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Bioremediation of Pharmaceutical Wastewater using *Oscillatoria subsalsa* and *Oscillatoria flos-aquae*: Efficiency in Contaminant Removal, Phytochemical Analysis and Antibacterial Activity against Biofilm-Forming Bacteria

J. SASIKALA and G. SUBRAMANIAN*

Department of Botany, Arignar Anna Government Arts College, Namakkal-637002, India

*Corresponding author: E-mail: gsubramanianbotany@gmail.com

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This study investigates the potential of cyanobacteria, specifically *Oscillatoria subsalsa* and *Oscillatoria flos-aquae*, for the bioremediation of pharmaceutical wastewater and their effectiveness in eliminating biofilm forming, multidrug-resistant bacteria. Cyanobacteria species were collected from the Mandapam coast in India and analyzed for their ability to treat pharmaceutical effluents. Phytochemical compounds in solvent extracts were assessed prior to remediation. The cyanobacteria were evaluated for their effectiveness in reducing pH, biochemical oxygen demand (BOD), chemical oxygen demand (COD), ammonia, nitrite, nitrate and phosphate levels in wastewater. Antibacterial activity was analyzed against biofilm-forming bacterial isolates from pharmaceutical effluents. Both *O. subsalsa* and *O. flos-aquae* demonstrated significant reductions in environmental contaminants. *O. subsalsa* was more effective in reducing COD, while *O. flos-aquae* excelled in reducing nitrate and nitrite levels. Phytochemical analysis revealed the presence of alkaloids, carbohydrates, flavonoids, phenols, saponins, tannins, terpenoids, sterols and quinones, indicating a rich profile of bioactive compounds. The antibacterial tests showed that both cyanobacterial species had significant antibacterial properties, with *O. flos-aquae* displaying stronger and more consistent activity, particularly against Gram-negative bacteria. The study highlights the potential of *O. subsalsa* and *O. flos-aquae* in wastewater treatment and their capability to combat multidrug-resistant pathogens. These findings suggest that cyanobacteria offer a promising, sustainable approach for effective bioremediation and management of pharmaceutical wastewater.

Keywords: Cyanobacteria, *O. subsalsa, O. flos-aquae*, Bioremediation, Pharmaceutical wastewater, Biofilm.

INTRODUCTION

In wastewater, a complex mixture of contaminants coexists, including organic matter, nitrogen and phosphorus nutrients, pathogens and emerging contaminants. This varied collection of emerging contaminants comprises a wide array of unregulated chemicals, originating from either synthetic or natural sources, presenting significant risks to both human health and the ecosystem. Pharmaceutical active compounds, a significant category of developing pollutants, survive in the environment for prolonged durations owing to their stable structure, which hinders breakdown. Their presence in wastewater originates from human or animal consumption, followed by excretion through urine and feces, as some of these compounds undergo only partial metabolism and absorption [1]. Infections from antibiotic resistant bacteria are linked to improper disposal of hospital waste, especially in places developing countries where antibiotics are overused and waste management is poor [2,3]. Liquid medical waste is often discharged into municipal sewage, contaminating aquatic environments [4]. Only 17% of wastewater is processed, allowing untreated sewage to spread drug resistant germs; moreover, treated wastewater has been linked to multidrug resistant pathogens [5]. Although literature has extensively documented antibiotic resistant bacteria and their mechanisms in hospital wastes [6-9], there remains an abundance of data regarding the biofilm formation of bacterial isolates and its correlation with multidrug resistance.

Several methods have been applied to remove pharmaceuticals from wastewater, with biological treatment being the most commonly researched and utilized [10,11]. However, due to the inherent limitations in the design of wastewater treatment plants (WWTPs), they are not equipped to eliminate persistent

pharmaceutical compounds entirely. Consequently, these compounds enter aquatic environments *via* the discharge of treated wastewater and through the use of biosolids. They pose a significant threat to the ecosystem, even in trace concentrations. Another issue in India is the presence of pharmaceuticals in treated water, which makes reuse more challenging as no strict policy has been enforced [12,13].

In recent years, there has been a growing recognition of cyanobacteria's potential as agents for the bioremediation and pollution control, whether in their natural form as mutants or through genetic engineering. Thus, the utilization of cyanobacteria for wastewater treatment might significantly enhance the sustainable recycling of wastewater. Cyanobacteria are very susceptible to sudden physical and chemical alterations of light, salinity, temperature and nutrient composition [14]. The application of cyanobacteria showed immense potential in wastewater and industrial effluent treatment, bioremediation of aquatic and terrestrial habitats, chemical industries, biofertilizers, food, feed, fuel, *etc.* [15].

Cyanobacteria have been effectively utilized worldwide as a cost-effective approach for treating wastewater by converting dissolved nutrients into biomass and for the biotreatment (removal) of dissolved inorganic nutrients from fish farms. This underscores their economic and low-maintenance suitability as a remediation technology for contaminated systems [16]. It is important not only to remove unwanted substances from water but also to eliminate microorganisms present in it. There is an urgent need for research into the use of cyanobacteria for treating pharmaceutical effluents and eliminating microbial contaminants, especially those forming biofilms and exhibiting multidrug resistance. Despite their potential for bioremediation and wastewater treatment, current methods inadequately address the persistent presence of pharmaceutical compounds and the spread of drug-resistant bacteria. Most wastewater treatment plants are ineffective at fully removing these persistent contaminants, leading to environmental risks and challenges in water reuse. Research focusing on optimizing cyanobacteria to tackle these issues could bridge significant gaps, improving the effectiveness of wastewater treatment and enhancing strategies for managing antibiotic-resistant pathogens in contaminated water systems.

EXPERIMENTAL

Collection of cyanobacteria species: Two cyanobacteria species, *O. subsalsa* and *O. flos-aquae* were collected from the Mandapam coast (9.2828º N, 79.1585º E), situated in the Ramanathapuram district of India. Upon collection, the cyanobacterial samples were carefully stored in plastic bags. Subsequently, the samples underwent cleaning procedures to remove any necrotic parts, followed by rinsing with sterile distilled water to eliminate associated debris. The identification of cyanobacterial strains was conducted using a specialized key. The isolated cyanobacterial species were then cultured and maintained in BG 11 media, with a temperature range of 25 ± 10 ºC and light intensity between 2000-3000 lux [17].

Preparation of solvent extracts: Centrifugation was used to remove antimicrobial compounds from algal cultures after the log phase ended. The pellets obtained from the process were subsequently measured and collected. Each algal pellet (1 g) was separately subjected to extraction using chloroform and methanol in a mortar and pestle, followed by overnight incubation at 4 ºC to ensure the complete extraction. The supernatant was collected post-centrifugation at 10,000×g for 10 min. Subsequently, the solvent extracts were concentrated under reduced pressure at 40 ºC. The resulting dry residue was then redissolved in the respective solvents and stored at 4 ºC until needed for bioassay.

Sampling of industrial effluent: Samples were obtained from the effluents of local pharmaceutical industries located in Hosur district, India. The wastewater collected from two industries with safety cans comprised a variety of substances like inorganic acids and bases, organic solvents, metals, unused chemicals and byproducts of chemical reactions. These samples encompassed rinses from various production departments, such as solids, syrups, antibiotics, instant products and powders. Following collection, the samples underwent analysis using standardized procedures outlined by APHA 2012 [18]. Effluent water was refrigerated at 4 ºC for preservation. Subsequently, the primary treated wastes were stored in a deep freezer at -20 ºC until further use.

Remediation bioassay: The two selected species were inoculated individually into 50 mL of BG-11 culture medium and incubated for one week to achieve heavy growth. For each species, 100 mL of BG-11 medium was prepared in four conical flasks, sterilized and then inoculated with 5 mL of one week old cyanobacterial suspensions. Additionally, 5% of 50 mL effluent water was added to each flask. The flasks were incubated under the specified conditions until reaching the mid-late log phase of growth (approximately 10 days). A negative control, without cyanobacterial inoculation, was also included. Two ecological factors *viz*. light intensity (8000 lux) and temperature (25 ± 2 ºC) were tested to assess their role in pharmaceutical wastewater bioremediation [19,20]. After 10 days of incubation under optimal conditions, the contents of all flasks were filtered through filter paper. The treated wastewater was then analyzed for BOD, COD, ammonia and phosphorus using the APHA, 1998 [21].

Phytochemicals analysis: The preliminary phytochemical studies for the algal extracts were performed according to the reported method [22-24]. The presence of phytochemicals like alkaloids, carbohydrates, flavanoids, phenols, saponins, tannins, terpenoids, quinones, glycosides, proteins and steroids were analyzed.

Isolation of pathogenic isolates from effluent water: The pharmaceutical wastewater was mixed with distilled water and prepared with 10^{-1} to 10^{-7} dilution. After dilution, 0.1 mL of diluents was spread from each dilution tube to nutrient agar plates. A sterile glass spreader spread the samples on culture plates aseptically. Finally, all the Petri dishes were incubated at 37 ºC overnight. After overnight incubation, distinct bacterial colonies were picked and again subcultured for pure isolation on selective media (MSA, Mac Conkey agar, enterococcus selective media, cetrimide agar and EMB agar). A series of biochemical tests such as oxidase, catalase, IMViC and sugar fer-

mentation were used to identify the isolated bacteria [25]. All bacteriological media were procured from Hi Media Laboratories Ltd., India.

Isolation of biofilm producing isolates: Freeman *et al*. [26] procedure was employed to conduct the biofilm assay. The isolates were inoculated using the single streak method on sterile brain heart infusion agar media supplemented with 5% sucrose and 0.08 g/L Congo red. Incubation of all plates occurred at 37 ºC for 24 h, with the development of a black colour indicating a positive result.

Antibacterial activity of cyanobacterial extract against effluent waste isolates: The antibacterial activity was assessed using the well diffusion method as outlined by Manivannan & Subramanian [27]. The biofilm producing effluent waste containing isolates were subjected to antibacterial activity. Fresh bacterial cultures from 24 h-old broth were spread evenly onto sterile Muller-Hinton agar plates. Metallic bores were employed to create wells, into which algal extracts were dispensed at various concentrations and appropriately labeled. Following inoculation, the plates were then incubated at 37 ºC for 24 h. Upon completion of the incubation period, the plates were observed for the formation of inhibition zones, which were subsequently measured and recorded.

RESULTS AND DISCUSSION

Using microalgae for wastewater treatment is a sustainable method studied for over 50 years, effectively converting CO_2 into biofuels and reducing greenhouse gases while avoiding pollution. It overcomes the traditional treatment limitations, such as high costs and secondary waste [28,29]. Based on this phenomenon, two microalgae *O. subsalsa* and *O. flos-aquae* were collected from Mandapam area and subjected to remediation of pharmaceutical waste effluent.

The collected algal species were confirmed with microscopic and macroscopic observation. The *O. subsalsa* cultures form dense, greenish mats or filaments on BG-11 medium, which can appear slimy and are often floating or settled layers. *O. flos-aquae* also forms dense mats but with a more uniform distribution, potentially buoyant or forming a pellicle at the surface. *O. subsalsa* ranges in colour from light to dark green, with a mucilaginous and slimy consistency, while *O. flos-aquae* is uniformly green and less slimy. Microscopic observations show *O. subsalsa* as elongated, cylindrical filaments with oscillating movement and a clear, granular sheath, whereas *O. flos-aquae* filaments are straighter, show more width variation and have a less pronounced sheath. Both species were characterized using BG-11 medium, which provides the necessary nutrients for their growth and observation.

After confirmation, both algal species were utilized for the remediation assay pharmaceutical effluent water. This study observed a decrease in pH from 9.5 to approximately 7.0-7.1 with both cyanobacterial species. The pH reduction through cyanobacterial treatment can be attributed to the absorption of CO and production of organic acids [30]. The achievement of near-neutral pH is significant since high pH in the wastewater caused phosphorus precipitation [31]. The neutralization of pH in present study supports an environment that mitigates such issues and improves overall water quality.

The reduction in BOD from 280 mg/L to 147-155 mg/L and COD from 671 mg/L to 351 mg/L with *O. subsalsa* and BOD to 147 mg/L and COD to 475 mg/L with *O. flos-aquae*, indicating an effective removal of organic pollutants. Sarfraz *et al*. [32] reported similar decreases in BOD and COD due to microalgal treatment, emphasizing their role in organic matter reduction. The higher reduction in the COD value observed with Oscillatoria species is consistent with the findings of Vanithasree & Murugesan [33], who observed the better performance of certain microalgae species in degrading complex pollutants.

The reduction in ammonia (from 58 mg/L to 30-32 mg/L), nitrite (from 57 mg/L to 31-36 mg/L) and nitrate (from 147 mg/L to 87-98 mg/L) align with the findings of Shabana *et al*. [30] who also observed the effectiveness of algae in nitrogen removal. Tam & Wong [34] and Dubey *et al*. [35] highlighted the role of microalgae in assimilating ammonium and mitigating the nitrogenous compounds. The slightly better performance of *O. flos-aquae* in reducing the nitrate and nitrite compared to *O. subsalsa*. Present study demonstrated reductions in both inorganic phosphate (from 23 mg/L to 14-16 mg/L) and the organic phosphate (from 25 mg/L to 13-18 mg/L). The effectiveness of *O. subsalsa* in reducing inorganic phosphate and *O. flos-aquae* in reducing organic phosphate supports the potentiality of cyanobacteria in preventing the nutrient pollution and eutrophication [36].

The reductions observed in calcium (from 75 mg/L to 14-35 mg/L), magnesium (from 70 mg/L to 35-38 mg/L), chloride (from 1570 mg/L to 1204-1247 mg/L), TDS (from 2350 mg/L to 1213-1324 mg/L), potassium (from 12.40 mg/L to 7.81-8.21 mg/L) and sodium (from 840.20 mg/L to 650.21-671 mg/L) were consistent with the reported literature value [30] and thus enhancing the water quality. The significant decrease in calcium, chloride and total dissolved solids (TDS) related to *O. subsalsa* indicates its potential in regulating water hardness and mineral composition, consistent with other studies on the effects of cyanobacteria on dissolved solids (Table-1).

Phytochemical studies: In this study, the phytochemical compounds in solvent extracts were analyzed before using the collected algal species to determine their antibacterial activity. The preliminary phytochemical analysis of *O. subsalsa* and *O. flos-aquae* has highlighted distinct differences in their chemical profiles, providing valuable insights into their potential biological activities and applications. Alkaloids and carbohydrates were present in both *O. subsalsa* and *O. flos-aquae* across both solvents. This is consistent with the findings of Prarthana & Maruthi [37], who reported that cyanobacterial alkaloids exhibit significant bioactivity, suggesting that both Oscillatoria species may possess similar therapeutic potentials. Carbohydrates are crucial for cellular functions and have prebiotic properties [38]. This finding aligns with the published work of Deviram *et al*. [39], which emphasizes the importance of cyanobacterial carbohydrates in various biotechnological applications.

Flavonoids found exclusively in the methanol extracts of *O. flos-aquae* and *O. subsalsa* are recognized for their anti-

TABLE-1
PHYSICO-CHEMICAL DATA OF RAW EFFLUENT AND EFFLUENT TREATED WITH CYANOBACTERIA SPECIES

Parameter	Before treatment	After treatment with cyanobacteria species	
		O. subsalsa	*O. flos-aquae*
pH	9.5	7.1	7.0
BOD (mg/L)	280	155	147
COD (mg/L)	671	351	475
Ammonia (mg/L)	58	32	30
Nitrite (mg/L)	57	36	31
Nitrate (mg/L)	147	98	87
Inorganic phosphate (mg/L)	23	14	16
Organic phosphate (mg/L)	25	13	18
Calcium (mg/L)	75	14	35
Chloride (mg/L)	1570	1204	1247
Magnesium (mg/L)	70	35	38
TDS	2350	1213	1324
Potassium (mg/L)	12.40	8.21	7.81
Sodium (mg/L)	840.20	650.21	671

oxidant and anti-inflammatory properties [40,41]. Similarly, phenols were present only in methanol extracts of *O. subsalsa*, highlighting a difference in phenolic content between the two species. This observation corroborates the findings of Ghareeb *et al*. [40], who reported variability in phenolic content among Oscillatoria species in the methanol and water extracts.

The presence of saponins and tannins in microalgae indicates strength of these compounds' specific benefits, such as immune modulation and astringency. Proteins and glycosides are also present in methanol extract of both species. The presence of proteins is noteworthy, as proteins in cyanobacteria are typically significant for nutritional and metabolic functions, suggesting that these species may have different nutritional profiles compared to others with higher protein content. The glycoside also indicates potential differences in pharmacological benefits as reported by Senousy *et al*. [42].

Terpenoids were found in both solvents for *O. subsalsa* but only in methanol for *O. flos-aquae*. This finding is supported by the work of Senousy *et al*. [42], who reported that terpenoids are commonly found in *Oscillatoria* and contribute to their bioactivity. The varied solubility in *O. flos-aquae* may reflect the different extraction efficiencies or concentrations of terpenoids, influencing their potential bioactivity. Sterols are present in both solvents for *O. subsalsa* but only in methanol for *O. flos-aquae*. However, quinones were present only in the methanol extracts of *O. subsalsa* and absent in *O. flos-aquae*. The presence of quinones in certain cyanobacteria exhibit unique bioactive properties [43].

Biofilm producing isolates: The extensive use of antibiotics in humans and animals has amplified the spread of multidrug resistance (MDR) in the environment, leading to persistent infections associated with biofilms due to their resistance to antimicrobial agents. Biofilms, prevalent in pharmaceutical wastewater, serve as indicators of resistant bacteria and genes [44]. In present work, seven bacterial genera of biofilm producing isolates were observed and suppressed using two *Oscillatoria* sp.

Antibacterial activity: The *O. flos-aquae* exhibited its antibacterial activity against Gram-negative and Gram-positive bacterial isolates (Table-2). The high level of activity may be attributed to the specific bioactive compounds present in the cyanobacterial extract such as alkaloids and terpenoids [45]. The increasing inhibition zones with higher extract concentrations further support the previous findings that higher concentrations of natural extracts typically enhance antibacterial activity.

Similarly, the *O. subsalsa* extract exhibited antibacterial activity against all the tested strains. The observed activity against *S. aureus* is particularly significant, aligning with the studies that highlighted the efficacy of cyanobacterial extracts against Gram-positive bacteria. For instance, Amudha *et al*. [46] reported that cyanobacterial extracts often show strong inhibitory effects against *S. aureus*, which could be attributed to specific bioactive compounds present in the extracts. The relatively higher inhibition zones observed for Gram-negative and Gram-positive bacteria in this study suggest that *O. subsalsa* may contain potent antimicrobial compounds effective against this pathogen. Both *O. flos-aquae* and *O. subsalsa* extracts have demonstrated significant antibacterial properties against biofilm producing effluent water isolates. However, *O. flos-aquae* generally exhibited stronger and more consistent antibacterial activity across the tested concentrations except for *K. pneumoniae*, where *O. subsalsa* showed significant efficacy (Table-3).

Conclusion

The study highlights the significant potential of *O. subsalsa* and *O. flos-aquae* for the bioremediation of pharmaceutical wastewater, addressing both chemical contaminants and microbial threats. Both cyanobacterial species effectively reduced

TABLE-2
ANTIBACTERIAL ACTIVITY DATA OF *O. flos-aquae* AGAINST BIOFILM PRODUCING EFFLUENT WATER ISOLATES

Bacteria	Zone of inhibition (mm)				Methanol (control)	Ampicillin (10 µg)
	Concentration of extract (mg)					
	10	20	40	80		
E. faecalis	16	18	22	24	–	16
S. aureus	–	–	–	–	–	15
E. coli	16	18	21	23	–	14
K. pneumoniae	16	18	24	26	–	15
P. aeruginosa	–	13	18	20	–	13
Proteus sp.	12	14	16	20	–	19
A. baumannii	–	9	11	13	–	15

TABLE-3
ANTIBACTERIAL ACTIVITY DATA OF *O. subsalsa* AGAINST BIOFILM PRODUCING EFFLUENT WATER ISOLATES

Bacteria	Zone of inhibition (mm)				Methanol (control)
	Concentration of extract (mg)				
	10	20	40	80	
E. faecalis	–	9	10	12	–
S. aureus	12	14	19	22	–
E. coli	–	9	11	14	–
K. pneumoniae	–	9	11	13	–
P. aeruginosa	–	–	11	14	–
Proteus sp.	–	–	–	–	–
A. baumannii	–	–	12	15	–

major pollutants such as BOD, COD and nutrient levels, demonstrating their capability to improve water quality. Additionally, their significant antibacterial activity against biofilm forming multidrug resistant bacteria underscores their dual role in both pollutant removal and microbial control. This research provides a promising approach for enhancing the wastewater treatment processes, emphasizing the need for integrating cyanobacterial bioremediation into conventional methods to address persistent pharmaceutical contaminants and reduce environmental and public health risks.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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