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Short Communication

Novel lipopeptide biosurfactant produced by hydrocarbon degrading and heavy metal tolerant bacterium *Escherichia fergusonii* KLU01 as a potential tool for bioremediation

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ABSTRACT

Escherichia fergusonii KLU01, a propitious bacterial strain isolated from oil contaminated soil was identified to be hydrocarbon degrading, heavy metal tolerant and a potent producer of biosurfactant using diesel oil as the sole carbon and energy source. The biosurfactant produced by the strain was characterized to be a lipopeptide. The minimum active dose and critical micelle concentration of the biosurfactant were found as 0.165 ± 0.08 μg and 36 mg/L, respectively. In spite of being an excellent emulsifier, the biosurfactant showed an incredible stability at extremes of temperature, pH and at various concentrations of NaCl, $CaCl_2$ and $MgCl_2$. Also the bacterium manifested tolerance towards Manganese, Iron, Lead, Nickel, Copper and Zinc. The strain emerges as a new class of biosurfactant producer with potential environmental and industrial applications, especially in hydrocarbon degradation and heavy metal bioremediation.

1. Introduction

Biosurfactants are the extracellular or membrane bound surface active compounds that are mainly produced by microorganisms. These biosurfactants are amphiphilic and exist in a wide variety of chemical structures such as lipopeptides, glycolipids, phospholipids, fatty acids, neutral lipids and polymeric biosurfactants and are produced by microorganisms of diverse origin (Ron and Rosenberg, 2001). Despite the broad applications of chemical surfactants, they are environmentally hazardous and can lead to ecological imbalance when accumulated in excess. In such a scenario, the performance of biosurfactants is most promising and they can act as an effective alternative to the chemical surfactants.

Petroleum hydrocarbons are the major pollutants leading to environmental degradation as a consequence of terrestrial freshwater runoff, refuse from oil refineries, shipping industries and accidental spillage during petroleum refining and transport activities (Thavasi et al., 2011). According to Itoh and Suzuki (1972), the ability of a rhamnolipid-negative mutant strain of *Pseudomonas aeruginosa* to utilize hydrocarbons as its carbon source was restored, only after the addition of rhamnolipid to the medium. This confirmed the indomitable role of surface active agents in the ability of the microorganisms to grow on oils, *n*-alkanes or other hydrocarbons. Since then, a large number of hydrocarbon degrading microorganisms were reported to produce biosurfactants (Bordoloi and Konwar, 2009; Zhao et al., 2011).

With the rapid advancements in technology, the natural environment often gets exacerbated by harmful effects of industrial pollution. The heavy metals which are utilized in a vast array of industrial activities, when released into the environment via waste waters, were known to severely affect the micro and macro biota inhabiting the water bodies, thereby perturbing the entire ecosystem (Joseph et al., 2009). Further, the exposure to heavy metals through the uptake of contaminated drinking water and foods can result in bioaccumulation of heavy metals in animals and humans eventually leading to biomagnification. In such a situation, the microbial means of heavy metal bioremediation can act as a potential eco-friendly solution for environmental clean up.

Considering the biosurfactant production by *Escherichia* species, so far there is no scientific report and the strain *Escherichia fergusonii* had been primarily isolated from clinical specimens (Mahapatra et al., 2005). The potential biotechnological applications of this strain remain unexplored and to our surprise, we identified the presence of this strain in oil contaminated soil which substantiates its diverging natural habitats. Hence in the present study, the isolated strain *Escherichia fergusonii* KLU01 was evaluated for its ability to produce biosurfactants, utilizing diesel oil as its sole carbon source and to bioremediate heavy metals. To our knowledge this is

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the first report, unveiling the ability of a hydrocarbon degrading and heavy metal tolerant *E. fergusonii* strain to produce potent lipopeptide biosurfactants which exhibited extreme stability, striking surfactant activity and excellent emulsifying capability.

2. Methods

2.1. Microorganism and culture maintenance

E. fergusonii KLU01 (GenBank: HQ214033.1), a strain isolated from the crude oil contaminated soil sample obtained from a petrol bunk in Srivilliputhur town, Tamilnadu, India was screened for biosurfactant production, hydrocarbon degradation and heavy metal tolerance. The strain was purified and stored at −80 °C as master stock. The working culture was preserved at 4 °C in nutrient agar plates and subcultured every 2 weeks.

2.2. Preliminary screening assays for biosurfactant production

In order to determine the ability of the strain to produce biosurfactant, the pure culture was subjected to preliminary screening methods namely hemolytic activity, drop collapsing test, oil displacement test and emulsification assay. All the above mentioned experiments were carried out based on the methodology previously described in Sriram et al. (2011) and the only modification here was that the strain *E. fergusonii* KLU01 and its cell free culture supernatant were utilized. All the experiments were done in triplicates.

2.3. Production media composition and cultivation conditions

Using the nutrient broth (NB) media (HiMedia, Mumbai, India), inoculum was prepared and incubated at 37 °C for 12 h. The seed culture at 3% was utilized to inoculate the biosurfactant production media of 100 ml in a 500 ml Erlenmeyer conical flask and incubated at 160 rpm at 37 °C for 5 days. The production media contained (g/L): $(NH_4)_2SO_4$, 10; NaCl, 1.1; KCl, 1.1; $FeSO_4.7H_2O$, 2.8×10^{-4}; $K_2HPO_4.3H_2O$, 4.4; KH_2PO_4, 3.4; $MgSO_4$, 0.5; Yeast Extract, 0.5; trace elements solution, 0.5 ml/L and diesel oil, 2% (v/v) as the sole carbon source. The composition of trace elements solution involved (g/L): $CaCl_2$, 0.24; $ZnSO_4$, 0.29; $MnSO_4$, 0.17 and $CuSO_4$, 0.25. After the production media was autoclaved, the trace elements solution was added prior to inoculation by filtering it with a 0.22 μm pore membrane (Millipore, USA).

2.4. Isolation, extraction and purification of biosurfactant

The cell free supernatant was obtained by centrifuging the broth culture at 10,000 rpm at 4 °C for 10 min. After centrifugation, the supernatant might possess some trace amounts of residual diesel oil which can be easily separated with the help of separating funnel. The supernatant was acidified with 6 N HCl, thereby reducing its pH to 2.0 and then incubated overnight at 4 °C. The acidified supernatant was centrifuged at 15,000 rpm for 20 min and the acid precipitate was collected and redissolved using Milli-Q water (pH 7.0) for its maximal dissolution (Javaheri et al., 1985). It was further lyophilized and this pale pinkish material was extracted thrice with methanol (Merck laboratory, Mumbai, India). The crude biosurfactant was eventually purified using thin layer chromatography (TLC) on a silica gel plate with the solvent system containing chloroform–methanol–water (65:25:4, v/v/v). The single spot comprising the purified biosurfactant was scrapped off from the TLC plate and then evaluated for its surfactant activity by oil spreading test. Such TLC-purified biosurfactant was utilized for further analysis.

2.5. Chemical characterization of biosurfactant

The primary characterization of the biosurfactant was carried through TLC as per the methodology described above in purification and the separated compound was visualized under UV light. Further characterization was accomplished with the help of Fourier transform infrared spectroscopic (FTIR) analysis, in which the dried sample of biosurfactant was scanned in the range of 450–4000 cm^{-1} at a resolution of 4 cm^{-1}. The high performance liquid chromatography (HPLC) analysis was carried out using C18 column for additional characterization of the biosurfactant.

2.6. Critical micelle concentration and stability analysis of biosurfactant

Critical micelle concentration (CMC) is specified as the concentration at which the micelle formation was initiated in the solution containing the amphiphilic compound. CMC was determined as the intersection of linear component of the curve drawn between the surface tension and the concentration of biosurfactant. The stability of the biosurfactant at extremes of physical parameters like temperature and chemical parameters like pH and different salt concentrations (NaCl, $CaCl_2$ and $MgCl_2$) were evaluated utilizing the oil spreading test (Morikawa et al., 2000). All the experiments were repeated thrice and the average values were taken as results in order to ensure its reproducibility.

2.7. Screening for heavy metal tolerance of E. fergusonii KLU01

The ability of the strain to tolerate heavy metals was determined based on the Tube and Agar diffusion methods previously described in Sriram et al. (2011) with slight modification. The test tubes containing NB medium and heavy metal salt solutions inoculated with *E. fergusonii* KLU01 were subjected to qualitative measurement of turbidity after the incubation period, based on which the tubes displaying the growth were considered as tolerant to the corresponding heavy metal and those that lacked growth were deemed to be sensitive or non-resistant.

3. Results and discussion

3.1. Preliminary screening assays for biosurfactant production

In order to confirm the ability of the strain *E. fergusonii* KLU01 to produce biosurfactants, the preliminary screening assays were carried out. In the hemolytic test, significant zone of clearance was observed around the colony, grown over the spot inoculated blood agar plate. Considering the drop collapsing test, the culture supernatant was placed over the oil surface as a single drop and in less than a minute the drop became flattened which testified the presence of biosurfactant. In the case of oil displacement test, a clear halo zone of 2.65 ± 0.11 cm^2 was observed over the oil surface and the emulsification activity of culture supernatant against the *n*-hexadecane was 57.5 ± 0.61%. The convincing results obtained from the above confirmatory assays strongly substantiated the biosurfactant production by the strain.

3.2. Production and characterization of biosurfactant

The surface tension of the production medium decreased from its initial value of 66 to 32 mN/m at the end of incubation period, which affirmed the ability of the strain to grow utilizing diesel oil as a carbon source and to produce biosurfactants. Such a step-down in surface tension during the logarithmic and stationary phase has also been reported for several biosurfactant producing