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Biomedical and environmental application of biosurfactant produced by bacterial strain *Klebsiella* sp. KOD36

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Abstract: Soil microorganisms, particularly bacteria exhibit a lot of potential to produce biosurfactants which are helpful in biomedical and environmental applications. Biosurfactants produced by bacterial strain *Klebsiella sp.* KOD36 exhibited excellent functional properties, such as biofilm inhibition, cytotoxicity, and in facilitating role in bioremediation of phenanthrene (PHE) in soil matrix and soil-water system. The results of the present study illustrate that partially purified biosurfactant exhibited good hemolytic activity (83%), thrombolytic activity (50%), and biofilm inhibition activity (28%). Additionally, application of biosurfactant along with nutrients NPK (i.e., nitrogen, phosphorus and potassium) enhanced the PHE mineralization potential by *Klebsiella sp.* KOD36 in all the three different textured soils and soil water systems. These findings reflect the importance of produced biosurfactant in biomedical and environmental field applications.

Keywords: biosurfactants, *Klebsiella sp.*, biofilm inhibition, cytotoxicity, biodegradation.

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1. Introduction

Biosurfactants have attracted attention as agents of hydrocarbon dissolution since 1960. Biosurfactants have unique structures and a large range of characteristics that can exploited at commercial scales. Biosurfactants are present everywhere in nature and are capable to be utilized in various fields, including biomedicine and therapeutics. Some of the bacteria and yeast release ionic surfactants which are helpful in emulsification of hydrocarbon substrate in growth media. Other

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microbes alter the structure of their cell wall which can be achieved by synthesis of lipopolysaccharides or non-ionic surfactants within the microbial outer layer [2].

Distinctive advantageous properties for biosurfactants include enhancement of solubility, reduction of surface tension, and little critical micelle concentrations (CMC). The range of CMC of biosurfactants is from 1 to 200 mg/L [3]. Micelle formation plays an important role in microemulsion creation [4], which are clear and stable liquid mixtures of water and oil domains divided by a single layer or aggregates of biosurfactants. When one liquid phase is dispersed in drops in another liquid phase, microemulsions are formed. If oil is dispersed in water, it is termed as direct microemulsion; when water is dispersed in oil, it is termed as reversed microemulsion [5]. High molecular weight biosurfactants are mostly polyanionic heteropolysaccharides consisting of polysaccharides and protein, while low molecular weight biosurfactants are generally glycolipids [6]. As biosurfactants have diverse structures, that make them suitable for biomedical and environmental applications [7, 8]. Since they are environmentally friendly, hence are alternative to synthetic chemical surfactants used in biomedical field as antimicrobial and therapeutic agents. Currently, there is increasing trend in investigating biosurfactants effects on human and animal cells [9]. Biosurfactants produced by *Bacillus* sp. (lipopeptides), and mannosylerythritol lipids produced by *Candida antartica,* and rhamnolipids produced by *Pseudomonas aeruginosa*, exhibited excellent properties as antimicrobial agents [10].

The main objective of the study was to evaluate the biological activities and functional properties of biosurfactants produced by *Klebsiella sp.* KOD36 and their application in the biomedical and environmental fields.

2. Materials and Methods

2.1. Culture medium, chemicals, and microorganism

The strain already isolated and identified as *Klebsiella sp.* through 16s RNA (accession number KT364873) was used in present study [11]. Mineral salts (MSM), NaCl (1.0 g/l), $CaCl_2$ (0.1 g/l), KH_2PO_4 (1.0 g/l), $MgSO_4{\cdot}7H_2O$ (0.5 g/l), Na_2HPO_4 (1.0 g/l), and yeast extract (4.0 g/l) were used for the purification and streaking of bacterial strains already reported to produce biosurfactants. All solutions have been prepared in distilled water at 121°C for 20 minutes.

2.2. Cytotoxicity test (hemolytic activity)

Human blood (3 mL) was mixed thoroughly with purified sample of biosurfactant and then poured into a 15 mL screw capped tube to centrifuge for 5 min according to the method of Tabasum et al. [12]. The supernatant was collected after centrifugation and the viscous pellets obtained were subjected to washing thrice with phosphate buffer saline (PBS) (5 mL) solution. The cells (after washing) were then suspended in sterile PBS (final volume 20 mL), and later counted on hemocytometer. Ice was used to maintain the blood cell suspension and diluted with sterile PBS. Peptide aliquots (20 mL) were placed into 20 mL microfuge tubes. Diluted blood cell aliquots (180 mL), the suspension collected were placed in each two milliliter tube aseptically and mixed smoothly thrice with pipette tip. Tubes were incubated at 37°C with agitation for 35 min. Immediately the tubes were placed for 5 min on ice and centrifuged for five minutes. 100 ml aliquots of supernatant were collected carefully and placed into 1.5 ml centrifuge tube. It was further diluted with 900 ml chilled sterile PBS. After dilution, all centrifuged tubes were placed on wet ice. Three replicates were run for each. Absorbance was measured at 576 nm.

2.3. Thrombolytic activity

Twenty healthy volunteers were selected, and their venous blood was transferred to a pre-weighed sterile microcentrifuge tube (500 µL/tube) and incubated at 37°C for 45 minutes. When the clot formation occurred, the serum was removed completely (care was taken while aspiration to avoid disturbance of clot formation) and then weight of each tube was measured to determine the weight of clot (which was equal to the difference between the clot weight) containing tube and the weight of the tube alone). Each clot-containing microcentrifuge

tube was labeled and 100 µL of crude extract and its polar fractions collected were then added to respective tube. For a negative thrombolytic control, water was added to one of the tube (having clot formation). For clot formation, this experimental set up were then placed in temperature (at 37°C) controlled incubator for 90 minutes. After incubation, the fluid formed were removed and each tube were gain weighed to calculate the difference in weight after the clot disruption. The % of clot lysis were measured as difference in weight before and after the clot lysis.

2.4. Biofilm inhibition activity

The inhibition in bacterial biofilm formation (Bacillus subtilis) was calculated by adopting microtiter-plate method previously described by Stepanovic et al. [13]. The wells of 24-well sterile tissue culture plate were then filled with nutrient broth of volume 100 microliter (Oxoid, UK). The testing sample with concentrations of 2.5 µg was dissolved in one milliliter of DMSO, and the added in different wells. Uniform bacterial culture each with 1×10^9 cell density was inoculated. The treatment as positive control having Rifampicin and nutrient broth and negative control having only the bacterial culture and nutrient broth was set. The plates were covered and incubated in temperature controlled incubator (37 ^{0}C) for 24 hours. Later the wells were beheaded and washed thrice with 220 µL phosphate buffer solution. Plates were vigorously shaken to remove all the nonadherent bacteria. The leftover attached leftover bacteria were later fixed with 99% methanol (220 mL) and left for drying for 15 minutes. After that the wells were stained with 220 mL of crystal violet dye (50%). Distil water was used for rinsing the extra stain and plated were air dried. 220 µL glacial acetic acid (33% v/v) was used for re-solubilization of bound dye. The optical density was the measured using microplate reader at 630 nm (Biotek, USA). Experiment was run in triplicate and mean values were taken. Biofilm inhibition activity was measured using the following formula:

$$\text{INH\%}=100-\frac{(\text{OD630 sample}\times100)}{\text{OD630 control}} \quad (1).$$

2.5. Biodegradation lab studies using PHE as model compound

The experiment was carried out to investigate the influence of the biosurfactant and fertilizers on the biodegradation of PHE in soils (Hanford coarse sandy loam, Dello loamy sand, and Willow silty clay). For this purpose, 250 mL conical flasks were used. In each flask, 50 g soil was used. Each conical flask containing sterilized soil was spiked with non-radiolabeled PHE (100 mg/kg) and 0.005 mCi PHE. Biometer flasks were kept overnight to allow evaporation of the added acetone and then inoculated with *Klebsiella sp.* KOD36 (cell density 1% v/v, CFU 1x105 bacterial cells mL^{-1}) along with biosurfactants at CMC (124 mg/kg) were added for PHE degradation. After inoculation, the gravimetric water content was adjusted with 0.2 M PBS. The effect of biosurfactants and fertilizer on PHE degradation was examined using the treatments presented in Table 1.

Table 1. The types of treatments for PHE degradation.

Sample	Biosurfactant	Fertilizer	Inoculation
B1N1I1	+	+	+
B1N0I1	+	-	+
B0N1I1	-	+	+
B0N0I1	-	-	+
B1N1I0	+	+	-
B0N1I0	-	+	-
B1N0I0	+	-	-
B0N0I0	-	-	-

The biosurfactant used at CMC and sub CMC was produced by the same strain *Klebsiella sp.* KOD36. A 2% fertilizer (urea and K_2HPO_4 1:1, w/w) was used. All other procedure was the same as described in the above sections.

2.6. Development of soil suspension using nutrient solution for soil-water system biodegradation study

Suspension (10 g/L) of each soil, Hanford coarse sandy loam, Dello loamy sand, and Willow silty clay soil was prepared in MSM. The soil suspensions and amendments were separately autoclaved (121°C for 30 min) three times, with a 1 day interval between each autoclaving. Soil suspensions were spiked with 0.001 mCi 14C-labeled PHE (volume of acetone 0.5%, v/v) to give a final PHE concentration of 100 mg/kg soil at a specific radioactivity of 13.3 mCi/mmol of PHE. The PHE-spiked soil suspensions were then stirred for 7 days before adding biosurfactant-producing bacteria along with fertilizer and additional biosurfactant. For each soil type, unamended controls consisting of only soil without any amendment were also prepared. Different (un)amended soil treatments were further shaken for 10 days on a horizontal shaker at 120 rpm at 30°C in the dark before use in the mineralization experiments, to allow for a phenanthrene (PHE) surface equilibration between the dissolved and solid phases. Similar set of treatment was used (except MS medium) for making soil-water systems. PHE mineralization in all three different textured soils was investigated by measuring $14CO_2$ evolved from the biometric flasks that was trapped in a vial containing 2 ml 1M NaOH. The vials were replaced periodically and the NaOH trapped solution inside the vial was collected to measure the $14CO_2$ evolved using liquid scintillation counter.

3. Results and Discussion

3.1. Biofilm inhibition

Figure 1 indicate biofilm inhibition when 5 µg of biosurfactant was dissolved in 1 mL DMSO showed good activity against *Bacillus subtilis* biofilm. About 28% reduction was observed in biofilm activity (Table 2) compared to its positive control Rifampicin which showed higher (98%) biofilm inhibition activity.

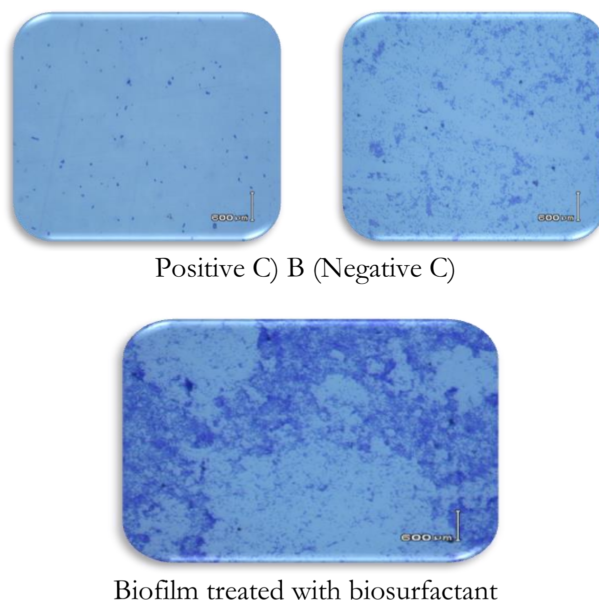

Figure 1. Biofilm inhibition of Bacillus subtilis by produced biosurfactant by bacterial strain KOD36.

3.2. Hemolytic and thrombolytic activity

The biosurfactant produced by *Klebsiella sp.* KOD36 showed a significant higher hemolytic activity (83.7%) than the commercial and synthetic nanoparticles. Similarly, thrombolytic activity was noted up to 50.2% (Table 2). It is quite high and can compete with other commercial synthetic thrombolytic agents because of its organic nature.

Table 2. Functional properties of the biosurfactant.

	Activity (%)	Significance
Hemolytic activity	83.70 ± 1.85	+++
Thrombolytic activity	50.20 ± 1.87	++
Biofilm inhibition	28.20 ± 0.15	+
Rifampicin (Positive control)	98.20 ± 0.15	+++

Biomedical application and potential of produced biosurfactant by *Klebseilla sp.* KOD36 investigated revealed that it had a unique hemolytic, thrombolytic, and biofilm inhibition activity. Such biosurfactants may have practical applications in the biomedical and therapeutical field. Recently, it has been reported that biosurfactants have the potential to act as antitumor therapeutic agents [14-17]. Application of biosurfactant in biomedical field has increased tremendously for the past couple of years. Functional properties like antifungal, antiviral and antibacterial activities make them suitable in application against various diseases and therapeutic agents [18, 19].

3.3. PHE biodegradation in soil system

Biodegradation of PHE in soil matrix microcosm experiment showed (Figure 2) that maximum (61%) PHE mineralization occurred in treatment group 4, where inoculation was performed along with biosurfactant and fertilizer in Dello loamy sand textured soil, followed by Hansford coarse sandy loam textured soil (49%), and Willow silty clay textured soil (43%). Application of biosurfactant in treatment T2 significantly improves the PHE mineralization. Maximum PHE mineralization (43%) was observed in the case of Dello loamy sand, followed by Hansford coarse sandy loam (30%), and Willow silty clay (27%). It was interesting to find out that the application of fertilizer alone to bacterial cells in treatment T3 have almost similar results as biosurfactant treatment was applied. Maximum PHE mineralization (45%) was observed in Dello loamy sand, followed by Hansford coarse sandy loam (37%), but their combination with fertilizer has significantly enhanced the total PHE mineralization. This indicated that the main factor improving the degradation was the presence of the biosurfactant rather than fertilizer. Biosurfactant application in facilitating biodegradation processes is well documented [20, 21]. The most commonly used biosurfactants are rhamnolipids from *Pseudomonas* species in biodegradation of various hydrocarbons like polycyclic aromatic hydrocarbons, pesticides, and other organic pollutants [22-24]. In our present state of knowledge, no study ever reported the production of rhamnolipid biosurfactant from *Klebsiella sp.* and its application in biodegradation of PHE.

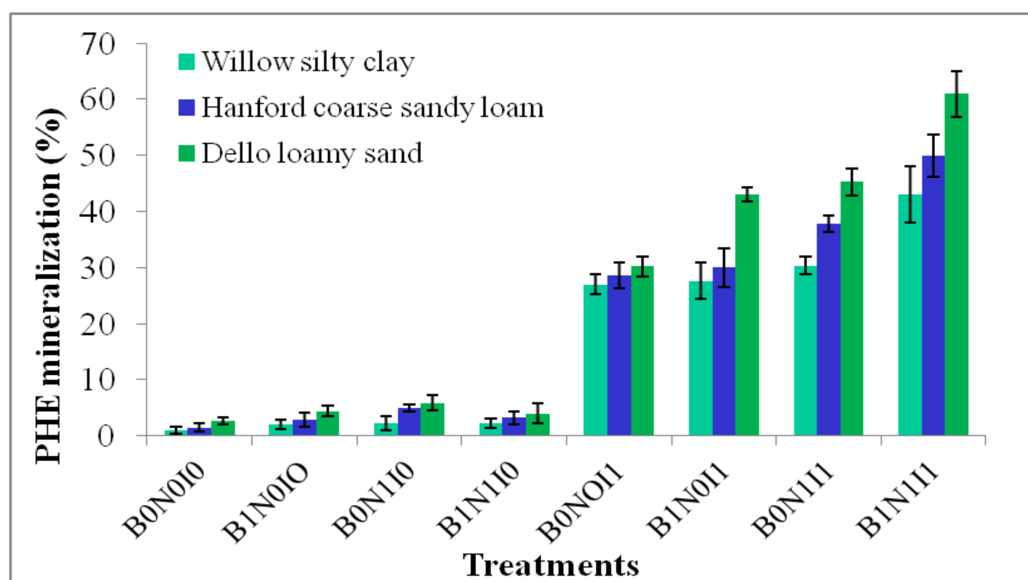

Figure 2. Biostimulation-biosurfactant assisted biodegradation of PHE in different textured soils.

3.1. Biodegradation of PHE in soil matrices

The results from microcosm soil system study showed improvement in PHE mineralization. It was observed (Figure 3) that maximum PHE mineralization (68%) was observed in Dello loamy sand soil-water systems, where the fertilizer was applied along with the biosurfactant and inoculation in treatment T1, followed by Hansford coarse sandy loam (67%), and Willow silty clay (59%), compared to the treatment T4 where no fertilizer and no biosurfactant were applied in inoculation. Contrary to soil matrix system experiment, biosurfactant application significantly improved the PHE mineralization, but application of fertilizer without biosurfactant to bacterial cells showed no increase in overall mineralization as in soil matrix system. Maximum PHE mineralization with biosurfactant application alone to bacterial cells in treatment T2 were observed in Dello loamy sand (47%), followed by Hansford coarse sandy loam (44%), which is equal to the PHE mineralization in Willow silty clay (43%). Fertilizer application alone to bacterial cells showed maximum mineralization in Dello loamy sand (53%), followed by Hansford coarse sandy loamy (35%), and Willow silty clay (33%).

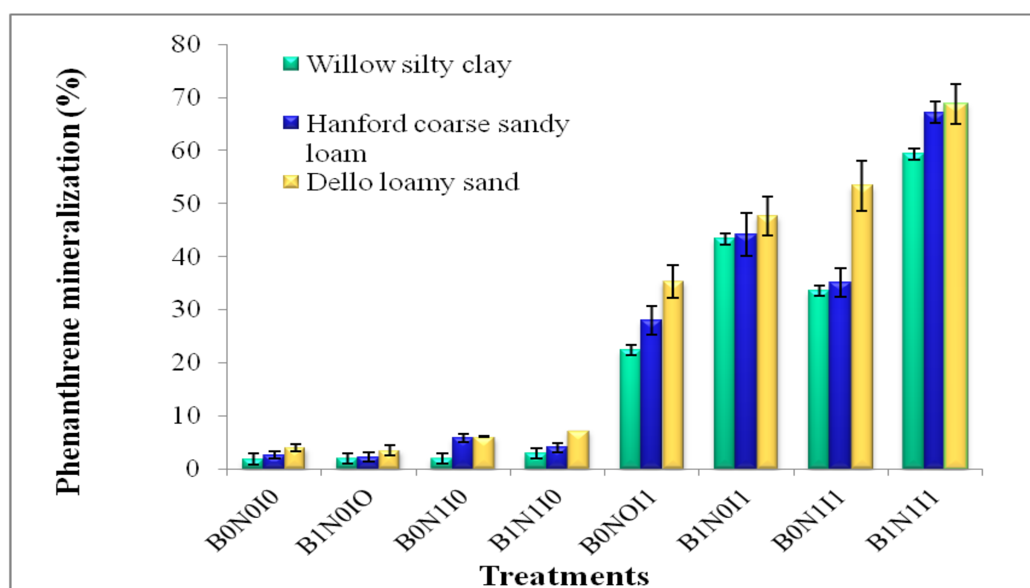

Figure 3. Biostimulation-biosurfactant assisted biodegradation of PHE in different textured soils water systems.

Biostimulation experiments were conducted to investigate the effect of fertilizer in biosurfactant facilitated bioremediation in three different textured soils and soil-water systems. It was obvious from the results that biodegradation varied with the soil texture. It is likely that biosurfactants activity may vary depending on chemical and physical characteristics of soil. As Dello loamy sand textured soil has 0.7% organic matter (OM) with 79% sand contents which are higher than the Hanford coarse sandy loam (0.75% OM, 68% sand), and Willow silty clay (2.2% OM, 5% sand). It is clear that a higher OM content tends to increase the adsorption capacity of the contaminants. Also, sand content may affect adsorption and desorption capacity of the soil. Moreover, successful bioremediation of contaminants can be achieved at sufficient number of microorganisms. As microorganisms increase in number, they can enhance the mineralization rate. Microorganisms require an optimum nutrient balance required for hydrocarbon remediation of C:N:P=100:10:4. Biostimulation has been considered as an accelerating strategy in the past few years. It is believed to promote the growth and activity of microorganisms capable of degrading pollutants. Previous studies suggest that nutrient supplementation stimulates bioremediation by increasing microbial biomass [25, 26]. When a major oil spill occurs in the environment, the supply of carbon is dramatically increased, and the availability of nitrogen and phosphorus generally becomes the limiting factor for oil degradation [27, 28].

4. Conclusions

Soil microorganisms, particularly bacteria, exhibit a lot of potential to produce biosurfactants which are helpful in biomedical and biodegradation of hydrocarbons. Partially purified biosurfactant exhibited good hemolytic (83%), thrombolytic activity (50%), and biofilm inhibition activity (28%). Application of biosurfactant along with NPK fertilizer enhanced the PHE mineralization potential by *Klebsiella sp.* KOD36 in all the three different textured soils. Biosurfactant addition alone to bacterial cells had no significant difference in PHE mineralization compared to treatments where biosurfactant and fertilizer were added in soil and slurry system. Thus, biosurfactant produced by *Klebsiella sp.* reflects capability to be used in biomedical and environmental field applications.

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Conflicts of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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