positive bacteria *Bacillus subtilis* and *Staphylococcus aureus* were effectively inhibited by the crude extract while *E. coli* was found to be most susceptible among gram negative bacteria. The extract could display 45% of antifungal activity. Similarly, ethyl acetate extract showed antimicrobial activity inhibiting at least one of the test pathogens. Among the strains extract of *Cylindrospermum muscicola* showed antimicrobial activity against all the tested pathogens. The extract effectively inhibited two gram positive bacteria i.e. *Bacillus subtilis* and *Staphylococcus aureus* (Table 3). Comparatively less activity was detected against gram negative. The extract displayed 54% antifungal activity inhibiting almost all the test fungal pathogens. Minimum inhibitory concentration (MIC) was carried out in selected cyanobacterial strains that showed good zone of inhibition against the test pathogens during agar cup diffusion assay. The MIC ranged from 1000 µg ml^{-1} to 125 µg ml^{-1} (Table 4).

Cyanobacteria are recognized as a rich but not yet extensively examined source of

Table 1. Antimicrobial activity of Methanol extract of some freshwater cyanobacteria

Cyanobacteria	Solvent used : Methanol Zone of inhibition (mm)										
	Bs	Sa	Se	Ec	Kp	Sd	Vc	Sf	Ca	Ct	Ck
Oscillatoria sp.	11.3±0.58	-	-	10.7±0.58	-	-	ND	ND	09.3±0.58	-	-
Oscillatoria curviceps	09.0±1.0	09.3±0.58	-	10.3±0.58	08.3±0.58	-	ND	ND	10.7±0.5	08.3±0.58	-
Phormidium sp.	10.7±0.58	-	09.7±0.58	09.7±0.58	09.7±0.58	-	ND	ND	10.0±1.0	-	-
Aphanothece microscopica	12.3±0.58	14.0±1.0	-	12.0±1.0	10.0±1.0	-	ND	ND	09.7±0.58	09.3±0.58	ND
Nostoc piscinale	12.0±1.0	09.0±1.0	08.3±0.58	-	ND	11.0±1.0	ND	ND	11.3±0.58	ND	-
Nostoc calcicola	-	-	-	-	-	-	ND	ND	-	-	-
Anabaena variabilis	10.3±0.58	10.3±0.58	-	09.0±1.0	-	-	ND	ND	-	-	ND
Anabaena spiroides	11.0±1.0	-	09.7±1.15	10.0±1.0	-	ND	ND	ND	-	-	-
Cylindrospermum muscicola	12.0±1.0	10.7±0.58	-	08.7±0.58	11.3±0.58	-	ND	ND	09.7±0.58	-	-
Microcoleus sp.	-	-	-	-	-	-	ND	ND	-	-	-
Microcystis sp.	09.7±0.58	10.0±1.0	10.3±0.58	-	-	10.0±1.0	ND	ND	09.0±1.0	-	-

*ND- not determined; – no zone of inhibition; Bs- *Bacillus subtilis*; Sa-*Staphylococcus aureus*; Se- *Staphylococcus epidermidis*; Ec- *Escherichia coli*; Kp- *Klebsiella pneumoniae*; Sd- *Shigella dysentriae*; Vc- *Vibrio cholerae*; Sf- *Shigella flexneri*; Ca- *Candida albicans*; Ct-*Candida topicalis*; Ck- *Candida krusei*; Values are means of three replicates (n=3); ± SD

Table 2. Antimicrobial activity of chloroform extract of some freshwater cyanobacteria

Cyanobacteria	Solvent used : Chloroform Zone of inhibition (mm)										
	Bs	Sa	Se	Ec	Kp	Sd	Vc	Sf	Ca	Ct	Ck
Oscillatoria sp.	13.3±0.58	09.3±0.58	-	11.3±0.58	-	08.3±0.58	14.7±0.58	-	ND	ND	ND
Oscillatoria curviceps	09.7±0.58	10.3±0.58	-	11.7±0.58	09.3±1.52	-	-	ND	11.3±1.52	09.0±1.0	-
Phormidium sp.	12.0±1.0	15.7±1.52	15.0±1.0	13.7±0.58	12.7±1.52	12.3±0.58	ND	ND	13.0±1.0	10.3±0.58	ND
Aphanothece microscopica	11.3±0.58	10.0±1.0	-	13.3±1.52	10.0±1.0	12.0±1.0	ND	-	-	10.7±0.58	ND
Nostoc piscinale	15.3±0.58	10.0±00	-	11.0±1.0	ND	10.0±2.64	13.3±0.58	ND	ND	ND	ND
Nostoc calcicola	-	-	-	-	ND	-	-	ND	ND	-	-
Anabaena variabilis	10.0±1.0	09.0±1.0	-	09.3±0.58	ND	-	-	-	-	ND	ND
Anabaena spiroides	12.7±0.58	10.7±0.58	-	ND	09.0±1.0	-	10.0±1.0	-	ND	ND	-
Cylindrospermum muscicola	11.3±0.58	11.7±1.52	12.0±2.0	14.3±0.58	-	10.0±2.0	ND	ND	12.0±2.64	13.0±1.0	-
Microcoleus sp.	-	-	-	-	-	ND	-	-	-	-	ND
Microcystis sp.	-	09.7±0.58	10.0±1.0	12.0±1.0	ND	09.7±1.15	-	ND	ND	-	09.0±1.0

*ND- not determined; - no zone of inhibition; Bs- *Bacillus subtilis;* Sa-*Staphylococcus aureus;* Se- *Staphylococcus epidermidis;* Ec- *Escherichia coli;*
Kp- *Klebsiella pneumoniae;* Sd- *Shigella dysentriae;* Vc- *Vibrio cholerae;* Sf- *Shigella flexneri;* Ca- *Candida albicans;* Ct-*Candida topicalis;*
Ck- *Candida krusei;* Values are means of three replicates (n=3); ± SD

Table 3. Antimicrobial activity of Ethyl acetate extract of some freshwater cyanobacteria

Cyanobacteria	Solvent used : Ethyl acetate Zone of inhibition (mm)										
	Bs	Sa	Se	Ec	Kp	Sd	Vc	Sf	Ca	Ct	Ck
Oscillatoria sp.	-	11.3±0.58	09.7±0.58	-	09.3±1.52	ND	ND	08.0±1.0	11.0±1.0	-	ND
Oscillatoria curviceps	10.3±0.58	09.7±1.15	12.0±1.0	ND	-	-	ND	09.7±0.58	10.0±1.0	09.3±2.1	-
Phormidium sp.	12.0±1.0	12.7±1.52	-	09.0±2.0	-	-	ND	11.3±1.15	-	ND	10.7±0.58
Aphanothece microscopia	12.3±0.58	10.7±1.52	10.7±1.52	-	ND	08.7±1.52	-	-	-	09.3±1.52	11.0±1.0
Nostoc piscinale	11.3±0.58	11.0±1.0	-	09.7±1.52	ND	-	ND	-	-	ND	ND
Nostoc calcicola	-	-	-	ND	-	ND	ND	-	-	ND	ND
Anabaena variabilis	11.0±1.0	08.7±1.52	-	10.7±1.52	ND	ND	10.0±1.73	-	-	ND	-
Anabaena spiroides	10.7±1.52	10.3±1.52	-	10.0±1.0	ND	-	09.3±1.52	ND	ND	-	-
Cylindrospermum muscicola	12.7±0.58	10.3±1.52	10.3±1.15	12.7±1.52	14.0±1.0	10.0±1.73	ND	ND	09.3±1.52	10.3±0.58	-
Microcoleus sp.	-	-	-	-	-	-	ND	ND	-	-	ND
Microcystis sp.	-	11.3±0.58	10.0±1.0	11.3±1.52	-	11.0±1.0	ND	ND	10.7±1.15	08.3±0.58	-

*ND- not determined; - no zone of inhibition; Bs- *Bacillus subtilis;* Sa-*Staphylococcus aureus;* Se- *Staphylococcus epidermidis;* Ec- *Escherichia coli;* Kp- *Klebsiella pneumoniae;* Sd- *Shigella dysentriae;* Vc- *Vibrio cholerae;* Sf- *Shigella flexneri;* Ca- *Candida albicans;* Ct-*Candida topicalis;* *Ck- Candida krusei;* Values are means of three replicates (n=3); ± SD

Table 4. Minimum inhibition concentration of the crude extracts against three bacterial pathogens

Test pathogens	MIC values (µg/ml)															
	Chloroform extract					Methanol extract					Ethyl acetate extract					
	Os	Oc	Ps	Am	Np	Cm	Os	Oc	Ps	Am	Np	Cm	Os	Oc	Ps	Am	Np	Cm
Bs	250	500	250	500	250	250	500	500	250	500	250	250	1000	500	500	250	500	250
Sa	500	500	125	500	500	250	1000	500	1000	250	500	500	500	500	250	500	500	500
Ec	500	500	250	250	500	125	500	500	500	500	1000	500	1000	1000	500	1000	500	250

Bs- *Bacillus subtilis* Os- *Oscillatoria* sp. Am- *Aphanothece microscopia*
Sa-*Staphylococcus aureus* Oc- *Oscillatoria curviceps* Np- *Nostoc piscinale*
Ec- *Escherichia coli* Ps- *Phormidium* sp. Cm- *Cylindrospermum muscicola*

pharmacological as well as structurally interesting secondary metabolites (Carmichael, 1992). Screening for a wide variety of potentially useful bioactives, including cytotoxic, multidrug resistance reversal, antifungal and antiviral effects has led to the discovery of numerous novel bioactive metabolites including peptides, macrolides and glycosides from various cyanobacterial strains. Most of the bioactive metabolites identified so far have been derived from marine cyanobacteria. However, freshwater cyanobacteria remain less explored for bioactive metabolites. Recent investigation reveals that freshwater cyanobacteria could also be a potential source of important bioactive metabolites for therapeutic applications (Skulberg, 2000). In the present investigation out of 11 cyanobacterial strains collected from locally occurring rice field, crude extract of 9 cyanobacteria were capable of inhibiting clinically significant microorganisms. Such results clearly agree with the assumption that rice field cyanobacteria harbour active metabolites of therapeutic values.(Kreitlow *et al.,* 1999); while examining crude extracts of different cyanobacterial strain found that none of the extracts showed activity against gram negative bacteria. However, (Ordog *et al*., 2004); have reported that secondary metabolites excreted by cyanobacteria are active against gram positive and gram negative microorganisms. Similar results were also obtained in our present study where crude extracts of different cyanobacterial strains not only showed activity against both gram positive and gram negative bacteria but also against pathogenic fungi. Such finding suggests that cyanobacteria produce diverse secondary metabolites with antimicrobial activity. Further, production of secondary metabolites might vary among different cyanobacterial strains based on different habitats and ecological niches. Such variations have been noticed from freshwater cyanobacteria collected from different parts of the world. In the present study three organic solvents (methanol, chloroform and ethyl acetate) were used for extraction of metabolites. In general all the crude solvent extracts showed antimicrobial activity. In most cases organic solvent like methanol has been commonly used for extraction of metabolites and it has proved to be efficient in inhibiting both bacterial and fungal pathogens

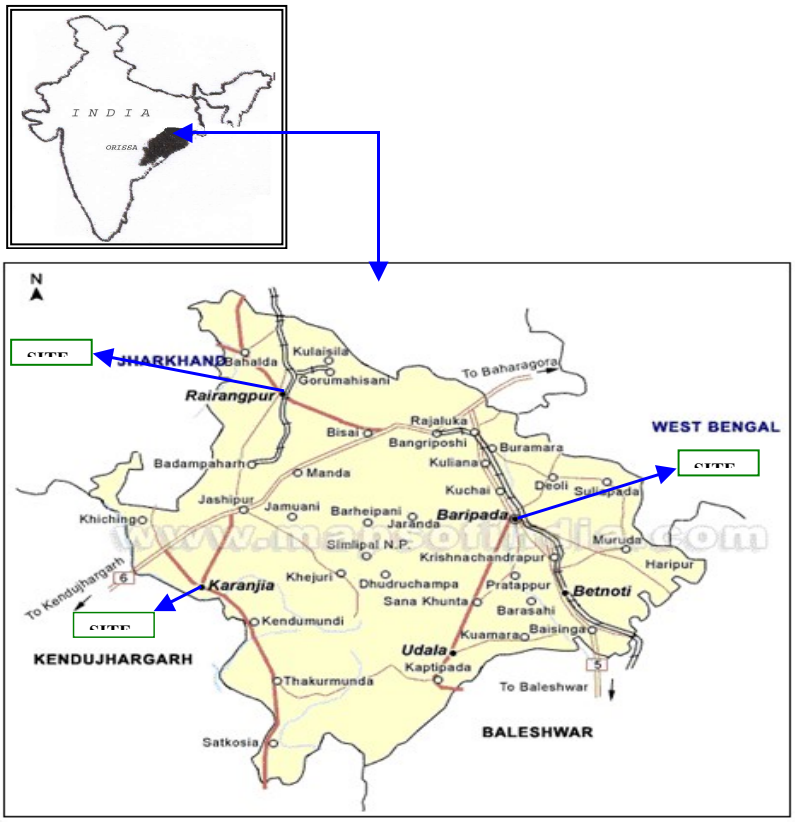

Fig. 1. Map showing collection site

(Ostensvik *et al*., 1998; Raveh and Carmeli, 2005). Similar results were obtained in our study where methanolic extract displayed both antibacterial and antifungal activity. Various workers have reported active metabolites from freshwater cyanobacteria of the family Nostocaceae (Rezanka and Dembitsky, 2006). The antimicrobial activities of four species of cyanobacteria under family Nostocaceae in our present study agree with such findings. In nature all organisms need to compete in order to survive in their habitat. This task is achieved by developing secondary metabolites, enzyme and toxins that inhibit other organisms. The production of antimicrobial metabolites in crude extracts of cyanobacteria may be due to interaction with other strains or to have antagonistic behaviours towards pathogens. Such phenomenon is term as allelopathy and has been well documented in various cyanobacterial strains (Leflaive and Ten-hage, 2007). Some of these allelopathic compounds have been shown to inhibit various competitors or predators. Very few workers have used solvent like chloroform and ethyl acetate for extraction of cyanobacterial metabolites. However, our results suggest that these solvent could also been used for extraction of antimicrobial metabolites. Although various freshwater cyanobacteria were documented to produce active metabolites, species like *Aphanothece microscopica* has not been reported yet. The present study reports antimicrobial activity of this freshwater cyanobacterium for the first time.

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