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# *Pseudomonas aeruginosa* KVD1 an Efficient Biosurfactant Producing Bacteria Isolated from Krishna Delta Mangrove Sediments

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## ABSTRACT

Biosurfactant-producing bacteria, isolate KVD1, was isolated from Krishna river delta mangrove sediments. The taxonomic identification of KVD1 strain was done by sequencing of 16S rRNA gene showed that it formed a coherent cluster with the clad that comprised of *Pseudomonas aeruginosa* to be its closest phylogenetic neighbour. Strikingly, for the first time *Pseudomonas aeruginosa* strain KVD1 isolated from mangroves was shown to produce highest biosurfactant (4.08 g $L^{-1}$) in optimised MSM containing 1.5% diesel as sole carbon source, 1% $(NH_4)_2SO_4$ as nitrogen source and 1 g $L^{-1}$ NaCl, at pH (7±2), 30°C, 150 rpm. The biosurfactant was obtained by cold acetone extraction method showed emulsification activity of $E_{24\%}$ = 61±2% with n-hexadecane. The stability of the biosurfactant at different salinity, pH and temperature was also investigated. This biosurfactant also exhibited significant efficiency in oil recovery sand experiment. The present study emphasizes that biosurfactant produced by strain KVD1 is critical to open new biotechnological applications in enhanced oil removal from spillages and also as cleaning and emulsifying agents in the industry.

**Key words:** Biosurfactant, mangrove sediments, *Pseudomonas aeruginosa*, emulsifying activity, enhanced oil removal

## INTRODUCTION

Mangrove ecosystems are noteworthy intertidal estuarine wetlands along the coastlines have been considered as significant carbon sinks for pollution from fresh water and tidal water discharges. Mangrove's exceptional features of high primary productivity, abundant detritus, rich organic carbon and reduced conditions make them a preferential site for uptake and preservation of hydrocarbons from anthropogenic inputs (Bernard *et al*., 1996). Continuous deposition of these hydrocarbons makes these preserved marine environments highly vulnerable to an ecological disaster. Therefore, efficient strategies to monitor oil spills in such environments must be developed, especially in pristine mangrove wetland ecosystems (Santos *et al*., 2010). Physical and chemical cleaning processes for decontamination of oil polluted areas have been scanty in their application to synthetic counterparts. One of the most effective methods to treat oil-related contamination is the use of biosurfactants that disperse the oil and accelerate its mineralization (Lima *et al*., 2011; Makkar *et al*., 2011). The principle processes for their effective removal are presently believed to be microbial transformation and biodegradation (Gibson *et al*., 1975). Biosurfactants, produced by microorganisms are amphipathic surface active molecules containing hydrophilic and hydrophobic

moieties that act by emulsifying hydrocarbons, increasing their solubilisation and subsequently rendering them available for biodegradation (Nerurkar *et al.*, 2009; Lima *et al.*, 2011). Biosurfactants can be glycolipids, lipopeptides, lipolysaccharides, polysaccharide-protein complexes, fatty acids and lipids (Makkar *et al.*, 2011). These microbial derived compounds are desirable alternatives to synthetic counterparts because of their selectivity, biodegradability, low toxicity and stability at extreme temperatures, pH levels and salt concentrations (Nerurkar *et al.*, 2009). The objective of the present study was to isolate and characterize biosurfactant produced by bacteria isolated from mangrove sediments and their evaluation for potential applications in microbial enhanced oil recovery.

## MATERIALS AND METHODS

**Study area and sampling:** Mangrove sediment samples were collected from Krishna Delta Mangrove sediments. Sediment samples (0-10 cm) were collected with a soil corer and transferred to precleaned polycarbonate glass bottles stored at -20°C and kept frozen prior to further analysis. Three replicates were collected at each station.

**Isolation and screening of biosurfactant-producing bacteria:** Bacteria were isolated by adding 1 g of sediment sample to the Mineral Salts Medium (MSM) (Saikia *et al.*, 2012). Crude oil (2% v/v) was provided as the sole carbon source to the medium and the enriched flask was incubated at 35°C for 4 days in a rotary shaker (Orbitek) with 150 rpm. From the enrichment culture, morphologically different bacterial colonies were isolated by plating on Zobell Marine Agar (ZMB) and pure cultures of the isolates were obtained by multiple subcultures. Biosurfactant activity of the bacterial isolates was measured by growing them in MSM containing diesel as the sole carbon source. The flasks were kept in a shaking incubator at 35±2°C and 150 rpm for 6 days. Emulsification activity ($E_{24}$%) was determined following the standard protocol of Wu *et al.* (2008). n-hexadecane was used as a positive control in the experiment.

**Identification of the bacterial isolate:** The most efficient bacterial strain was identified by (a) studying the morphological and physiological characteristics (Cappuccino and Sherman, 1999) and by (b) sequencing 16S rDNA. The genomic DNA was extracted from a pure culture and using Taq DNA polymerase, PCR amplification of 16S rRNA gene was carried out with universal bacterial primers as followed by Nilsson and Strom (2002). The amplified sequence was aligned in NCBI GenBank and RDP databases and analyzed to find the closest homolog of the isolated bacterial strain and submitted in GenBank.

**Production of biosurfactant:** Biosurfactant production was carried out in optimized MSM with 1.5% diesel as carbon source, 1% $(NH_4)_2SO_4$ as nitrogen source and 1 g $L^{-1}$ NaCl, at pH (7±2), 30°C, 150 rpm. Production of biosurfactant was measured in terms of dry cell weight, biosurfactant production and emulsification index (%). The dry cell weight was determined as followed by Raza *et al.* (2007). Biosurfactant production was estimated by the method described by Das and Mukherjee (2007).

**Stability characterization:** Thermal stability of the biosurfactant was determined by heating the cell free culture supernatant at 30,40,50,60,70,80,90,100,110 and 121°C for 15 min. The effect

of pH and salinity on stability of the biosurfactant was evaluated by altering the pH (2.0-10.0) and concentration of NaCl (2-10%) of the cell free culture supernatant and measuring the $E_{24\%}$ and surface tension.

**Application of the biosurfactant in oil removal from contaminated sand:** Enhanced oil recovery by the biosurfactant was investigated using contaminated sand added with fractions of aqueous solutions of the SDS, Triton X-100 and KVD1 biosurfactant solutions (Saimmai *et al.*, 2013). The amount of oil residing in the sand after the impact of biosurfactant and synthetic surfactants was gravimetrically determined as the amount of material extracted from the sand by hexane (Sobrinho *et al.*, 2008).

**Statistical analysis:** All measurements were expressed as Mean±Standard Deviation (SD) and Standard Error (SE) with each experiment conducted in triplicate. SPSS 11.0 software version was used in the statistical analyses. The correlation between stability of biosurfactant and oil recovery assay was examined using Pearson's correlation. The criterion for significance was set at $p<0.05$.

## RESULTS AND DISCUSSION

A total of 10 selected bacterial isolates were obtained from the mangrove sediment samples of Krishna estuary, out of which, only one isolate showed significant emulsification activity. Based on standard morphological characteristics and biochemical tests strain KVD1 was preliminarily identified as *Pseudomonas aeruginosa* and confirmed by 16S rRNA sequence analysis. In the screening experiment the cell free supernatant of strain KVD1, showed the highest emulsification activity ($E_{24\%}$) of 66.5% compared to other isolates. Subsequently, based on the maximum $E_{24\%}$, the bacterial isolate KVD1 was selected for further experiments. Highest biosurfactant production of up to 4.080 g $L^{-1}$, dry cell weight of 6.94 g $L^{-1}$ and emulsification activity of 68.2% were attained using optimized MSM (Table 1). The biosurfactant produced by *P. aeruginosa* KVD1 was shown to be thermostable. Autoclaving it at 121°C could not reduce its emulsification properties. The surface tension measured was observed as 30.02 mN $m^{-1}$ before autoclavation. However, the same activity was retained and recorded as 30.6 mN $m^{-1}$ after autoclavation. The $E_{24}$ index was recorded as 66 and 62% before and 62% after autoclavation respectively. The effect of addition of NaCl and pH on surface tension and E24 index of biosurfactant is shown in Fig. 1. However, only considerable changes were observed for both parameters on addition of up to 10% (w/v) NaCl. The biosurfactant was also stable at different pH ranging from 2.0 to 10 and increase of pH had a positive effect on $E_{24}$ index (Fig. 1). The ability of biosurfactant from *P. aeruginosa* KVD1 to enhance oil removal from contaminated sand was examined in comparison with synthetic surfactants (Fig. 1). The biosurfactant from *P. aeruginosa* KVD1 and Triton X-100 showed enhanced recovery of 25-30% of crude oil at 25°C. The synthetic surfactant SDS was found tobe least efficient.

The mangroves receive large inputs of crude oil from anthropogenic sources and potentially sequester a huge fraction in its sediments. Despite the fact that the mangrove ecosystems of Krishna estuary is away from the major ports, still the oil spill risk is moderate due to ship breakers, offshore oil wells, spillage from oil tankers, accidents and cleaning of cargo vessels. The enrichment culture technique using crude oil showed the lowest percentages of positive isolates. These results may be ascribed to the fact that high concentrations of crude oil did not select for bacteria able to

Table 1: Biosurfactant production in optimized MSM

| Optimized MSM parameters | Biosurfactant concentration (g $L^{-1}$) | Dry cell biomass (g $L^{-1}$) | Emulsification activity (%) |
|---|---|---|---|
| Diesel (1.5%) | 4.08 | 6.94 | 68.2 |
| $(NH_4)_2SO_4$ (1.0%) | | | |
| NaCl (1 g $L^{-1}$) | | | |
| pH (7±2) | | | |
| Temperature (30°C) | | | |

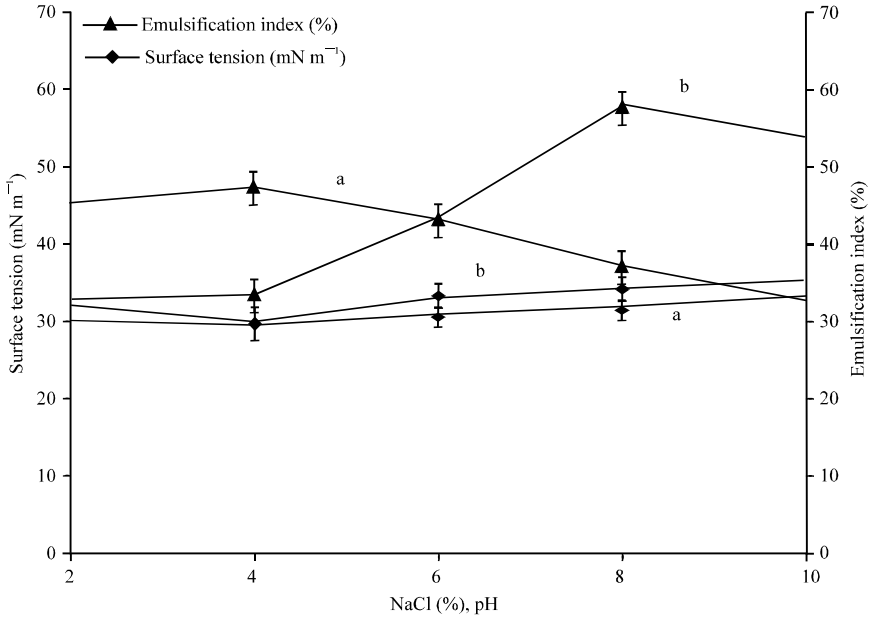


Fig. 1: Stability study of KVD1 biosurface

produce biosurfactants. This is consistent with the previously reported dominance of used lubricant oil resistant bacteria in mangrove sediments (Saimmai *et al*., 2013). However, to our knowledge, there has been no attempt on the study of biosurfactant producing bacteria from mangrove wetland ecosystems of coastal Andhra Pradesh. Strains of *Pseudomonas* sp., were also isolated from mangrove wetland ecosystem, contaminated with various petroleum hydrocarbons and oil spillages (Saimmai *et al*., 2013; De Sousa and Bhosle, 2012). Taccari *et al*. (2012) has recently shown that surfactant mediated increases in hydrocarbon bioavailability can lead to increased biomass and biodiversity in oil contaminated sites. This suggests that certain taxa of surfactant-producing species may play unique roles in determining the composition and population sizes of petroleum-degrading species in oil-degrading communities. These results have implications for the potential use of a biosurfactant produced by *P. aeruginosa* KVD1 to enhance sorbed motor oil from environment. The biosurfactant production of strain KVD1 was dependent on the media composition, affecting its production yield. Optimized MSM media was used by involving variations in carbon, nitrogen sources, pH, temperature and NaCl% with the aim of increasing biosurfactant productivity and emulsification activity. Similarly Rajkumar *et al*. (2007) also demonstrated the optimized parameters for the production of arabinolipid biosurfactant from *Serratia marcescens* DT-1P. The preferred carbon source diesel produced the highest biosurfactant yield. The ability of

KVD1 strain to utilize diesel as carbon source is a significant advantage as it provides an alternative use for glucose, provided that its biosurfactant production can be enhanced to economically viable values (Panesar *et al*., 2011). Biosurfactant production reported in this study (4.08 g $L^{-1}$) was comparatively higher than previous reports on use of diesel as carbon source for biosurfactant production. Preferences for complex compounds over simpler counterparts have been previously reported in *P. putida* strains (Basu *et al*., 2006). The growth of microorganisms on hydrocarbons is frequently associated with the production of biosurfactants that can assist in emulsification of these hydrophobic substrates in the growth medium (Calvo *et al*., 2002). This biochemical property is highly imperative in the containment of environmental pollution, due to their use in degradation of oil derived environmental polluters (Ozturk *et al*., 2012). Moreover, a variety of different hydrophobic substrates were efficiently emulsified by *P. aeruginosa* strain KVD1 supernatant. It was reported in a study that n-hexadecane was attracted to the cell surface of the 21 BN strain of *P. putida* at a rate of 72% (Tuleva *et al*., 2002). In a different study, by Wouter and Dick (2002), it was found that n-hexadecane was attracted to the cell surfaces of the strains of biosurfactant producer *P. aeruginosa* 18 UG2, *Acinetobacter calcoaceticus* RAG1, *Rhodococcus erythropolis* DSM 43066 and *R. erythropolis* ATCC 19558 at rates of 42, 81, 30 and 12%, respectively. The results indicated that the strain KVD1 biosurfactant was capable of effectively emulsifying both aromatic and aliphatic hydrocarbons, suggesting that it could be used for hydrocarbon remediation and oil recovery (Ilori *et al*., 2005; Sifour *et al*., 2007; Thampayak *et al*., 2008; Techaoei *et al*., 2011). The KVD1 biosurfactant was found to be thermostable at higher temperatures, pH and NaCl concentrations. These results are in agreement with previous reports (Techaoei *et al*., 2011).

PAH derivatives bind to soil and sediment componentsand are deemed to be challenging in terms of removal and degradation (Sobrinho *et al*., 2008). Biosurfactants can emulsify hydrocarbons augmentingtheir water solubility, decreasing surface tension and increase the displacement of oil substances from soil particles (Banat *et al*., 2010). The ability of biosurfactant from KVD1 to enhance motor oil removal from contaminated sand was studied in comparison with those of synthetic surfactants, this is a non-ionic surfactant TritonX-100 and anionic surfactants SDS (Fig. 2). Biosurfactant from KVD1 and Triton X-100

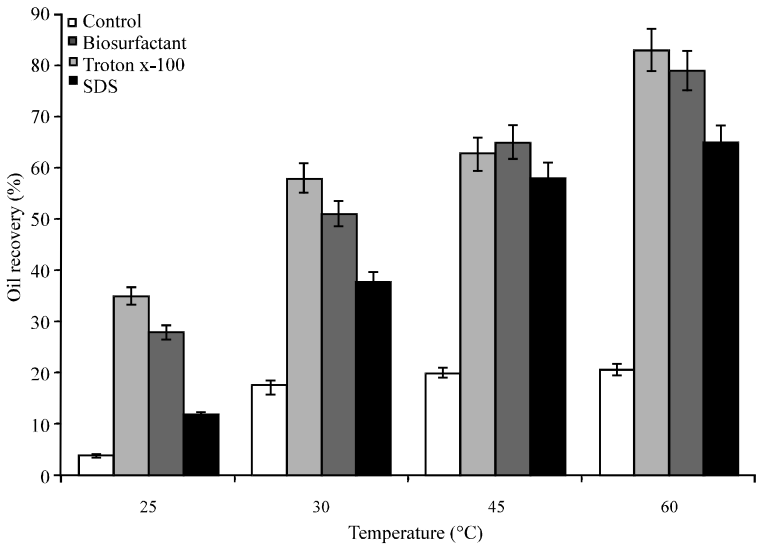


Fig. 2: Oil recovery assay using surfactants

recovered 25-30% of motor oil from contaminated sand at 25°C, 50% at room temperature (30±2°C), 65% at 45°C and 85% at 60°C. The synthetic surfactant SDS was found tobe less efficient.

Currently there is an escalating interest in the identification of novel biosurfactants for environmental cleanup and bioremediation (Muthusamy *et al*., 2008; Rahman and Gakpe, 2008). The frequency and occurrence of biosurfactant producers in PAH's and hydrocarbon contaminated mangrove sediments has not been well researched, however strain KVD1 could play a pivotal role in biodegradation of PAH's. The biosurfactant obtained from *P. aeruginosa* strain KVD1 employing diesel as substrate having high emulsification activities may provide a promising focus for further investigations on its application as a compound with efficient biological activity for enhanced oil recovery management in mangrove sediments. A precise chemical and structural analysis of strain KVD1 biosurfactant is currently in progress.

## CONCLUSION

In this study, we demonstrated the screening of biosurfactant producing bacteria, molecular strain characterization, production and stability studies of biosurfactant, emulsification activity and efficiency of oil removal by the biosurfactant produced by mangrove sediment bacterium *P. aeruginosa* strain KVD1 and its importance in bioremediation of oil from pristine mangrovesediments. Strain KVD1 therefore holds good potential for effective bioremediation of hydrocarbons in the mangrove wetland ecosystem and oil spill management.

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