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BIOSURFACTANT PRODUCTION BY *Pseudomonas* sp. ISOLATED FROM HOSPITAL CLINICAL SAMPLES

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ABSTRACT

Biosurfactants are amphiphilic bioactive compounds synthesized extra-cellularly by various microbes from various substrates like sugar, oil and other waste materials. In present study a biosurfactant producing bacterium was isolated from clinical samples and characterized morpho-biochemically. The isolated bacterium was tentatively identified as *Pseudomonas* sp. and evaluated for its oil degrading ability. The growth and biomass production by the bacterium was optimized using crude oil at different concentrations at variable incubation periods. The maximum growth was observed after 8 days of incubation on minimal medium containing 20% crude oil while maximum biomass (0.1 g) was noticed after 10 days incubation on basal medium containing 25% crude oil. Based on Rf value in TLC, the biosurfactant produced was of rhaminolipid type I with Rf value 0.736. The isolated bacterium can be employed in bioremediation processes.

Keywords: Bioremediation, biosurfactant, crude oil degradation, *Pseudomonas*

INTRODUCTION

In refineries, fuel storage areas and other service stations, fossil fuel spills often lead to the deposition of undesired petroleum products which if added in higher amounts consequently cause environmental pollution. Substantial efforts are on to devise inexpensive and practical methods for the cleanup of such polluted sites. Bioremediation is a promising and relatively inexpensive cleanup technique so has become a common alternative for the cleanup of readily degradable pollutant-affected sites through biosurfactant producing microbes (Karlapudi *et al.*, 2018). Petroleum is a multifaceted mixture of several thousands of chemical compounds. These are classified into four major groups *viz*., alkanes, aromatics, resins and asphaltenes. In general, alkane fractions are easily biodegradable, while resins and asphaltenes are prone to degradation only in polar fraction. Aromatic compounds especially the polycyclic aromatic hydrocarbons are biodegradable but are of major concern due to their toxicity and bioaccumulation (Heidarrezaei *et al.*, 2020). Petroleum hydro-carbons through various pathways typically mix up with soil and ground water in huge quantities leading to strong environmental problem in the form of its spillage from underground facilities. A wide range of remediation methods like vapour extraction, incineration, flushing, washing, thermal desorption, solvent extraction etc., have been used to treat polluted areas affected with petroleum spills. These methods are expensive and do not completely eradicate contaminants (Lee *et al.*, 2018).

Several microbes are able to decompose complex organic materials for deriving energy. Adjuvants, bio-emulsifiers or bio-surfactants are the microbial synthesized compounds that modify the environment prevailing on a surface. A range of microorganisms (bacteria, fungi and yeasts) synthesize biosurfactants. Bacterium *Pseudomonas,* known as "Superbug", degrade crude oil through their biosurfactant producing ability (Gakpe *et al.*, 2007; Xu *et al.*, 2020). Most of these biosurfactant are chemically well-known and classified based on molecular mass. Glycolipids and lipopeptides, such as rhamnolipids and surfactin, are among the low molecular mass biosurfactants. Proteins and bioproteins or complex mixtures of these polymers are among the high molecular mass compounds. They all are amphiphilic in nature, while biosurfactants are structurally diverse as they contain both hydrophobic and hydrophilic groups. The hydrophobic amino groups, such as leucine and isoleucine, are typically saturated, unsaturated, or hydroxylated fatty acids, or polar amino acids. Mono-, di-, or poly-saccharides, carboxylic acids, polar amino acids or peptides form the hydrophilic moieties (Borah and Yadav, 2017; Silva *et al.*, 2018). One major parameter that enhances the biodegradation efficacy is their bioavailability and this is the primary research objective in the concept of bioremediation. Hydrocarbon elastic microbes mostly produce many surface-active agents collectively known as biosurfactants or bioemulsifiers. Any novel or unexamined hydrocarbon elastic microbial isolates offer a significant discovery of a novel biosurfactant compound (Patowary *et al.*, 2017). The present study was aimed to isolate *Pseudomonas* sp. from clinical samples and investigate the hydrocarbons bioremediation process. The degradation efficacy of biosurfactant synthesizing *Pseudomonas* species was determined.

MATERIALS AND METHODS

Collection and processing of clinical samples

The clinical samples (swabs) were collected from the District Hospital, Erode, India and processed for the isolation of bacteria on MacConkey and nutrient agar media plates. The bacterium was tentatively identified on the basis of cell morphology and biochemical characteristics and the identity compared with *Bergey's Manual of Determinative Bacteriology* (Bergey and Holt, 2000). The isolates were screened by using enrichment medium to select crude oil degrading microorganisms. The mineral salt medium (MSM) containing crude oil as a sole carbon source (Zhang and Miller, 1992) was used for the enrichment of crude oil degrading bacterium.

Isolation and characterization of crude oil degrading bacterium

To MSM (300 mL) in conical flasks, different concentrations of crude oil (5, 10, 15, 20 and 25%) were added as carbon source and kept in a shaker (150 rpm) at 30^{o}C for 48 h for uniform distribution of oil in the medium (Santa Anna *et al*., 2002). The medium was then inoculated with the isolated bacterium and incubated at room temperature for 3-4 days. After incubation, the broth culture was then streaked onto MSM so as to isolate oil degrading bacterium. The colonies on agar medium were picked up and subjected to Gram-staining, motility test, **i**ndole production, H_2S production, methyl red, Voges-Proskauer, citrate utilization, oxidase, catalase, carbohydrate fermentation and TSI tests for tentative identification of bacteria as per Bergey and Holt (2000).

Biomass measurement

The biomass of bacterium was measured as per the method of Pena-Gomar *et al.* (2012). For this, 2 mL broth culture from the medium containing crude oil of different concentrations was taken in a cuvette and optical density measured at 600 nm by using UV-Vis spectrophotometer (UV-1900i UV-VIS spectrophotometer, Shimadzu, USA). MSM broth culture (10 mL) was taken in pre-weighed centrifuge tube and centrifuged at 8000 rpm for 10 min. The supernatant was discarded and the pellets in centrifuge tube dried in a hot air oven at 100^{o}C for 2 h. The pellets were weighed by subtracting the weight of centrifuge tube.

Total viable count

For viable count determination, 0.1 mL broth culture was placed on nutrient agar plates, spread by using sterile L-rod and incubated at 37°C for 48 h to count the total colony forming units (CFU).

Production and extraction of biosurfactant

Biosurfactant production medium (300 mL) was prepared in a conical flask and sterilized. Bacterial broth culture (I mL) was inoculated into the medium and incubated at 37°C for 4 days. Then bio-surfactant was extracted from production medium by precipitation and dissolution method (Bordoloi and Konwar, 2008). Broth culture (20 mL) was centrifuged at 12000 rpm for 10 min. The supernatant was transferred to another tube and biosurfactant extracted from it by a series of consecutive steps of acid precipitation and dissolution in 50 mM of sodium bicarbonate (Zhang and Miller, 1992).

Thin layer chromatography analysis of biosurfactant

Thin layer chromatography (TLC) was performed to assess the characteristics of the biosurfactant produced. The homogeneity of dissolved biosurfactant in solution was checked and biosurfactant analyzed by using TLC (Dlamini *et al*., 2020). The samples were spotted on TLC plates and the chromatogram developed. The solvents used were either chloroform: methanol: acetic acid (85:15:2) or chloroform: methanol (5:1) for type I biosurfactant, and chloroform: methanol (5:1) or chloroform: methanol (2:1) for type II biosurfactant.

Statistical analysis

All the experiments were conducted in a completely randomized design and each treatment replicated three times. The data was analysed using one-way analysis of variance for significant differences, if any, among the treatments. The optical density and biomass were expressed as the average ± standard deviation. The statistical analysis was performed using Microsoft Office Excel 2016 software.

RESULTS AND DISCUSSION

Isolation and identification of crude oil degrading bacterium

The success of biosurfactant production relies on the type of microbe involved, the development process followed and the raw material used. The bacterium degrading crude oil was isolated by streak plate method using enrichment agar medium. The colonies on agar medium were analyzed on the basis of colony and cell morphology and biochemical tests. The morpho-biochemical characterization revealed that the bacterium was Gram negative motile rod, negative for indole production, methyl red and Voges-Proskauer tests, and positive for citrate utilization, H_2S production, nitrate reduction, catalase, oxidase and TSI tests. It easily fermented glucose, maltose, mannitol, lactose and sucrose. The characteristics of bacterium were compared with the *Bergey's Manual of Determinative Bacteriology* (Bergy and Holt, 2000). The isolate was tentatively identified as *Pseudomonas* sp.

Growth and biomass

Broth culture (2 mL) from MSM medium containing variable crude oil concentration were taken in cuvettes and optical density of cultures noted spectrophotometerically. The maximum OD of 1.56 was noticed in the medium containing 20% crude oil after 8 days incubation (Table 1). The biomass of bacterium in different crude oil containing MSM is given in Table 2. The maximum biomass (0.1 g) was noted in the medium containing 25% crude oil after 10 days incubation. Broth culture (0.1 mL) was placed on nutrient agar plates, spread by sterile L-rod and incubated at 37°C for 48 h to assess the total viable count. In all the concentrations tested, the broth cultures yielded the colonies on agar medium too numerous to count.

In production medium, biosurfactant of *Pseudomonas* was noticed after 3rd day of incubation (Table 2). Initially, the degradation of crude oil was slow *viz*., up to 6th day of incubation, then increased from 7th day onwards and decreased after 10 days of incubation in terms of OD and biomass.

Characterization of biosurfactant

The quality and quantity of biosurfactant are affected by C and N substrates, concentration of elements, pH, temperature, agitation and dilution rate in culture. In present study, optimum production conditions were achieved when pH of 7.0 ± 1, temperature of 37°C, agitation at 100 rpm and incubation time of 10 days were maintained through-out the study period. The biosurfactant extracted from *Pseudomonas* broth culture was characterized by OD and TLC. The OD of biosurfactant was found to be 0.111. A single spot was observed on TLC plate had Rf value of 0.736.

Table 1: Optical density of *Pseudomonas* sp. grown on variable concentrations of crude oil at different incubation time

Incubation time (days)	Concentration of crude oil (%)				
	5	10	15	20	25
2	0.78 ± 0.50	0.52 ± 0.70	0.69 ± 0.15	0.38 ± 0.10	0.44 ± 0.33
3	0.39 ± 0.10	0.27 ± 0.60	0.28 ± 0.21	0.20 ± 0.05	0.23 ± 0.14
4	0.50 ± 0.00	0.43 ± 0.42	0.46 ± 0.25	0.29 ± 0.17	0.38 ± 0.08
7	1.50 ± 0.23	1.48 ± 0.35	1.49 ± 0.30	1. 46 ± 0.23	1.47 ± 0.60
8	1.55 ± 0.14	1.51 ± 0.41	1.55 ± 0.18	1.56 ± 0.70	1.49 ± 0.44
9	1.55 ± 0.56	1.46 ± 0.60	1.52 ± 0.24	1.05 ± 0.61	1.29 ± 0.38
10	1.48 ± 0.11	1.40 ± 0.80	1.45 ± 0.36	1.02 ± 0.53	1.20 ± 0.22
15	1.33 ± 0.34	1.27 ± 0.19	1.35 ± 0.52	1.00 ± 0.90	1.08 ± 0.09

The values given are mean of three observations ± standard error

Table 2: Biomass of *Pseudomonas* sp. (g 10 mL^{-1}) grown on variable concentrations of crude oil at different incubation time

Incubation time (days)	Concentration of crude oil (%)				
	5	10	15	20	25
2	0.02 ± 0.05	0.03 ± 0.05	0.05 ± 0.02	0.06 ± 0.04	0.07 ± 0.05
3	0.04 ± 0.03	0.03 ± 0.01	0.02 ± 0.06	0.02 ± 0.01	0.01 ± 0.02
4	0.01 ± 0.02	0.03 ± 0.04	0.04 ± 0.04	0.06 ± 0.06	0.07 ± 0.06
7	0.07 ± 0.06	0.07 ± 0.09	0.08 ± 0.07	0.09 ± 0.07	0.09 ± 0.08
8	0.07 ± 0.05	0.08 ± 0.07	0.08 ± 0.06	0.09 ± 0.08	0.09 ± 0.06
9	0.05 ± 0.01	0.06 ± 0.04	0.07 ± 0.04	0.07 ± 0.05	0.08 ± 0.05
10	0.04 ± 0.02	0.06 ± 0.02	0.06 ± 0.02	0.06 ± 0.04	0.10 ± 0.07
15	0.03 ± 0.01	0.05 ± 0.03	0.05 ± 0.03	0.06 ± 0.06	0.09 ± 0.07

The values given are mean of three observations ± standard error

The biosurfactant produced by *Pseudomonas* sp. was rhaminolipid type I biosurfactant. The isolated strain showed oil degrading ability and gave good biosurfactant yield as compared to previous reports (Rashedi *et al.*, 2005). George and Jayachandran (2013) have reported that biosurfactant produced by *Pseudomonas* sp. is rhaminolipid type of biosurfactant. *P. aeruginosa* has widely been studied for biosurfactants production (Silva *et al*., 2010). Micelle-forming amphiphiles are considered surface active materials or surfactants that can theoretically be used in surface chemical work. The properties of soap and detergents are identical to surfactants (Karlapudi *et al*., 2018). Biosurfactants improve the consumption of insoluble substrates. There are three strategies for hydrocarbon intake *viz*., direct interface absorption through hydrophobic cell membranes, interfacial uptake by means of biosurfactants emulsification and solubilization of hydrocarbons by biosurfactants. Two of these methods are directly influenced by biosurfactants [emulsification and solubilization] (Patowary *et al.*, 2017). Currently significant work is conducted to develop low-cost, practical clean-up solutions for contaminated areas. In specific, bioremediation using indigenous microbial communities show promise as a reasonably low-cost method.

Conclusion: The isolated bacterium *Pseudomonas* sp. grew in medium containing crude oil as a sole source of carbon. Initially, crude oil degradation by the isolated species was slow up to 6 days of incubation, then increased upto 10 days of incubation in terms of OD and biomass. The biosurfactant of *Pseudomonas* sp. was found in the production medium after 3 days of incubation. The biosurfactant of type-I (rhaminolipid) as per TLC analysis and the isolate can be used for bioremediation processes.

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Conflict of interest: Authors declare that they have no conflict of interest.

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