

Antibacterial properties of selected freshwater microalgae against pathogenic bacteria

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ABSTRACT

Aqueous, ethanolic and methanolic extracts from four selected freshwater microalgae, *Euglena viridis*, *Chlorella vulgaris*, *Microcystis aeruginosa* and *Spirulina platensis* were examined for antibacterial activity by single disk diffusion method. All four algae revealed antibacterial properties. Best results were shown by the ethanolic extracts of *E. viridis* against *Vibrio alginolyticus*, *Vibrio harveyi*, *Pseudomonas putida* and *Escherichia coli*. Aqueous extracts of *C. vulgaris* also possessed antibacterial properties.

Keywords: Antibacterial properties, *Chlorella vulgaris*, *Euglena viridis*, *Microcystis aeruginosa*, *Spirulina platensis*

Introduction

A variety of biologically active constituents have been isolated from various species of microalgae. The increased use of antibiotics and chemotherapeutics for disease treatment leads to problems of emergence of drug resistant forms and also impact adverse effects on the ecosystem (Aoki, 1992). A decreased efficacy of antibiotics regardless of their mechanism of action, leads to the need for suitable alternatives. Microalgae are extremely diverse group of organisms yielding an almost unlimited range of chemicals. One potential commercial application of microalgae derived compounds that has, as yet, received little attention is in the area of pharmaceuticals. Both cell extracts and extracts of the growth media of various unicellular algae (e.g., *Chlorella vulgaris*, *Chlamydomonas pyrenoidosa*) have been proved to have antibacterial activity *in vitro* against both Gram-positive and Gram-negative bacteria. It has also been reported that a wide range of *in vitro* antifungal activities are obtained from extracts of green algae, diatoms and dinoflagellates. Various strains of Cyanobacteria are known to produce intracellular and extracellular metabolites with diverse biological activities such as antibacterial, antifungal, antiviral and antialgal activity (Noaman *et al.*, 2004; Volk and Furkert, 2006; Valdor and Aboal, 2007; Rania *et al.*, 2008).

Screening of lipophilic and hydrophilic extracts from cultured cyanobacteria or waterbloom material, isolated from German lakes and the Baltic sea for antiviral, antibiotic, immunomodulating and enzyme inhibiting activity in different *in vitro* systems revealed strains with interesting effects (Mundt *et al.*, 2001). Secondary

metabolites from various microalgae are associated with toxic, hormonal, antineoplastic and antibacterial effects (Patterson *et al.* 1994; Goldin, 2003). Secondary metabolites influence other organisms in the vicinity and are thought to be of phylogenetic importance. There has been an increasing interest in cyanobacteria as a potential source for new drugs (Skulberg, 2000). There are numerous reports of compounds derived from *Chlorella* with a broad range of biological activities, such as antibacterial (Pratt *et al.*, 1944; Matusiak *et al.*, 1965), antiviral (Ibusuki and Minamishima, 1990), antitumor (Tanaka *et al.*, 1998; Hasegawa *et al.*, 2002) as well as immunomodulatory (Halperin *et al.*, 2003) effects. The free radical scavenging, anti-oxidative and anti-inflammatory activities of *Chlorella* have also been discovered in *in vitro* studies (Vijayavel *et al.*, 2007). *Microcystis aeruginosa* shows antibacterial activity against the Gram-positive bacterium *Staphylococcus aureus* (Ishida *et al.*, 1997). The objective of the present study was to develop and apply bacterial bioassays for the observation and quantification of possible antibacterial effects from algal extracts, which are active against fish pathogenic bacteria. It should be possible to develop specific microalgal based biologically active compounds for aquaculture in order to avoid development of resistance against antibiotics used in human medicine.

Materials and methods

Collection and culture of microalgae

Samples of freshwater algae (*Euglena* and *Microcystis*) were collected from ponds of Central Institute of Freshwater Aquaculture and from Bindusagar, Bhubaneswar, India in

the month of September and January 2008 respectively. All samples were brought to the laboratory in plastic bags containing pond water and then washed three times with distilled water to separate potential contaminants. These two algae were identified as belonging to families Euglenophyceae and Chlorococcaceae respectively following Records of Botanical Survey of India (Biswas, 1949).

Axenic cultures of *C. vulgaris* and *S. platensis* (procured from Algal Culture Unit, CIFA, Bhubaneswar) were grown in freshly prepared culture medium. For growth, a temperature of $28 \pm 2^\circ\text{C}$ and illumination by cool white fluorescent lamps of intensity 25 Wm^{-2} was provided. The cells of 20 d old cultures were then harvested by centrifugation (5000 g) at 4°C and washed with Millipore water.

Dry weight determination

The cells were separated from the culture filtrate by centrifugation and then washed several times with distilled

water. Biomass were transferred to a pre-weighed dry filter paper using a clean spatula and placed in an oven at 60°C overnight to reach constant weight.

Preparation of the extracts

Harvested samples were dried at room temperature and ground in an electric grinder. Resulting powder was submitted to lipid soluble polar solvents (ethanol, methanol) as well as aqueous medium for extraction, using a soxhlet extractor at $55-60^\circ\text{C}$. All samples were refluxed until saturation (24 h) and the respective extracts were dried in rota-evaporator. Subsequently the residual extracts were suspended in the respective solvents to a final concentration of $10 \mu\text{g} \mu\text{l}^{-1}$.

Test organisms

Antibacterial sensitivity was tested against the pathogenic Gram-negative strains of *Aeromonas hydrophila* (AH1, AH2, AH3 and AH4), *Pseudomonas putida* (PP1,

Table 1. Inhibition zone in mm of ethanolic extracts of freshwater microalgae

Microorganisms	Code	Algae			
		<i>Euglena viridis</i>	<i>Chlorella vulgaris</i>	<i>Microcystis aeruginosa</i>	<i>Spirulina platensis</i>
<i>Pseudomonas putida</i>	PP1	14.0 ± 0.57 ^a	10.6 ± 0.3 ^b	11.3 ± 0.3 ^b	14.3 ± 0.3 ^a
	PP2	15.33 ± 0.33 ^a	12.0 ± 0.5 ^b	11.6 ± 0.3 ^b	13.0 ± 0.57 ^b
	ATCC(49128)	13.33 ± 0.3 ^a	11.6 ± 0.3 ^b	11.3 ± 0.3 ^b	14.3 ± 0.33 ^a
<i>Pseudomonas aeruginosa</i>	PA1	13.0 ± 1.15 ^a	09.6 ± 0.3 ^b	10.3 ± 0.3 ^b	13.3 ± 0.3 ^a
	PA2	13.0 ± 0.57 ^a	11.3 ± 0.3 ^a	12.6 ± 0.6 ^a	12.3 ± 0.3 ^a
	ATCC(27853)	14.6 ± 0.3 ^a	12.0 ± 0.3 ^{bc}	11.3 ± 0.3 ^c	12.6 ± 0.3 ^b
<i>Pseudomonas fluorescens</i>	PF1	10.3 ± 0.3 ^b	12.3 ± 0.3 ^a	10.3 ± 0.3 ^b	13.3 ± 0.3 ^a
	PF2	13.6 ± 0.3 ^a	13.0 ± 0.5 ^a	9.6 ± 0.6 ^b	12.3 ± 0.3 ^a
<i>Aeromonas hydrophila</i>	AH1	11.0 ± 0.5 ^{bc}	13.0 ± 1.1 ^{ab}	13.6 ± 0.3 ^a	10.0 ± 0.5 ^c
	AH2	12.3 ± 0.3 ^c	16.3 ± 0.3 ^a	14.3 ± 0.3 ^b	15.6 ± 0.3 ^a
	AH3	10.0 ± 0.5 ^b	12.0 ± 1.0 ^b	11.3 ± 0.3 ^b	14.3 ± 0.3 ^a
	AH4	12.3 ± 0.3 ^b	11.3 ± 0.3 ^b	12.0 ± 0.5 ^b	15.3 ± 0.3 ^a
	ATCC(49140)	12.3 ± 0.3 ^c	14.6 ± 0.3 ^a	13.6 ± 0.34 ^b	15.3 ± 0.3 ^a
	MTCC (646)	10.6 ± 0.45 ^b	13.6 ± 0.26 ^a	12.0 ± 0.3 ^b	10.65 ± 0.34 ^b
<i>Vibrio alginolyticus</i>	VA	20.0 ± 1.1 ^a	14.3 ± 0.3 ^b	14.3 ± 0.3 ^b	13.3 ± 0.3 ^b
<i>Vibrio parahaemolyticus</i>	VP	16.6 ± 0.3 ^a	12.0 ± 1.0 ^b	15.6 ± 0.3 ^a	13.3 ± 0.3 ^b
<i>Vibrio harveyi</i>	VH	21.0 ± 0.5 ^a	13.0 ± 1.1 ^b	11.6 ± 0.6 ^b	11.6 ± 0.3 ^b
<i>Vibrio fluvialis</i>	VF	10.0 ± 0.5 ^d	14.3 ± 0.3 ^b	16.3 ± 0.3 ^a	11.6 ± 0.3 ^c
<i>Vibrio fisheri</i>	VFS	19.0 ± 1.1 ^a	11.3 ± 0.6 ^c	9.6 ± 0.3 ^c	15.3 ± 0.3 ^b
<i>Vibrio anguillarum</i>	VAN	16.33 ± 0.3 ^{ab}	15.3 ± 0.3 ^b	16.3 ± 0.3 ^a	15.3 ± 0.3 ^b
<i>Escherichia coli</i>	O1	16.6 ± 0.3 ^a	15.3 ± 0.3 ^b	10.3 ± 0.3 ^d	15.6 ± 0.3 ^{ab}
	O115	17.6 ± 0.3 ^a	14.3 ± 0.3 ^c	11.3 ± 0.3 ^d	15.6 ± 0.3 ^b
	O156	13.3 ± 0.3 ^a	13.3 ± 0.3 ^a	12.3 ± 0.3 ^{ab}	11.3 ± 0.3 ^b
	O164	09.3 ± 0.3 ^c	12.0 ± 0.0 ^b	9.6 ± 0.3 ^c	13.3 ± 0.1 ^a
	O111	18.3 ± 0.3 ^a	10.6 ± 0.3 ^b	10.3 ± 0.3 ^b	-
	O109	17.6 ± 0.3 ^a	12.3 ± 0.3 ^b	4.3 ± 0.2 ^c	-
<i>Edwardsiella tarda</i>	ETA	12.3 ± 0.3 ^a	11.3 ± 0.3 ^a	11.3 ± 0.3 ^a	12.3 ± 0.3 ^a

Values represent mean ± S.D.,

Values bearing common superscript are not significantly different ($p < 0.05$)

PP2), *Pseudomonas fluorescens* (PF1, PF2), *Pseudomonas aeruginosa* (PA1, PA2), *Vibrio alginolyticus* (VA), *Vibrio anguillarum* (VAN), *Vibrio fluvialis* (VF), *Vibrio parahaemolyticus* (VP), *Vibrio harveyi* (VH), *Vibrio fisheri* (VFS), *Escherichia coli* (O115, O1, O156, O164, O111 and O109) and *Edwardsiella tarda*.

Inhibitory effect by the disc diffusion method

Single disc diffusion method as described by Chabbert (1963), was used for antibacterial sensitivity test of the above selected algal extracts. The bacterial pathogens used in this work (other than *E. coli*) were isolated from diseased fish and prawns which are being maintained the Fish Health Management Division, Central Institute of Freshwaters Aquaculture (CIFA), Bhubaneswar.

All bacteria were grown in nutrient broth (10^7 cells ml^{-1}), incubated at 37 °C for 24 h and plated using a sterile swab, on to petridishes containing Antibiotic Assay Medium (Hi media, Mumbai). At the same time, sterile discs of

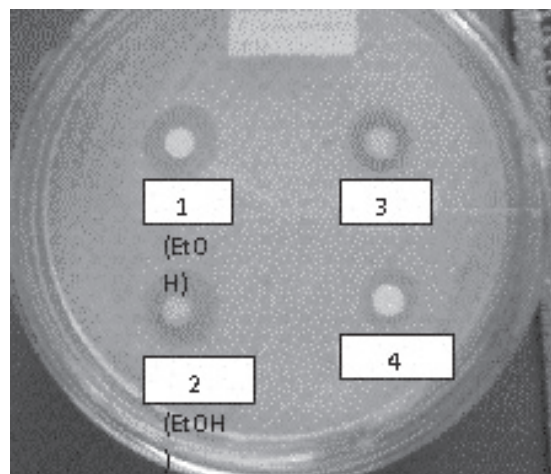


Fig. 1. Antibacterial activity of ethanolic extracts of *Euglena* (1), *Microcystis* (2) *Chlorella* (3) and Control (4) against *P. aeruginosa* (PA2)

Table 2. Inhibition zone in mm of aqueous extracts of freshwater microalgae

Microorganisms	Code	Algae			
		<i>Euglena viridis</i>	<i>Chlorella vulgaris</i>	<i>Microcystis aeruginosa</i>	<i>Spirulina platensis</i>
<i>Pseudomonas putida</i>	PP1	7.6± 0.3 ^b	8.6± 0.3 ^a	-	-
	PP2	-	9.3± 0.3 ^a	-	-
	ATCC(49128)	7.0± 0.5 ^b	9.3± 0.3 ^a	-	-
<i>Pseudomonas aeruginosa</i>	PA1	-	12.6± 0.3 ^a	-	8.6± 0.3 ^b
	PA2	-	13.6± 0.3 ^a	-	-
	ATCC(27853)	-	13.6± 0.3 ^a	-	9.6± 0.3 ^b
<i>Pseudomonas fluorescens</i>	PF1	-	10.3± 0.3 ^a	-	8.3± 0.3 ^b
	PF2	6.01± 0.01 ^b	10.6± 0.3 ^a	-	-
<i>Aeromonas hydrophila</i>	AH1	-	11.6± 0.3 ^a	-	-
	AH2	-	11.0± 0.5 ^a	-	9.6± 0.3 ^b
	AH3	-	15.0± 0.5 ^a	-	10.3± 0.3 ^b
	AH4	7.3± 0.3 ^c	15.3± 0.3 ^a	6.3± 0.3 ^c	8.6± 0.3 ^b
	ATCC(49140)	-	12.3± 0.3 ^a	-	-
	MTCC (646)	-	14.3± 0.3 ^a	-	10.0± 0.5 ^b
<i>Vibrio alginolyticus</i>	VA	0	-	-	-
<i>Vibrio parahaemolyticus</i>	VP	7.3±0.3 ^b	11.3± 0.3 ^a	-	-
<i>Vibrio harveyi</i>	VH	8.0± 0.3 ^b	6.3± 0.3 ^c	7.3± 0.3 ^{bc}	11.3± 0.3 ^a
<i>Vibrio fluvialis</i>	VF	-	-	-	9.6± 0.3 ^a
<i>Vibrio fisheri</i>	VFS	-	-	-	-
<i>Vibrio anguillarum</i>	VAN	7.6± 0.3 ^b	12.0± 0.5 ^a	-	-
<i>Escherichia coli</i>	O1	-	-	10.0± 0.0 ^a	-
	O115	-	-	11.0± 0.5 ^a	-
	O156	7.6± 0.3 ^{bc}	7.3± 0.3 ^c	12.3± 0.3 ^a	8.6± 0.3 ^b
	O164	-	-	10.3± 0.3 ^a	10.0± 0.5 ^a
	O111	-	-	10.3± 0.3 ^a	-
	O109	-	-	-	-
<i>Edwardsiella tarda</i>	ETA	8.6± 0.0 ^b	-	10.3± 0.3 ^a	-

Values represent mean±S.D.

Values bearing common superscripts are not significantly different (p<0.05)

5 mm diameter were embedded with 10 µl of the algal solvent extracts. After solvent evaporation, the discs were put on the above agar plates inoculated with the test bacteria and incubated at 37 °C. Discs with solvent (10 µl) used for dissolution were taken as control after evaporation of the solvent. Activity of the microalgae extracts against bacterial pathogens was determined after 24 h at 37 °C by measuring the diameter of the halo around the discs (Izzo *et al.*, 1995). The results were analyzed using one way analysis of variance (ANOVA) and significant difference among the four microalgae were compared using Duncan's multiple range test (DMRT) (Duncan, 1955). The antibacterial activities of algal extracts were compared with inhibition zones around three commercial antibacterial discs *i.e.*, Clotrimazole, Tetracycline and Furazolidone (Hi Media, India) that were used as references.

Results and discussion

From the screening test conducted it was observed that the ethanolic extract of *E. viridis* showed the highest zone of inhibition against VH (21 mm) followed by VA, VFS and two serotypes of *E. coli i.e.*, O111 and O115 (Table 1). Aqueous extract of *E. viridis* was not effective against the above selected pathogens. In the case of *C. vulgaris*, both ethanolic as well as aqueous extracts were active against these pathogens (Table 2, Fig.1). Ethanolic extract of *C. vulgaris* showed maximum zone of inhibition (15.3-16.3mm) against *A. hydrophila* (AH2), *V. anguillarum* (VAN) and *E. coli* (O1), whereas, aqueous extract of *Chlorella* was also highly active (15.3mm) against *A. hydrophila* (AH3, AH4). It was found that ethanolic extracts of the other two groups of algae (*M. aeruginosa* and *S. platensis*) showed maximum zone size, ranging from 15.6 to 16.3 mm against AH2, VF, VAN and two strains of *E. coli* (O1, O115). Aqueous extract of *Microcystis* showed antibacterial activity against five strains of *E. coli*, whereas the other selected pathogens were resistant to the above extract. It was further noticed (Table 1) that the antibacterial activity of ethanolic extracts of all the four microalgae tested were significantly different ($p < 0.05$) from each other, when tested against VFS and *E. coli* (O115).

Several different organic solvents have been used for screening algae for antibacterial activity. Recently, Das *et al.* (2005) reported the antibacterial activity in the chloroform, acetone, methanol and ethanol extracts of *E. viridis* against different fish pathogens. Singh *et al.* (2001) and Mian *et al.* (2003) reported antibacterial and antialgal activities from terrestrial and freshwater Cyanobacteria. Antiherpes and antiinfluenza activities have also been reported (Serkedjieva, 2000; Serkedjieva *et al.*, 2000). Sastry *et al.* (1994), showed antibacterial activities against Gram-negative pathogenic strains after successive

extraction of marine algae with benzene, chloroform and methanol. Likewise, Naviner *et al.* (1999) have shown antibacterial activity in organic extracts of *Skeletonema costatum* against nine fish pathogens. Recently, Pradhan *et al.* (2004) reported antibacterial activities of *Chlorella vulgaris* against various strains of fish and shellfish pathogens. Antimicrobial effects from Cyanobacterial aqueous and organic extracts were visualized in bioassays using selected microorganisms (*Micrococcus luteus*, *Bacillus subtilis*, *Bacillus cereus* and *E. coli*) as test organisms (Frankmolle *et al.*, 1992; Falch *et al.*, 1995).

The chemical nature of active principles in lipid soluble extracts of algae is not so far totally identified. Udea *et al.* (1991) noticed methyl jasmonate and jasmonic acid in *Chlorella* and *Spirulina*, which were found to be plant inhibitors. Our preliminary results suggest that antibacterial activity observed in ethanolic extract of four microalgae against Gram-negative bacteria could be due to more than one active principle. Purification of the crude ethanolic and aqueous extracts by fractionation or chromatography would be beneficial in the long run for preparing commercial microalgae based products. The use of natural products in disease control is recommended these days, in preference to antibiotics and chemotherapeutants. Most synthetic chemicals are more hazardous due to their long persistence, non-target toxicity and carcinogenic as well as mutagenic activities.

Some of the crude algal extracts, have shown better antibacterial activity than selected commercial antibiotics in the present study (Table 3). These bioactive compounds could become promising agents for disease control in aquaculture.

Table 3. Antibacterial sensitivity of different antibiotics against fish bacterial pathogens (inhibition zone in mm)

Antibiotics	Bacterial strain						
	AH1	AH2	PA1	PP1	PF1	VP	VAN
Clotrimazole (10 mcg)	11	11	10	10	10	10	10
Tetracycline (25 mcg)	20	29	19	15	19	19	19
Furazolidone (50 mcg)	23	23	18	15	15	ND	ND

ND-Not detected; VAN: *Vibrio anguillarum*; VP: *Vibrio parahaemolyticus*; PA: *Pseudomonas aeruginosa*; PP: *Pseudomonas putida*; AH: *Aeromonas hydrophila*

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