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ORIGINAL ARTICLE

Growth characteristics and antimicrobial activity of *Pithophora* sp. isolated from wastewater

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ABSTRACT
Wastewater has been recognized as potential resource for production of microalgal biomass. In the present study a branched, filamentous green algae Pithophora sp. was collected from the municipal wastewater bodies of Titagarh area. The growth characteristics was conducted in varying gradient mixture of modified Bold's Basal Medium and municipal wastewater (pH 8.2) to study the extent of morphometric changes and capability of biomass production of Pithophora sp. The results showed that the increase of cell size (8.98±0.83 cm²) and better biomass production (O.D. 0.618±0.03) was suitable in modified Bold's Basal Medium comparison to the experimental natural wastewater medium. Different solvents such as methanol, butanol, chloroform and petroleum ether were used for solvent extraction and screening of antimicrobial property of the isolated Pithophora sp. Among all the solvents used, highest antimicrobial activity was detected in butanol extracts followed by methanol, chloroform and petroleum ether. Butanol crud extract of Pithophora sp. showed highest zone of inhibition against Escherichia coli (20±2.33 mm), followed by Bacillus subtilis (19±1.69 mm) and Staphylococcus aureus (19±1.37mm). None of the four organic solvents crud extracts of Pithophora sp. showed zone of inhibition against Pseudomonas aeruginosa.
Keywords: Growth characteristics, Antimicrobial activity, Solvent extracts, *Pithophora* sp.

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INTRODUCTION

Microalgae are well known for their multiple use in heavy metal accumulation, production of biodiesel, bioactive compounds etc [1, 29-31]. Wastewater is a potential resource for growth of micro and macro algal biomass. The growth of algae is a function of many factors, including nutrients, pH, salinity, temperature and light [2]. Among these factors, nutrients and pH that directly influences the algal growth are important factors in defining optimal conditions for the culture. In the natural habitats, algae grow predominantly without any knowledge of specific condition required for its growth; hence cultures should be placed in the defined medium at controlled light and incubation temperature to study the proper growth requirement of the isolated organism. In its optimum growth condition an organism can able to produce maximum biomass and desired secondary metabolites. Algal organisms are rich source of structurally novel and biologically active secondary and primary metabolites which may be potential bioactive compounds of interest in the pharmaceutical industry [3,4]. Many authors had found antimicrobial activities of microalgae due to fatty acids [5, 6, 7, 8]. From biotechnological point of view, cell suspension culture still seems the most appropriate system for the production of secondary products on an economically feasible scale [9]. The prolonged use of synthetic drugs develops resistance against pathogens. Hence the uses of green medicines are healthier and safer than synthetic medicines because of their limited side effects [10, 14, 18]. The ability of microalgae to produce antimicrobial compounds could be used as a defence agent against pathogens and also as used as a pharmaceutical bioactive natural compound [11, 17-19]. There is an urgency to screen microalgal species from diverse ecological niches

since microbial diversity are least explored and therefore, probability of finding new and interesting microbes are great, which might produce important bioactive metabolites for applications in the field of medicine, agriculture and industries.

Hence, the objectives of the present work is to isolate microalgal species from the native environment, identify and culture them in suitable growth medium and to screen their antimicrobial properties of cell extracts against clinically significant microorganisms

MATERIAL AND METHODS

Sample collection

Sample was collected from local wastewater bodies beside burial ground, Titagarh municipality, West Bengal, India with the help of fine forceps, scalpels and polythene sample collection bottles. Geographical location, temperature, pH of the wastewater and mode of occurrence of the collected sample were recorded as 22°44'12.8"N and 88°23'03.3"E, 28^{o} C, 8.2, free-floating and mat-forming on the surface of the water body respectively. The sample was brought to the laboratory for microscopic observation and as experimental specimen.

Slide preparation, identification and culture

To obtain unialgal culture, algal mats were thoroughly washed with distilled water to remove dirt's, epiphytes and debris and maintained in Bold's Basal Medium (BBM)

g/L: KH_2PO_4 17.5; $CaCl_2.2H_2O$ 25; $MgSO_4.7H_2O$ 7.5; $NaNO_3$ 25; K_2HPO_4 7.5; NaCl 25; $Na_2EDTA.\ 2H_2O$ 10; KOH 6.2; $FeSO_4.7H_2O$ 4.98; H_3BO_3 2.86; $MnCl_2.4H_2O$ 1.81; $ZnSO_4.7H_2O$ 0.222; $Na_2MoO_4.2H_2O$ 0.390; $CuSO_4.5H_2O$ 0.079; Co $(NO_3)_2.6H_2O$ 0.0494 and H_2SO_4 1ml/L; pH 8.2) [12] as described by Aneja [13]. From The above concentration of stock solutions were prepared separately and from each stock respective quantity of solution were taken following the standard method described [27]. Microscopic observation was carried out for taxonomic characterization of the isolates by comparing the cell size, shape, and color with a microalgae database according to their morphology Guri and Guri, [14].

Growth characterization

Once the selected algal species was identified that shifted to the suitable culture medium to study their growth characteristics. The isolated algal strain was inoculated aseptically in triplicate as follows (A) In 30 ml of wastewater (pH 8.2), (B) In 15ml of wastewater and 15 ml of modified BBM (pH 8.2), and (C) In 30 ml of modified BBM (pH 8.2) and growth measurement were carried out after 1, 3, 7, 15 and 30 days of inoculation respectively at 25±2^{0}C in 16:8 light-dark periods with illumination of 30-35 µ mol photons m^{-2} s^{-1} white continuous light in culture room. Samples of each culture were collected and measured cells size (changes in Length and breadth) under bright field microscopy. For measurement of the growth and biomass production, Optical Density (OD) i.e. of the culture tubes inoculated with the *Pithophora* sp. were taken after 1, 3 7, 15 and 30 days of inoculation respectively at 680 nm.

Solvent extraction

The clean shade dried pure algal biomass collect from natural wastewater bodies was made into fine powdered form then extracted using four different organic solvents (i.e. methanol, chloroform, butanol and petroleum ether) following the standard method described by Cowan [32]. The extracts were centrifuged at 4000 rpm for 10 min, and filtered through Whatman no.1 filter paper. After filtration, the filtered solvents were further concentrated in vacuum rotary evaporator. The sticky layers formed at the end of the evaporation were powdered and then stock solutions of extract were prepared in DMSO at 50 mg/ml for the evaluation of antimicrobial activity [12,16].

Screening for antimicrobial activity

The crude extracts were screened for their antimicrobial activity against some clinically significant microorganism using agar cup diffusion method as described by Patra et al., (2009). The test organisms include two Gram positive bacteria namely *Bacillus subtilis* (Bs) and *Staphylococcus aureus* (sa), two Gram negative bacteria *Escherichia coli* (Ec) and *Pseudomonas aeruginosa* (Pa) and one pathogenic fungi namely *Candida krusei* (Ck). The agar cup wells plate streaked in triplicate with respective pathogen and filled with 100 µl of extract and DMSO was used as negative control. Plates were incubated 24 hours at 37 ± 1 ºC for bacterial strains and 72 hours at 25 ± 2 ºC for fungal strains. The diameter of inhibition zones was measured in triplicates.

Statistical Analysis

The results obtained were subjected to statistical analysis as mean and standard deviation. The mean values and standard deviations were calculated from the data obtained from three different experiments. Statistical difference at $p < 0.05$ was considered to be significant.

RESULTS

Isolation and Identification of microalgae from wastewater

A single microalgal strain was isolated from local wastewater bodies beside burial ground, Titagarh municipality, West Bengal, India and named as Titagarh waste water algae-1 (TWWA-1). During collection, the sample appeared as a free-floating and mat-forming species with fishy odor that ranged in color from yellowish to dark green or greenish brown. Under bright field microscope the specimen appeared as green, filamentous, undifferentiated into base and apex (Fig.1). Individual filaments show apical growth with extensive branching. Branching arises from just below the septa, solitary or sometimes in opposite pairs. Cells are cylindrical, joined end-to-end, longer (806.4±13.33 ųm) than the breadth (70.9±5.37 ųm), multinucleate. Chloroplast is parietal and reticulate with several pyrenoids. Rhizoid-like processes are at two opposite sides of the filament. Large terminal and intercalary akinetes are densely packed with food reserves. Based on distinguishable morphological characters under light microscopic examination, the strain was preliminary ascribed to the genera *Pithophora* species. This genus is green algae that belong to division Chlorophyta.

Growth Characteristics

Changes of cell size in respect to length and breadth of cell were observed under 10 x objectives and 15 x eye-pieces of compound and light microscope and the results are presented in Table-1. Photographs for each observation were taken and respective data were recorded carefully. Maximum growth (8.98±0.83cm^2) and biomass production (O.D. 0.613±0.03) has been observed in modified BBM medium after 30 days of incubation, whereas very least growth (4.31±0.73 cm^2) and biomass production (O.D. 0.148 ± 0.08) were found with pure wastewater culture.

Screening of antimicrobial activity

The results of primary screening of anti-microbial activity of green algae *Pithophora* sp. are summarized in **Table-2.** The assay showed that the four different organic solvent (butanol, methanol, chloroform, petroleum ether) extracts of *Pithophora* sp*.* tested exhibited significant antimicrobial effect against pathogenic strain *Bacillus subtilis, Staphylococcus aureus, Escheriachia coli* and *Candida krusei*. However, none of the solvent extract could exhibit antimicrobial properties against the pathogenic strain *Pseudomonas aeruginosa.* Among the four solvent extracts highest antimicrobial activity has been detected in butanol extract followed by methanol, chloroform and petroleum ether (Fig. 2). The butanol crud extract of *Pithophora* sp. showed highest zone of inhibition against the pathogen tested in comparison to other solvent extracts (Table-2). No zones of inhibition were observed in any of the four control test (Only solvents).

DISCUSSION

In the present study *Pithophora* sp. was isolated from the local wastewater bodies beside burial ground, Titagarh municipality, West Bengal, India. Isolation of *Pithophora* sp. from wastewater was also reported earlier [16]. The cell size increments has been conspicuously observed in BBM (C) in comparison to wastewater medium (A) and mixed combination of wastewater and BBM (B) probably because of suitable pH and balanced nutrient richness. In most of the vegetative cells in maintained pH condition in the BBM culture media, cell size increased by the increment of the age of the culture up to a certain incubation period. Chloroplasts inside the cells also thickened with reticulate morphological form and dark green to green in color. Akinetes were found in very few filaments. In pure wastewater medium, comparative algal growth has been observed which prove that the isolated *Pithophora* sp. could grow in stress condition in the polluted water and might have some phycoremediation properties. In B culture medium, the pH was maintained to 8.2 with 50 % of the medium were added externally in form of BBM, and observed better growth than the pure wastewater culture medium which implies that the strain was at stress or due to deficiency of proper nutrients showed comparatively less growth. The maximum biomass production (O.D. 0.618) observed in the present investigation. In contrast, the present algal growth rate is lower than Hayashida *et al*. [11] who observed higher algal growth rate (OD680 = 0.84).

The production of secondary metabolites by green algae has an interesting scientific and commercial potential. Besides biomass production and growth characteristics, presence of antimicrobial compound also makes these organisms, interesting. Antimicrobial activity depends on both algal species and the solvents used for their extraction [23, 25]. Antimicrobial activity from algal extracts reported earlier was confirmed in the present investigation [1, 2, 18, 6]. The antimicrobial potential of the algal strain of *Pithophora* sp. against tested pathogen. The result showed in Table-2 pointed to better sensibility of diffusion method. In our present investigation we found that crude extracts of filamentous green algal strains showed activity against both Gram positive and Gram negative bacteria as well as against

pathogenic fungi. Such finding suggests that green algae produced diverse secondary metabolites with antimicrobial activity.

Table 1 Growth characteristics of *Pithophora* sp.

Set	**Growth characteristics**	Day-1	Day-3	Day-7	Day-15	Day-30
A	Cell size (cm^2)	4.31 ± 0.73	4.70 ±0.69	5.45 ±0.33	5.99 ±0.39	5.97 ±0.33
	Biomass (O.D.)	0.148 ±0.08	0.157 ±0.03	0.192 ±0.07	0.286 ±0.08	0.283 ±0.03
B	Cell size (cm^2)	5.71 ±0.63	5.79 ±0.39	6.22 ±0.83	7.94 ±0.57	7.76 ±0.93
	Biomass (O.D.)	0.178 ±0.06	0.211 ±0.04	0.289 ±0.02	0.463 ±0.33	0.439 ±0.02
C	Cell size (cm^2)	6.47 ±0.91	6.66 ±0.73	7.02 ±0.19	8.38 ±0.49	8.98 ±0.83
	Biomass (O.D.)	0.297 ±0.08	0.359 ±0,05	0.423 ±0.13	0.588 ±0.01	0.618 ±0.03

(A) 30 ml of wastewater, (B) 15ml of wastewater and 15 ml of modified BBM and (C) 30 ml of modified BBM

TABLE 2: Antimicrobial activities (mm) of the crude extracts of *Pithophora* sp.

Pathogen	Control (DMSO)	Chloroform	Methanol	Butanol	Petroleum Ether
E. coli	ND	11 ± 1.67	18 ±2.57	20 ± 2.33	12 ±1.13
B. subtilis	ND	14 ±1.13	17 ±1.83	19 ±1.69	11 ±0.93
S. aureus	ND	9.0 ±0.89	12 ±2.31	19 ±1.37	9 ±0.87
P. aeruginosa	ND	ND	ND	ND	ND
C. krusei	ND	9 ±0.11	11 ±1.31	14 ±1.13	9 ±0.47

ND: No zone of inhibition detected

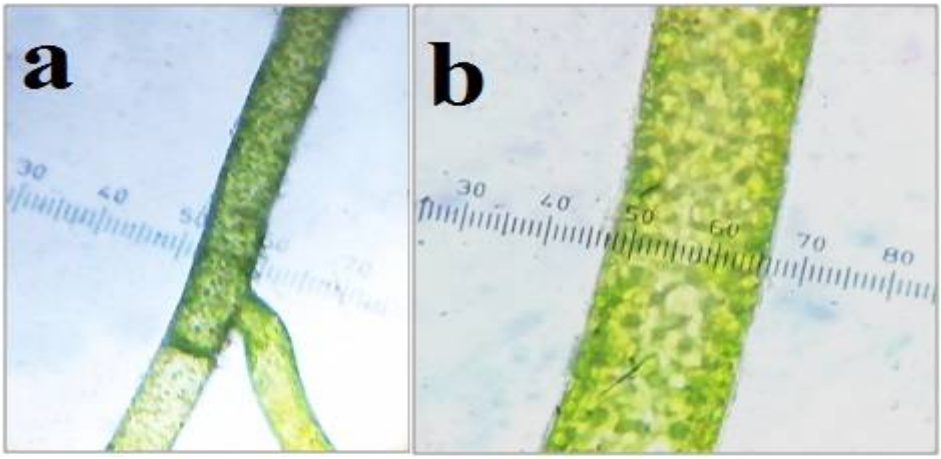

Fig. 1 Microscopic image of *Pithophora* sp. isolated from waste water (a) 10x- Objective, (b) 40x-Objective

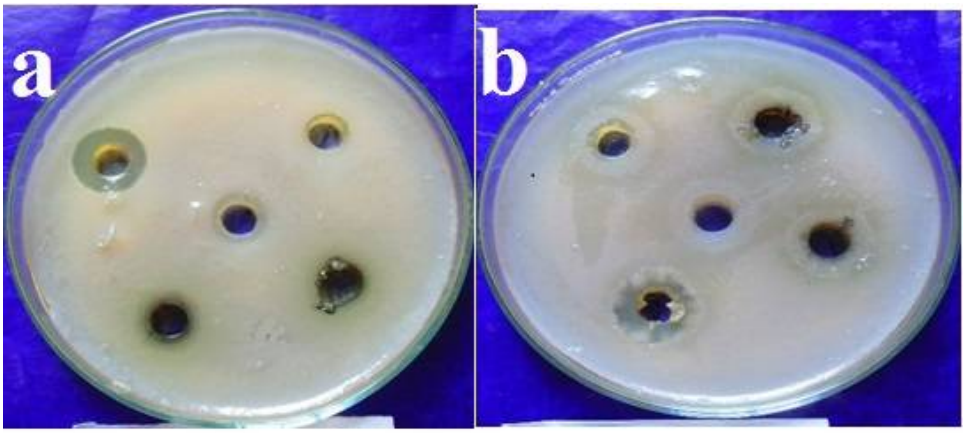

Fig 2: Crud extract of *Pithophora* sp. showing antimicrobial activity against pathogenic (a) Bacteria, (b) Fungi

CONCLUSION

The present experiment signifies that the experimental composition of BBM (modified) is suitable for the production of large biomass of the experimental strain of the green alga *Pithophora* sp. compared to the experimental wastewater medium. Herbal drugs are gaining momentum at a very fast pace all world over for the cure of various human ailments due to ready availability and their cheapness without any side effect. In view of this *Pithophora* sp. found in the mono algal from as mats in natural freshwater habitats, not only help mitigating nuisance and pollution load in the concerned water body, but also profitably used for the development of new pharmaceuticals addressing the novel therapeutic needs of mankind.

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