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STUDY ON SECONDARY METABOLITES OF FUNGI AS POTENTIAL ANTIALGAL COMPOUNDS

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ABSTRACT – Algae cause deterioration, colored patinas (green disease), incrustations, formation of paint blisters, and degradation of support polymers or of glues and binders resulting in detachment of the paint layer from the support.

Samples of greenish or grayish black algal biofilms were taken from the external surfaces of old buildings in Mumbai (India) to analyze the algae present, and were found to belong to genera; *Phormidium, Chroococcus* and *Oscillatoria* The fungal isolates with antialgal activity were isolated from natural fresh or marine water bodies showing algal growth on their surface. The fungi with antialgal activity belonged to genera *Aspergillus* and *Fusarium*. Secondary metabolites from these fungi were extracted using various solvents. Cell free supernatant and purified extracts of these fungi were found to inhibit growth of algal lawns of *Phormidium* sp, *Oscillatoria* sp and *Chroococcus* sp. as demonstrated in the paper disc method. Further an *in situ* method was conceptualized to evaluate the effectiveness of the extracted antialgal compounds by inoculating the isolated algae into white cement or white distemper paint holding the inhibitory molecules. Chloroform extract of *Fusarium* sp showed maximum activity against *Phormidium* sp whereas, Hexane extract of *Aspergillus* sp showed maximum activity against *Oscillatoria* sp.

The lyophilized extracts were subjected to GC-MS analysis. The chloroform extract of *Fusarium* sp revealed the presence of Benzene,1,4-dichloro-, Dibutyl phthalate while its methanol extract showed the presence of 3,4 Dihydrothieno-(3,4-b)-5-carbox and Fenmetramide. The hexane extract revealed the presence of; 16-Octadecanoic acid, Hexadecanoic acid and Benzene,1,4-dichloro- compounds.

Key words : Algae, fungi, antialgal, white cement and white distemper paint.

INTRODUCTION

Microorganisms found on external walls could be diverse including algae, fungi, bacteria, actinomycetes, myxomycetes and protozoa. Of these, cyanobacteria are more frequently present and indicate an ecologically isolated group (Crispim *et al,* 2006). Algae are extremely diverse in morphology and size ranging from unicells about one micrometer in diameter up to complex seaweeds many meters long (Acreman, 1994). However, cyanobacteria can survive repeated drying and rehydration cycles and high UV levels giving them a distinct advantage over many other organisms on exposed surfaces (Gracia *et al*, 1992; Roy *et al*, 1997). Phototrophic microorganisms growing on modern and historic buildings cause deterioration of the structure. They cause degradation of structures through physical penetration, colored patinas (green disease), incrustations, degradation of support polymers or of glues and binders resulting in detachment of the paint layer from the support (Gaylarde *et al*, 2005; Macedo *et al*, 2009; Ciferri, 1999). Algal growth usually does not occur on interiors, instead is normally seen externally in wet or humid environments, typically an area of the building which is wetted by a leaking drainage. The most widespread and commonly reported alga on external walls belongs to the genera of *Gloeocapsa, Phormidium, Chroococcus* and, among chlorophyta to *Chlorella, Stichococcus* and *Chlorococcum* (Macedo *et al,* 2009).

Lytic fungi from myriad environments were isolated and fungi representing genera *Acremonium* sp and *Emericellopsis* sp were reported. The lytic activity was associated with a diffusible heat-stable extracellular molecule which, evidence suggests, could be cephalosporin antibiotic(s).Thus frequent isolation of lytic fungi from algal habitats suggests a possible natural algal-destroying role for such fungi, and can be exploited for control of algal bloom (Redhead and Wright, 1978).

The current study focuses on screening, isolation and analysis of secondary metabolites from fungi which exhibit antialgal activity. Further it demonstrates the potential use of these molecules in environments where algae are likely to infest.

MATERIALS AND METHODS

Sampling methods:

Samples of greenish or grayish black algal biofilms

were taken from the external surfaces of buildings (50 years old) in Mumbai (India) by scraping the dried growth in an empty sterile petri plate. The samples were transferred to sterile tap water containing 0.025% $CaCO_3$ and $NaNO_3$, incubated in light away from direct sunlight till its purification process was initiated. Further these algae were cultivated in a 1:2 diluted Algae Culture broth [0.1% sodium nitrate, 0.025% K_2HPO_4, 0.0513%$MgSO_4$, 0.005% NH_4Cl, 0.0058% $CaCl_2$, 0.0003% $FeCl_3$]. Dilution and repeated sub culturing in the same medium isolated and purified the algal cultures.

Paper disc assay was done using either the neutralized crude cell free supernatant (CFS) or lyophilized extracts on algal lawn, developed by incorporating algal isolates into solid algae culture medium (1.5%). These algal plates were incubated at RT in presence of white light for desired period maintaining the day night system (Safferman and Morris, 1962).

Detection, isolation, identification and maintenance of antialgal fungi:

The fungal isolates were isolated from fresh or marine water bodies in Mumbai showing algal growth on their surface. Sabouraud's Broth was used for enrichment of fungi while Potato Dextrose Agar (PDA) was used for their isolation and purification. Temperature tolerance, ballistospore production and microscopic identification were used to identify the cultures (Koneman and Roberts, 1985; Barnett, 1969).

Extraction and analysis of molecules with antialgal activity:

Aspergillus sp and *Fusarium* sp being the effective fungal isolates were inoculated at 10^5 spores/ml into Sabouraud's broth and incubated under shaker conditions for 2 days and subsequently under static conditions for another 3days. CFS was harvested, neutralized and extracted by two methods using different organic solvents (Young *et al*, 2009; Alamsjah *et al,* 2005). Hence, four different fractions (hexane, chloroform, 60% methanol, and water) were collected from each fungal isolate. The extracts were lyophilized (Micromodulyo freeze dryer) and surrendered to paper disc assay and GC-MS Analysis (IIT- Mumbai).

Application studies :

Two tiles (2.5" x 2.5") were held in place on a design mount. 3g of white cement was mixed separately with 1.5ml of each extract and 0.5ml of 1:2 diluted sterile algae culture broth, and the paste was smeared between the two adjacent sides of the joints. Control paste was made by replacing sterile water for extract. After allowing the cement to completely dry at RT for a day, fixed amount of each of the algal isolate was uniformly inoculated over the dried cement. The entire mount was covered with a moist filter paper and incubated in natural light. After 7days of incubation, the filter paper was removed and the alga was reinoculated. For testing in paints, 10g of white distemper was mixed with 5ml of each of the fungal extract and 2.5ml of 1:2 diluted algae culture broth. This mixture was applied on two sides (duplicates) of each concrete brick pre-coated with plaster of Paris. Control was made by replacing sterile water for extract. The remaining methodology was as for cement study. Results were finally noted after 21 days. All studies were done in duplicates. The total area with algal growth was calculated using a graph paper, thus establishing a quantitative relationship.

RESULTS AND DISCUSSION

Screening of algae and fungi:

Samples of algal biofilms taken from the external surfaces of buildings were purified and microscopically identified to belong to genera *Phormidium, Chroococcus* (Komárek, 1992) and *Oscillatoria* (Bergey and Holt, 1994). Microalgae, cyanobacteria and algae are reported on exterior of buildings and rock surfaces. However, species of *Gloeocapsa, Chroococcus, Chroococcidiopsis, Phormidium, Leptolyngbya, Nostoc, Scytonema, Chlorella* and *Trentepholia* show global occurrence (Gaylarde and Gaylarde, 2005; Samad and Adhikary, 2008;Crispim *et al*, 2006).

Five species of fungi were isolated from fresh and marine water bodies with algal growth and 3 were found to exhibit inhibition of algal growth. The

Table 1 : Detection of antialgal activity of extracts from fungi by the paper disc assay.

Name of Fungus	Solvent used for antialgal activity	Test algae	Zone of inhibition (mm)
Aspergillus fumigatus	Water extract	*Oscillatoria* sp	6
	Water extract	*Chroococcus* sp	7
	Hexane extract	*Chroococcus* sp	8
Fusarium equiseta	Water extract	*Phormidium* sp	6
	Chloroform extract	*Chroococcus* sp	9
Aspergillus niger	Chloroform extract	*Phormidium* sp	5
	Methanol extract	*Oscillatoria* sp	5

Table 2 : Quantitative measurement of algal inhibition by fungal extract on white cement between the tiles

Algae inoculated	Source of antialgal compound	Area covered by algae(cm2)	% reduction in algae
Oscillatoria sp	Control	2.05	-
	Methanol extract of *Aspergillus niger*	1.05	48.79
Chroococcus sp	Control	2.59	-
	Methanol extract of *Aspergillus niger*	1.25	51.74
	Chloroform extract of *Aspergillus niger*	1.62	37.46
Phormidium sp	Control	2.98	-
	Chloroform extract of *Fusarium equiseti*	0.7	76.52
	Hexane extract of *Aspergillus fumigatus*	1.25	58.06

Key - : No reduction in algal growth

Table 3 : Quantitative measurement of algal inhibition by fungal extract on white distemper paint

Algae inoculated	Source of antialgal compound	Area covered by algae(cm2)	% reduction in algae
Oscillatoria sp	Control	7.19	-
	Methanol extract of *Aspergillus niger*	3.72	48.26
	Hexane extract of *Aspergillus fumigatus*	1.63	77.32
	Chloroform extract of *Aspergillus niger*	3.55	50.62
	Water extract of *Aspergillus fumigatus*	2.87	60.08
Chroococcus sp	Control	6.16	-
	Methanol extract of *Aspergillus niger*	2.08	51.74
	Chloroform extract of *Aspergillus niger*	3.33	37.46
	Water extract of *Aspergillus fumigatus*	3.18	48.37
	Hexane extract of *Aspergillus fumigatus*	3.22	47.72
Phormidium sp	Control	2.98	-
	Chloroform extract of *Fusarium equiseti*	0.7	76.52
	Hexane extract of *Aspergillus fumigatus*	1.25	58.06

Key - :No reduction in algal growth

fungi were identified to *Fusarium equiseti, Aspergillus niger* and *Aspergillus fumigatus* (Koneman and Roberts, 1985). Out of approximately 60 fungal isolates screened from Zijin Mountain, 8 belonging to Ascomycota and 5 to Basidiomycota exhibited algicidal activity (Han *et al*, 2011).

Analysis for antialgal activity :

The antialgal activity of different extracts from the three effective fungal isolates was initially evaluated by the paper disc assay (Table 1). The chloroform and hexane extracts of *Fusarium equiseta* and *Aspergillus fumigatus* respectively exhibited significant inhibition. It should be noted, however, that others exhibited moderately small inhibitory zones on the algal paper disc assay plates (Safferman and Morris, 1962; Shinya *et al*, 2002).

Application studies :

As evident from table 3 and table 4, chloroform extract from CFS of *Fusarium equiseti* gave maximum reduction of *Phormidium* sp, while methanol extract from CFS of *A. niger* was apparently equally effective against *Oscillatoria* and *Chroococcus* in both cement and distemper. The GC-MS analysis of the latter revealed the presence of 3,4-Dihydrothieno-(3,4-b)-5-carbox, Fenmetramide and Ethane, 1,1,2,2-tetrachloro compounds. Polychlorinated alkanes are reported to be toxic to algae (US EPA) however; no relevant data is available regarding the other two compounds.

Hexane extract from CFS of *A. fumigatus* showed a significant reduction in inoculated *Oscillatoria* sp in both the applications. The GC-MS analysis of the same revealed the presence 16-Octadecanoic acid, Hexadecanoic acid, Benzene, 1,4-dichloro compounds, all of which are algicidal. Although, the algicidal mechanism of fatty acids still remains

unclear, nevertheless unsaturated fatty acids have been reported to exhibit algicidal activity (Kakisawa *et al*, 1998; Alamsjah *et al*, 2007). Benzene, 1, 4-dichloro is known to cause acute toxicity due to bioaccumulation. Three different algae *Chaetocerous.neogracilis*, *Phaeodactylum tricornutum* and *Dunaliella tertiolecta* from marine plankton were reported to produce isoprene, halogenated compounds, chlorobenzene and dichlorobenzene to varying extents (Colomb *et al*, 2008). Also chlorobenzene was found to be toxic to *Cyclotella meneghiniana* (Figueroa and Simmons, 1991) and Tetrahymena (Zhang *et al*, 2012), while dibutyl phthalate detected in chloroform extract is reported to be toxic to algae (Kuang *et al*, 2003).

Although the antialgal activity was studied, it becomes important to evaluate the stability of these compounds within the medium which is exposed to different day conditions, seasonal variations as they would find use on exterior walls or swimming pools where algae usually are associated with.

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