Characterization of the Biocidal Spectrum of Extracellular Filtrates of *Microcystis aeruginosa*

Pranita Jaiswal · Radha Prasanna ·
Pawan K. Singh

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Abstract The effect of dichloromethane (DCM) and ethyl acetate (EA) extract of a cyanobacterium, *Microcystis aeruginosa* was evaluated against unicellular cyanobacteria and the phytopathogenic fungus *Rhizoctonia solani*. Fractionation of the filtrate showed the presence of five spots of different Rf values on silica gel coated plates indicating the presence of a number of compounds in the extract. A marked reduction in growth (52%) of the fungus was recorded on the plates supplemented with cyanobacterial extract, indicating the involvement of anti-fungal metabolite(s). The extract did not show any negative influence on seed germination and growth of seedlings of wheat, rice and mung, emphasizing the suitability of the compound for use in agriculture.

Keywords Cyanobacteria · *Microcystis* · Fungicidal · *Rhizoctonia*

Introduction

Cyanobacteria constitute an ancient, diverse and widely distributed group of oxygen evolving photosynthetic

prokaryotes having properties in common to both higher plants and bacteria. They show remarkable metabolic flexibility and biochemical diversity and successfully colonize almost all kinds of terrestrial and aquatic habitats. They are equipped with effective protective mechanisms against different biotic stresses and have remarkable adaptability to varying environmental conditions. Under favorable nutritional and environmental conditions, they proliferate into cyanobacterial blooms, which are becoming more common with the increasing eutrophication of fresh water bodies. The concern over the outcome of cyanobacterial blooms has increased during the past decades with increasing information on the detrimental power of the toxins produced by these organisms [1–3]. They are known to be a rich source of secondary metabolites. Such secondary metabolites produced by cyanobacteria are of special interest, not only as disease causing agents but also as bioactive molecules in applied and basic studies. Globally, they have been frequently associated with various kinds of water management problems, including many cases of human poisoning, animal sickness and death due to ingestion of water contaminated with toxic cyanobacteria [2, 4, 5]. Ecologically, these antimicrobial compounds of cyanobacteria are important as they may play a critical role in the allelopathic interactions among microorganisms in any ecological community/niche. Only recently the chemical, pharmaceutical, immuno-modulatory, bio-regulatory and therapeutic potential of these exciting bio-molecules have been recognized internationally [6, 7]. Scientists all over the world are now looking for the commercial exploitation of these bioactive molecules with novel chemical and molecular structures [8].

The present research work is being undertaken to bring to light the utility of the bioactive molecules produced by the cyanobacterium, *Microcystis aeruginosa*, which is

P. Jaiswal
Division of AS and EC, Central Institute of Post Harvest
Engineering and Technology (CIPHET), Ludhiana 141004, India

R. Prasanna (✉)
Division of Microbiology, Indian Agricultural Research Institute
(IARI), New Delhi 110012, India
e-mail: radhapr@gmail.com

P. K. Singh
Department of Botany, Banaras Hindu University,
Varanasi 221005, India

notorious in creating nuisance in ponds/water bodies and the possible use of the toxic compounds produced by *Microcystis aeruginosa* as a biocontrol agent against phytopathogenic fungi.

Materials and Methods

Organism and Growth Condition

Microcystis aeruginosa used in this investigation was isolated from Laxmikund pond, Varanasi [9]. The strain was cultivated and maintained in the laboratory in Parker's medium [10]. Two unicellular cyanobacterial strains (*Synechococcus* sp., *Synechocystis* sp.) used as test organisms were maintained in N⁺ BG-11 medium [11]. The cultures were incubated at 27 ± 1°C, 3,000-lux light intensity and 16:8 L/D cycles. The fungal strains (*Pythium debaryanum*, *Rhizoctonia solani* and *Rhizoctonia bataticola*) used for evaluating fungicidal activity were obtained from Division of Agricultural Chemicals, IARI, New Delhi. The cultures were maintained on potato dextrose agar medium. The fungal strains were incubated at 28 ± 2°C in a BOD incubator.

Mass Culture of *Microcystis aeruginosa*

The axenised culture of the cyanobacterium was set for mass multiplication in 3 l flasks and incubated under optimal growth promoting conditions for the cyanobacterium [10]. The cultures were harvested in exponential phase of growth by pelleting at high speed (15,000 rpm) for 20 min. The cell free extracts were pooled for the extraction of secondary metabolites.

Extraction of Secondary Compounds

The culture filtrates were partitioned with non-polar, polar and highly polar solvents (*n*-Hexane, Dichloromethane and Ethyl acetate) sequentially to extract all the compounds present in culture filtrates. The organic phase was then separated from the aqueous phase and dried over anhydrous sodium sulphate. The extracts were then subjected to rotary evaporation under pressure. The solid extract was dissolved in acetone and stored at 4°C until further analyses.

Biocidal Activity of the Compounds

The bioactivity of the cyanobacterial filtrate, crude extracts and the partially purified compounds as tested on the lawn of two unicellular cyanobacteria (*Synechococcus* sp. and *Synechocystis* sp.) and three phytopathogenic fungus by disc diffusion technique [9]. The diameter of the inhibition

zone was taken as the measure of extent of toxicity. The percent inhibition of the fungal strain was also calculated by food poisoning technique and the colony size of the fungal strain was measured.

Minimum Inhibitory Concentration (MIC)

The inhibitory effect of the concentrated extracts was examined in agar-based media using 96 well ELISA plates [12]. The extracts obtained after rotary evaporation of the extracts were weighed and dissolved in desired volume of acetone and a series of different dilutions were prepared. Five replicates of each concentration were put on a sterilized medium (BG-11/PDA) placed on the wells of ELISA plates. Each well was inoculated with test organism (cyanobacterium/fungus) and incubated under suitable conditions along with suitable controls.

Effect of Cyanobacterial Extract on Important Crop Plants

The effect of crude extract of the cyanobacterium was tested on seed germination, length of coleoptile and radicle of wheat (*Triticum aestivum* var HD2689), Rice (*Oryza sativa* var Pusa Basmati 1) and Mung (*Vigna mungo* var Pusa Ratna). Seeds were surface sterilized with 0.1% mercuric chloride for 30 min and thereafter washed thoroughly with sterile water. They were imbibed overnight in sterile water in dark at room temperature and kept for germination on water agar (2% agar in distilled water containing MIC levels of cyanobacterial extracts). The experiments were conducted in triplicate with ten seeds per plate for each crop. The plates were incubated in dark at 25 ± 2°C. Percentage germination of the seeds was calculated after 24 h of incubation while coleoptile, radicle length was measured after 96 h of incubation.

Chromatographic Fractionation of the Compounds

Thin layer chromatography of the sample solution was done to separate the compounds present in the extracts of *Microcystis aeruginosa*. Silica gel coated plates (0.15 mm thick), activated in a hot air oven at 120°C were applied for TLC. The crude extract dissolved in acetone was spotted on the TLC plates and then passed through different solvent system(s) to achieve maximum separation of the compounds present in the extract.

Statistical Analysis

Statistical analysis was employed using Microsoft Excel. At least three independent experiments were conducted and readings were taken in triplicate for each set.

Results

Biocidal Activity

The cyanobacterial extracts obtained after extraction using non-polar, polar and highly polar solvents were tested for their biocidal activity. Algicidal activity was recorded in all the three extracts (Hexane, Dichloromethane and Ethyl acetate) but with varying degree of sensitivity (Fig. 1). Maximum activity was recorded in DCM extract followed by EA and *n*-Hexane extract. The *n*-Hexane extract did not show any fungicidal activity while both DCM and EA extract inhibited the growth of fungi. Among the three fungi tested, *Rhizoctonia solani* was inhibited by the cyanobacterial extract, but the potency of inhibition was more in DCM extract.

The bioactivity of the extracted compound when tested against the *R. solani* revealed significant reduction in growth (Table 1), which was observed from the third day of incubation onwards (6.25%) and the mycelial growth was reduced to 52% after 9 days of incubation.

Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration required for the algicidal and fungicidal activity were calculated by serial dilution of the concentrated extract. The MIC for algicidal activity was 31.25 ppm, while it was 62.5 ppm for fungicidal activity.

Fig. 1 Inhibition zone produced by cyanobacterial extract (in different solvent systems) on the lawn of unicellular cyanobacteria (*Synechococcus* sp. and *Synechocystis* sp.) and phytopathogenic fungus (*Rhizoctonia solani*)

Table 1 Effect of cyanobacterial extract (*Microcystis aeruginosa*) on colony size of *Rhizoctonia solani*

Incubation period (days)	Colony diameter (cm) control plates	Colony diameter on plates supplemented with crude cyanobacterial extract	Reduction in growth (%)
1	0.4	0.4	0.00
2	0.9	0.9	0.00
3	1.6	1.5	6.25
4	2.8	2.1	25.00
5	3.5	2.5	29.00
6	4.3	2.8	35.00
7	5.4	2.9	46.00
8	5.8	3.0	48.00
9	6.5	3.1	52.00

Effect of Cyanobacterial Extract on Important Crop Plants

The effect of cyanobacterial crude extract on seed germination of important crop plants was evaluated (Table 2). The concentration of the extract used for this study was 31.25 and 62.5 ppm (i.e. the concentration showing biocidal activity). It was observed that the extracts did not show any significant effect on germination of wheat and rice seeds, whereas the germination of mung seeds were reduced by 10% at 62.5 ppm (MIC). Similarly, the extracts did not show any negative influence on the length of coleoptile and radicle of wheat and rice plant, while in the case of mung seedling 23% reduction in coleoptile length and 6% reduction in radicle length was recorded at 62.5 ppm concentration (Table 3).

Chemical Characterization of Cyanobacterial Extract

The culture filtrate of the cyanobacterium *Microcystis aeruginosa* isolated from Laxmikund pond, Varanasi, showed algicidal activity [7]. Preliminary chemical characterization of the extract (in different solvent systems) was carried out through TLC. Combinations of different solvent systems were used for resolving the cyanobacterial extracts and hexane:benzene (1:1) proved to be most efficient for maximum resolution of extracts. The *n*-hexane extract resolved into three spots while dichloromethane (DCM) and ethyl acetate (EA) extract resolved into five

Table 2 Effect of cyanobacterial extract on seed germination of important crop plants

Concentration of cyanobacterial extract (ppm)	Seed germination (%)		
	Wheat	Rice	Mung been
31.25	100	100	100
62.5	100	100	90

Table 3 Effect of cyanobacterial extract on the length of coleoptile and radicle of important crop plants

Treatments	Length (cm)					
	Wheat		Rice		Mung bean	
	Coleoptile	Radicle	Coleoptile	Radicle	Coleoptile	Radicle
Control (water)	4.87 ± 0.15	3.0 ± 0.00	5.97 ± 0.58	3.03 ± 0.06	3.10 ± 0.10	1.93 ± 0.06
Control (acetone)	4.87 ± 0.12	2.97 ± 0.06	5.87 ± 0.15	3.00 ± 0.00	3.10 ± 0.00	1.90 ± 0.10
31.25 ppm	4.90 ± 0.10	2.97 ± 0.06	5.83 ± 0.15	2.93 ± 0.06	3.00 ± 0.10	1.90 ± 0.10
62.50 ppm	4.87 ± 0.12	2.90 ± 0.00	5.77 ± 0.12	2.93 ± 0.06	2.87 ± 0.06	1.87 ± 0.11

Table 4 Rate of flow of different fractions of crude extract of *Microcystis aeruginosa* after thin layer chromatography using silica coated plates

Solvent	No. of bands	Rf values							
		A	B	C	D	E	G	H	
<i>n</i> -Hexane	3	0.11	0.22	–	–	0.63	–	–	
Dichloromethane	6	0.11	0.22	–	0.49	0.63	0.86	0.92	
Ethyl acetate	6	0.11	0.22	0.26	–	0.63	–	0.92	

spots each. The three spots present in *n*-hexane extract were common to both DCM and ethyl acetate extract. The other spots for DCM and EA extracts differed from one another and the Rf values of the spots are given in the Table 4.

Biocidal Activity of Partially Purified Extract

The extracted toxin (DCM extract) was partially purified using preparatory thin layer chromatography. It was found that fraction 4 exhibited maximum activity followed by fraction 5 while the other fractions did not show any fungicidal activity.

Discussion

Cyanobacterial blooms, have now become very common due to increasing eutrophication of water bodies through nutrient enrichment from fertilizer run off from agricultural land, domestic and industrial effluents etc. Some of the common bloom-forming cyanobacterial genera are *Microcystis*, *Aphanizomenon*, *Anabaena*, *Nostoc*, *Nodularia*, *Oscillatoria* etc. Among them, *Microcystis* is the most common and well characterized genus, which produce microcystins, a group of heptapeptide hepatotoxins having a variety of forms with distinct toxicity levels [13]. The role of environmental, physical and nutritional factors on toxin production by this cyanobacterial genus has been the subject of intensive research since 1980s [10, 14–18, 25]. Also, the genetic aspect of toxin production i.e. the enzyme

responsible for toxin production, enzyme structure and genes involved in toxin production by this cyanobacterial genus has also been investigated by several workers [19–22]. Studies have revealed that there is significant influence of culture age on microcystin production. Most of the reports indicate that it is released into the medium in the late exponential phase after cell lysis [2, 25]. Dias et al. [23], recorded similar results with cyanobacterium *Aphanizomenon* sp. LMECYA 11. They reported that the amount of extracellular toxins increased with culture time, indicating the toxins are released in water through cell lysis and may be expected to remain there upon the collapse of toxic blooms. However, Ray and Bagchi [18] reported a positive correlation of biomass yield and algicide production in *Oscillatoria* sp.

During the course of the present investigation the presence of biocidal compound was recorded in the crude extract of *Microcystis aeruginosa*, which showed algicidal and fungicidal activity. In our earlier study with this cyanobacterium, algicidal activity was recorded at all the growth phases i.e. early exponential to decline phase, but the amount of metabolite present varied with culture age and was found to be directly related with the growth of the cyanobacterium [10], these results were similar to those of Ray and Bagchi [18]. Also, the cyanobacterium did not show toxicity against albino mice (data not shown). This reflects that the compounds might include other compounds besides toxin, which play a role in allelopathic interactions, thereby contributing to maintenance of cyanobacterial bloom.

The production of metabolites other than known toxins is not uncommon in cyanobacteria. Suikkanen et al. [24] also reported that the culture of *Nodularia spumigena* was more allelopathic in exponential phase and more hepatotoxic in stationary phase. Volk [25] also reported that the content of exometabolites of a cyanobacterium *Nostoc insulare* changes with growth phase. During the linear growth phase, a non toxic metabolite was prevalent while, in stationary phase the content of antimicrobial, cytotoxic metabolite increased. Other workers obtained similar results, which shows that there is no correlation between toxin production and antibiosis in cyanobacteria [26, 27].

The bioassay of crude extract of cyanobacterium (H, DCM, EA) indicated that DCM extract of the cyanobacterium was most active followed by EA extract. Hexane extract did not show any biocidal activity indicating the polar nature of the bioactive molecule. Although the complete chemical nature of the bioactive molecule could not be identified in the present study, bioassay (against *R. solani*) of different fractions of DCM extract showed that compound no. 4 is most active followed by compound no. 5. The other fractions did not show any bioactivity indicating the possible role of anti-fungal compound (compound no. 4) in the inhibition of the fungus. The presence of anti-fungal compounds has been reported in a few cyanobacterial genera [6, 7, 28, 29].

Effect of crude cyanobacterial extract, on seed germination and growth of important crop plant, revealed that the extract did not have any negative influence on seed germination and growth of seedlings. This can be of added advantage for the use of extract as a fungicidal agent.

The TLC separation of crude cyanobacterial extract in *n*-Hexane, Dichloromethane and Ethyl Acetate showed the presence of 3, 5 and 5 spots, respectively when Hexane: Benzene was used as solvent system. This confirms the presence of more than one metabolite in the extract(s); which is further confirmed by presence of more than one peak in the GLC analyses of the extracts. The presence of large no of metabolites with varying toxicity and host range has been reported in cyanobacteria [30–32]. Recently Volk and Mundt [33] also reported the presence of no. of metabolites in filtrate of cyanobacterium *Nostoc insulare* though all of them were not toxic.

There has been tremendous concern all throughout the world over the nuisance created by this bloom-forming cyanobacterium, i.e. *Microcystis*. The current investigation highlights the potential of this cyanobacterium as a source of compounds for application in agriculture and industry. Also, it emphasizes the need to characterize the cyanobacterial extract/filtrate at different growth phases, which may prove to be of tremendous biotechnological interest because of the broad spectrum of activity and their pharmaceutical significance.

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