Antibiofilm Potential of Microbial Biosurfactant against Clinical Isolates

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Abstract

Biosurfactants are eco-friendly surface-active compounds produced by microorganisms and are emerging as effective alternatives to synthetic agents. This study aimed to isolate biosurfactant-producing bacteria from oil-contaminated soils and evaluate their antibiofilm activity against clinical isolates. A total of 15 soil samples yielded 8 isolates identified as *Pseudomonas aeruginosa* based on morphological and biochemical characteristics.

Biosurfactant production was confirmed using phenol–sulphuric acid method, oil displacement assay, and emulsification index (36–60%). Clinical samples (n=15) revealed multiple pathogens, of which 8 isolates showed biofilm-forming ability. The extracted biosurfactant exhibited significant antibiofilm activity, producing clear zones of inhibition.

These findings demonstrate that *Pseudomonas aeruginosa* from oil-contaminated environments is a promising source of biosurfactants with potential applications in controlling biofilm-associated infections.

Key words:- Bio-surfactant, Oil Contaminated Soil and related Micro-flora, *P. aeruginosa*, Clinical isolates, Bio-film Inhibition.

Introduction

The increasing prevalence of antimicrobial resistance and biofilm-associated infections has created an urgent need for novel and eco-friendly therapeutic strategies. In this context, biosurfactants have emerged as promising biological molecules with significant applications in environmental and biomedical fields (Banat *et al.*, 2010; Marchant & Banat, 2012).

Biosurfactants are amphiphilic, surface-active compounds produced by microorganisms such as bacteria, yeasts, and fungi (Desai & Banat, 1997). Due to the presence of hydrophilic and hydrophobic moieties, they accumulate at interfaces and reduce surface and interfacial tension, facilitating emulsification and solubilization of hydrophobic compounds (Mulligan, 2005). Unlike synthetic surfactants, biosurfactants are biodegradable, less toxic, and remain stable under extreme environmental conditions, making them suitable for diverse industrial and medical applications (Banat *et al.*, 2014; Santos *et al.*, 2016).

Various microorganisms, including *Pseudomonas aeruginosa*, *Bacillus spp.*, *Candida spp.*, and *Rhodococcus spp*., are known producers of biosurfactants (Cooper & Paddock, 1984; Banat *et al.*, 2010). These compounds are broadly classified into glycolipids, lipopeptides, phospholipids, polymeric, and particulate biosurfactants (Ron & Rosenberg, 2001). Among these, glycolipids such as rhamnolipids and lipopeptides like surfactin are widely studied due to their strong surface activity and antimicrobial potential (Smyth *et al.*, 2010).

Biofilms are structured communities of microorganisms embedded in a self-produced extracellular polymeric substance (EPS) matrix composed of polysaccharides, proteins, lipids, and

extracellular DNA (Flemming & Wingender, 2010). Biofilm formation occurs through sequential stages including attachment, microcolony formation, maturation, and dispersion, and is regulated by quorum sensing mechanisms (Costerton *et al.,* 1999; Hall-Stoodley *et al.,* 2004). Biofilms provide protection to microorganisms against environmental stress, antibiotics, and host immune responses (Donlan & Costerton, 2002).

Biofilm-associated infections represent a major challenge in clinical settings, as microorganisms within biofilms exhibit significantly higher resistance to antimicrobial agents compared to planktonic cells (Costerton *et al.,* 1999). Pathogens such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, and *Klebsiella pneumoniae* are commonly involved in device-associated and chronic infections (Donlan, 2001; Hall-Stoodley *et al.,* 2004). These infections are difficult to treat due to limited antibiotic penetration, reduced metabolic activity, and the presence of persister cells within the biofilm matrix (Lewis, 2007).

Clinical samples serve as an important source for isolating pathogenic microorganisms and studying their virulence and resistance patterns (Brooks *et al.,* 2013). Clinical isolates are particularly relevant as they often exhibit higher levels of antimicrobial resistance and biofilm-forming ability compared to laboratory strains. Therefore, they provide a more realistic model for evaluating novel antimicrobial and antibiofilm agents (Römling & Balsalobre, 2012).

Among biofilm-forming pathogens, *Pseudomonas aeruginosa* is one of the most significant opportunistic pathogens associated with hospital-acquired infections. It exhibits remarkable metabolic versatility, intrinsic and acquired antibiotic resistance, and a strong ability to form biofilms (Gellatly & Hancock, 2013). These characteristics make it a critical priority pathogen and a suitable model organism for antibiofilm studies.

Biosurfactants have gained attention as natural antibiofilm agents due to their ability to disrupt microbial adhesion, alter cell surface hydrophobicity, and destabilize biofilm matrices (Rodrigues *et al.,* 2006; Rivardo *et al.,* 2009). They can also enhance the susceptibility of microorganisms to antibiotics and inhibit biofilm formation (Dusane *et al.,* 2010). Thus, biosurfactants can be considered natural bioactive agents with significant potential in controlling biofilm-associated infections.

Material and methods

Oil-contaminated soil samples were collected from petroleum hydrocarbon–affected sites, including automobile workshops, petrol pumps, roadside spill areas, and mechanical garages. Samples were collected from a depth of 1–5 cm after removing surface debris and were pooled from multiple points to obtain representative composite samples. Approximately 50 g of soil was collected in sterile containers, labeled, and transported immediately to the laboratory for analysis.

For isolation of bacterial strains, soil samples were inoculated on nutrient agar plates prepared using standard protocols and incubated at 37°C for 24 hours. Distinct colonies were selected based on morphological characteristics and further subcultured on selective Pseudomonas Isolation Agar. Identification of isolates was carried out using cultural, morphological, and biochemical characteristics with reference to Bergey's Manual of Determinative Bacteriology.

Selected isolates were screened for biosurfactant production. Cultures were grown in nutrient broth at 37°C for 4 days and centrifuged at 3000 rpm for 30 minutes to obtain cell-free supernatant. Biosurfactant production was initially confirmed by the phenol–sulphuric acid method, indicated by a color change from yellow to orange. Further evaluation was carried out using oil displacement assay and emulsification index (E24). Oil displacement activity was determined by measuring the diameter of the clear zone formed after adding culture supernatant to an oil layer on water. Emulsification index was calculated by mixing equal volumes of kerosene and supernatant, followed by vortexing and measuring the emulsion layer after 24 hours.

Clinical samples including blood, urine, pus, sputum, and wound swabs were collected aseptically from healthcare centers and processed within one hour. Biofilm production by isolates was screened using Congo Red Agar method and tube method. In the Congo Red Agar method, black colonies indicated biofilm producers, whereas in the tube method, visible film formation along the walls confirmed biofilm production.

The antibiofilm activity of the extracted biosurfactant was assessed using the agar well diffusion method. Standardized bacterial cultures were lawn cultured on nutrient agar plates, and wells were filled with different concentrations of crude biosurfactant. After incubation at 37°C for 24 hours, zones of inhibition were measured, indicating antibiofilm efficacy.

Antibiotic sensitivity testing was performed using the Kirby–Bauer disc diffusion method. Bacterial isolates were lawn cultured on nutrient agar, and antibiotic discs were placed on the surface. After incubation, zones of inhibition were measured and interpreted as sensitive, intermediate, or resistant based on standard guidelines.

Result and Discussion

Biosurfactants are emerging as effective natural alternatives to chemical antibiofilm agents due to their surface-active and antimicrobial properties. In the present study, oil-contaminated soil samples were collected from five automobile-associated locations, yielding a total of 15 samples. These hydrocarbon-rich environments favored the growth of biosurfactant-producing microorganisms. From these samples, 8 isolates were identified as *Pseudomonas aeruginosa*, indicating its prevalence in oil-polluted environments and its ecological role in hydrocarbon degradation.

Colonial and morphological characteristics of *Pseudomonas aerogenosa*.

Sr.No	Isolate	Colony characteristics	Gram character
1	S1	Green pigmented smooth colonies	Gram-negative rod
2	S3	Greenish blue colonies with metallic sheen	Gram-negative rod
3	S5	Flat colonies with green pigment	Gram-negative rod

Morphological and cultural characterization revealed that the isolates exhibited typical features of P. aeruginosa, including green to bluish-green pigmented colonies, smooth surface, and Gram-negative rod-shaped cells. Some isolates also showed metallic sheen and fruity odor, which are distinctive characteristics of this organism.

Biochemical analysis further confirmed the identity of the isolates, as they showed non-fermentative metabolism with negative sugar fermentation and a characteristic IMViC pattern, including citrate positivity.

The frequency distribution of isolates indicated variation among sampling sites, with the highest occurrence observed in Aanand Scooter Work, Bappu Garage, and Kissan Tractor Works (25% each). This variation may be attributed to differences in contamination levels and environmental conditions. These findings are consistent with previous reports suggesting that *Pseudomonas aeruginosa* is commonly isolated from petroleum-contaminated environments due to its metabolic versatility.

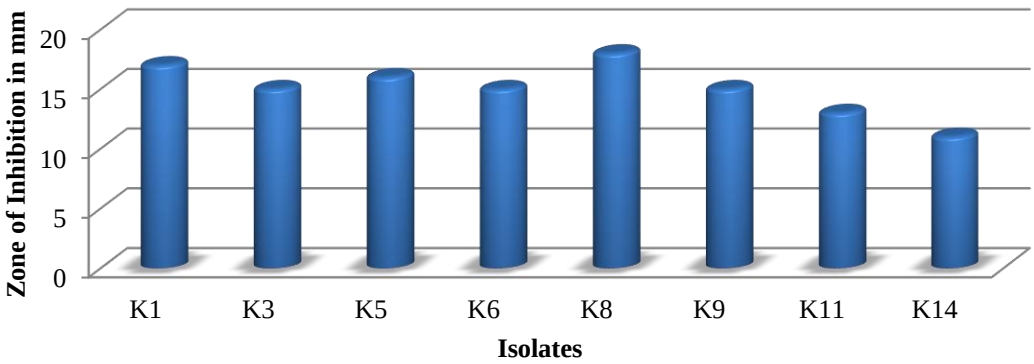
Confirmatory test for Biosurfactant production

Sr. no	Isolate	Oil displacement method	Emulsification index (%)	Phenol sulphuric acid test	Result
1	S1	2.5cm	60	Orange colour	Biosurfactant producer
2	S3	3.0cm	43	Orange colour	Biosurfactant producer
3	S5	3.0cm	40	Orange colour	Biosurfactant producer

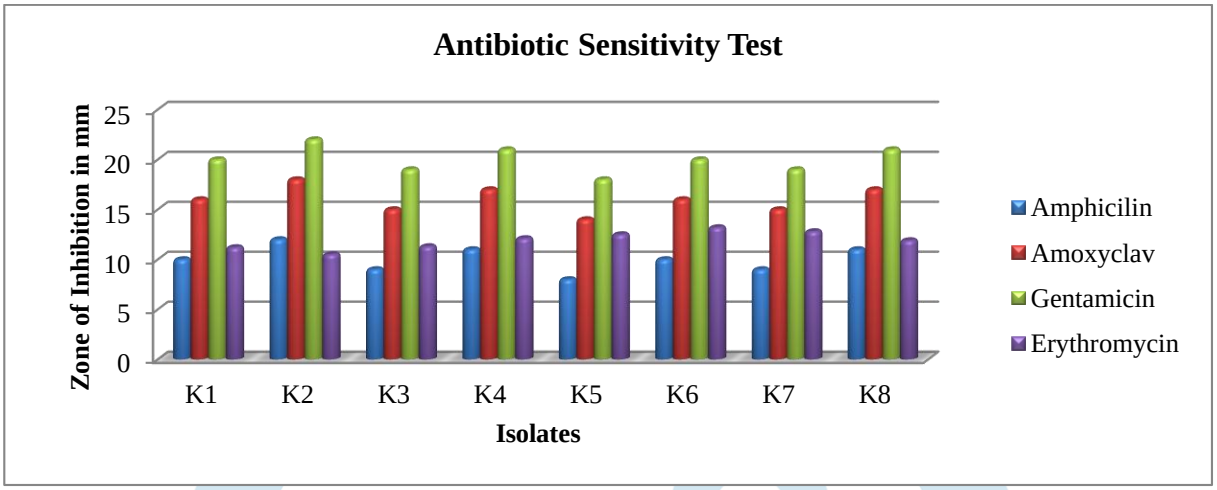
All isolates were screened for biosurfactant production using oil displacement assay, emulsification index (E24), and phenol–sulphuric acid method. The oil displacement zone ranged from 2.5 to 3.0 cm, while the emulsification index varied between 36% and 60%, indicating significant biosurfactant activity. Most isolates tested positive, with only one isolate showing weak activity. These results confirm the potential of *P. aeruginosa* isolates as efficient biosurfactant producers.

A total of 15 clinical samples (urine, pus, wound swab, sputum, and blood) were analyzed, yielding various pathogenic bacteria including *P. aeruginosa*, *E. coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Salmonella typhi*. Among these, biofilm formation was observed in 8 out of 15 isolates using Congo Red Agar and tube methods. Biofilm-producing isolates showed black colonies and visible film formation, indicating strong adherence capability and virulence potential.

Figure No 3. Antibiofilm efficacy of extracted Biosurfactants against clinical isolates



The antibiofilm activity of the extracted biosurfactant demonstrated clear zones of inhibition against biofilm-forming clinical isolates, suggesting its effectiveness in disrupting biofilm structures. The results indicate that biosurfactants can significantly inhibit microbial adhesion and biofilm formation, supporting their potential application in medical and industrial fields.

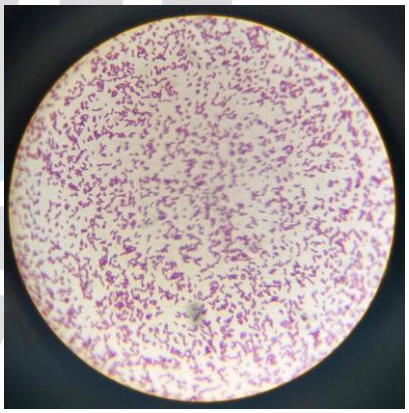


Overall, the study highlights that oil-contaminated environments are a rich source of biosurfactant-producing *Pseudomonas aeruginosa*. The produced biosurfactants exhibited promising antibiofilm activity against clinically relevant pathogens, suggesting their potential as natural therapeutic agents to combat biofilm-associated infections.

Conclusion

- *Pseudomonas aeruginosa* derived biosurfactant shows strong potential as antibiofilm agent against clinical pathogens.
- There natural origin, biodegradability and relatively low toxicity make them promising substance for use in medical, pharmaceutical and antimicrobial surface applications.

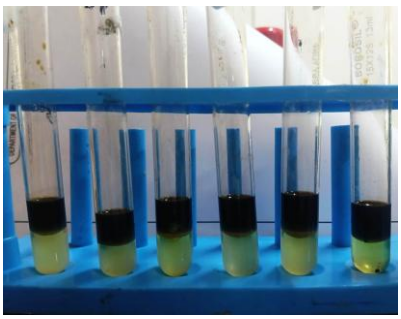
Growth of *P. aeruginosa* on Cetrimide Agar



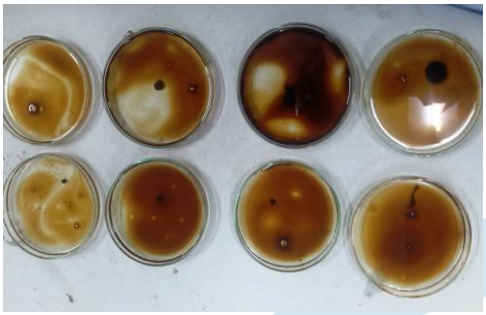
Microscopic view of *P. aeruginosa*

Biosurfactant Extraction and it's Confirmatory Test

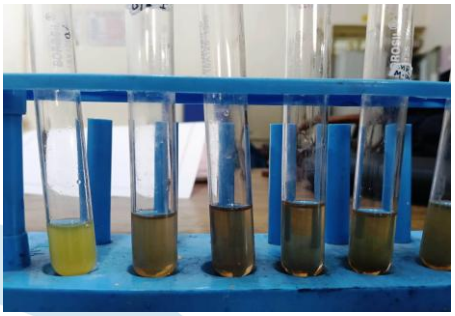
Extracted Biosurfactant from *P. aeruginosa*



Positive Emulsification Method

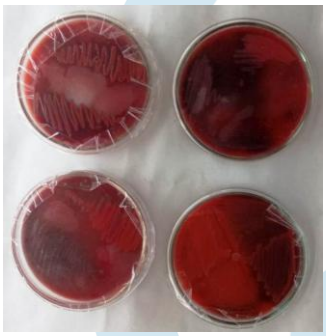


Positive Oil Displacement Method

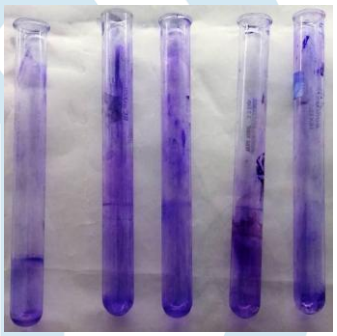


Positive Phenol Sulphuric Method

Confirmatory Test for Biofilm Formation

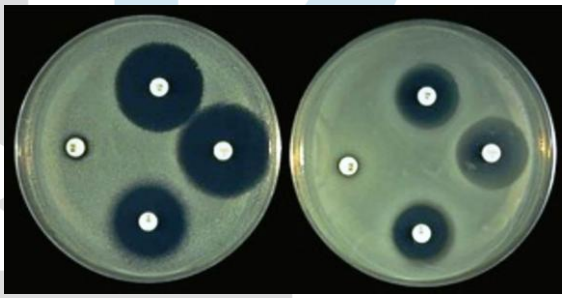


Congo Red Agar Method



Tube Method

Antibiofilm Efficacy of Biosurfactant against Clinical Isolates



Antibiotics Sensitivity / Resistance Pattern agains Clinical Isolates

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