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Characterization of dioxygenases and biosurfactants produced by crude oil degrading soil bacteria

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ABSTRACT

Role of microbes in bioremediation of oil spills has become inevitable owing to their eco friendly nature. This study focused on the isolation and characterization of bacterial strains with superior oil degrading potential from crude-oil contaminated soil. Three such bacterial strains designated as PA, PM and BM were selected and subsequently identified by 16S rRNA gene sequence analysis as *Corynebacterium aurimucosum*, *Acinetobacter baumannii* and *Microbacterium hydrocarbonoxydans* respectively. The specific activity of catechol 1,2 dioxygenase (C12O) and catechol 2,3 dioxygenase (C23O) was determined in PA, PM and BM wherein the activity of C12O was more than that of C23O. Among the three strains, BM exhibited superior crude oil degrading ability as evidenced by its superior growth rate in crude oil enriched medium and enhanced activity of dioxygenases. Degradation of total petroleum hydrocarbon (TPH) in crude oil was also high with BM followed by PA and PM. The three strains also produced biosurfactants of glycolipid nature as indicated d by biochemical, FTIR and GCMS analysis. These findings emphasize that such bacterial strains with superior oil degrading capacity may find their potential application in bioremediation of oil spills and conservation of marine and soil ecosystem.

Introduction

Oil pollution is a perpetual problem that affects terrestrial as well as marine ecosystems. Oil spillage may arise either accidentally or operationally during production, transportation, storage, processing or when used at sea or on land.[1] Crude oil is a multifarious mixture of many petroleum hydrocarbons of which polycyclic aromatic hydrocarbons (PAH) constitute a major fraction[2] which invite greater attention owing to their toxic, mutagenic and carcinogenic nature.[3] The recovery of spilled crude oil could be achieved by adopting physical and chemical methods but they can take care of only 10–15% of oil spillage. On the other hand,

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bioremediation by exploiting complex microbial communities can act as a self-driven, economical and eco-friendly method. Highly hazardous oily materials can easily be mineralized to harmless end products by introducing suitable microbial strains.[4] PAH can act as carbon source for certain microbial communities in oil-polluted environment.[5] Many bacterial species including *Pseudomonas aeruginosa, Bacillus subtilis, Bacillus megaterium, Corynebacterium kutscheri*, have been reported to possess oil degrading potential.[6,7] The biochemical process of crude oil degradation by microbes involves the action of several enzymes including oxygenases, dehydrogenases and hydroxylases that fragment aromatic and aliphatic hydrocarbons. A major constraint in biodegradation of oil is its hydrophobicity but biosurfactants produced by oil degrading bacteria facilitate uptake of hydrocarbons by the bacterial cells. Hence, those bacterial strains that possess the ability to produce biosurfactants along with enhanced oil degrading capacity are widely recommended for use in order to achieve fast degradation of crude oil.[8]

In this study, three bacterial strains with superior oil degrading potential isolated from an oil contaminated soil site were used. The competence of the bacterial strains that were designated as PA, PM and BM to utilize crude oil as the sole carbon source, determination of activity of enzymes – Catechol 1,2 dioxygenase (C12O) and Catechol 2,3 dioxygenase (C23O) and characterization of biosurfactants produced by them were carried out. Additionally, the extent of degradation of aliphatic and aromatic compounds of total petroleum hydrocarbon (TPH) in crude oil by these strains was also analyzed. Subsequently, the three bacterial strains were identified by 16S rRNA gene sequencing.

Materials and methods

Sampling and isolation of crude oil degrading bacterial strains

Crude oil and soil samples from crude oil contaminated sites were collected from Indian Oil Corporation Limited (IOCL), Chennai, India. For the isolation of crude oil degrading bacteria, 1 g of soil sample was inoculated into 100 mL of mineral salt medium (MSM pH 6.8 ± 0.2) supplemented with 1% crude oil[9] and incubated at room temperature for 1-week. From this, 1 mL of active inoculum was again transferred to fresh MSM containing 1% crude oil and was used for the isolation of crude oil degrading bacteria by serial dilution technique followed by spread plate technique using nutrient agar (NA) plates (pH 7.4 ± 0.2). The bacterial colonies were then cultured on fresh MSM agar plates supplemented with 1% (w/v) crude oil and those bacterial colonies grown were picked and preserved in liquid nutrient broth containing 70% glycerol at −80 °C. Among the 12 bacterial colonies isolated, 3 bacterial strains that exhibited superior growth by utilizing of crude oil were selected for this study and were designated as PA, PM and BM.

Determination of bacterial growth and activity of C12O and C23O enzymes

The ability of PA, PM and BM to utilize crude oil as carbon source was assessed by measuring their growth as colony forming units[9] (log CFU) in MSM containing different concentrations (1, 2 and 3%) of crude oil (incubated at 30 °C in an orbital shaker at 200 rpm) for 13 days at 24 h intervals. The enzyme activity of C12O and C23O were also determined in the cell free extracts of PA, PM and BM cultures at 24 h intervals for 13 days. For this purpose, cells were harvested by centrifugation at 5000 rpm (4 °C), washed twice with 0.01 M phosphate buffer (pH 7.0), subjected to sonication (15s) and the cell free extracts were collected by centrifugation at 20,000 rpm for 25 min (4 °C). The activity of C12O was determined by detecting the levels of *cis, cis*-muconate[10] and C23O based on the formation of 2-hydroxymuconic semialdehyde[11] by UV–Vis spectrophotometer (Shimadzu, UV-Pharmaspec 1700). Protein concentration was determined by the Bradford method.[12]

Purification of C12O

The partial purification of C12O from the cell free extracts of PA, PM and BM was carried out by ammonium sulphate precipitation followed by ion exchange chromatography (DEAE cellulose). The cell free extract was treated with cold saturated $(NH_4)_2SO_4$ solution (pH 7.5) to give 40, 60 and 80% saturation and the precipitates formed were collected by centrifugation at 10,000 rpm for 20 min (4 °C). The pellets were dissolved in Tris–HCl buffer 50 mM, (pH 7.5) and the resulting protein solutions were desalted by dialysis.[13] The dialyzed protein sample was then loaded on to a DEAE cellulose column (10 cm × 2 cm) that had been pre-equilibrated with 50 mM Tris–HCl (pH 7.5). Elution of protein was carried out with 50 mM Tris–HCl (pH 7.5) buffer containing NaCl in a linear gradient from 0.1 to 0.5 M.[14] The fractions collected were analyzed for enzyme activity and those fractions with highest activity were pooled and subjected to SDS PAGE analysis[15] to determine the molecular weight and the purity of the C12O. Molecular weight marker (97.4–14.3 kDa) (Merck bioscience) was used in this analysis.

Physicochemical and biochemical characterization of biosurfactants

To characterize the biosurfactants produced by PA, PM and BM, the bacterial strains were inoculated individually in to MSM broth containing 1% crude oil and incubated at 30 °C for 5 days in a shaker (200 rpm). The cell free supernatant was collected by centrifugation at 10,000 rpm for 20 min and was subjected to physicochemical and biochemical characterization. The emulsification index (E_{24})[16] and the oil displacement capacity[17] of the cell free supernatant was determined. Qualitative analysis to detect glycolipids and lipoproteins in the cell free supernatants of PA, PM and BM was carried out respectively by Phenol sulfuric acid[18] and Bradford Method.[12] The sugar content was quantitatively determined by orcinol assay.[19]

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