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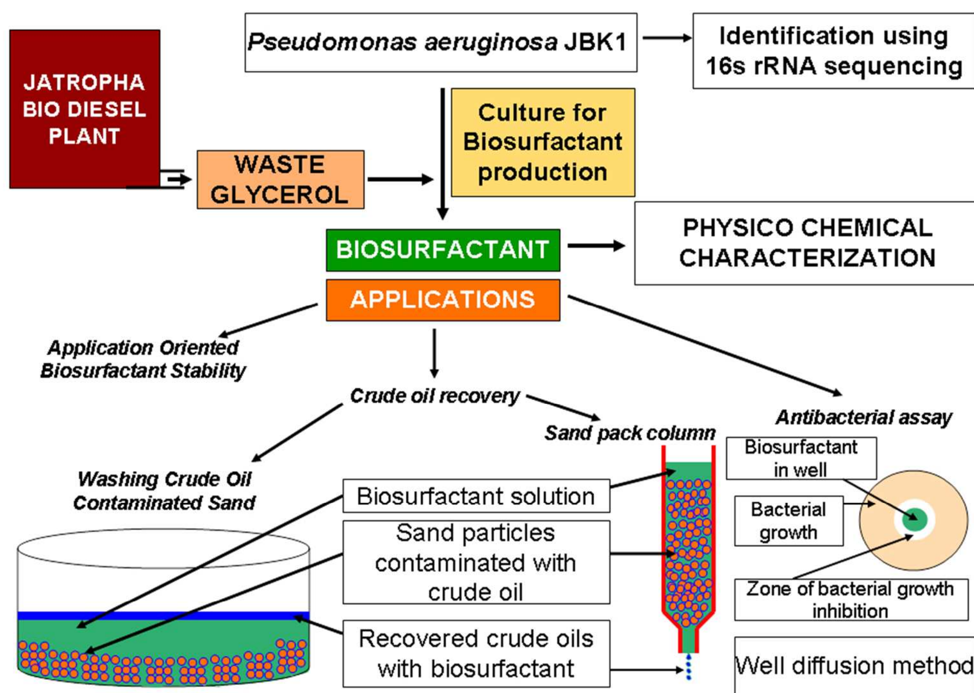

Graphical Abstract
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Biodiesel derived waste glycerol as an economic substrate for biosurfactant production using indigenous *Pseudomonas aeruginosa*

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Abstract

The present investigation demonstrates the utilization of biodiesel-derived crude glycerol as a low-cost substrate for the production of biosurfactant. *Pseudomonas aeruginosa* JBK1 was identified using 16s rRNA sequencing. A maximum of 3.9 g biosurfactant was obtained at 3% raw bio-glycerol having the critical micelle concentration (CMC) at nearly 540 mg/l. The bacterium lowered the surface tension (ST) of culture medium upto 33.7 mN/m within 60 h of fermentation and showed better adhesion to the hydrophobic substrate surfaces. The biosurfactant formed stable emulsion with various hydrocarbons and achieved a maximum emulsion index of 62% with kerosene and xylene. The cell free culture supernatant exhibited a stable surface as well as emulsifying activity, and remained unaffected to the exposure of high temperature, pH and salinity. Mass spectrometric studies indicated that the purified product is glycolipid in nature. Sand pack column experiment showed a 10.8% recovery of crude oil from the column after treatment with the cell free culture supernatant. A maximum of 54.6% residual crude oil was removed through washing by the biosurfactant solution. The biosurfactant also exhibited potential antimicrobial activity against a variety of bacterial and fungal strains. The results suggest that the biosurfactant

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produced by the bacterium on waste glycerol, may have potential application in hydrocarbon bioremediation.

Keywords: biodiesel; glycerol; biosurfactant; emulsion; recovery

1. Introduction

Biological macromolecules such as biosurfactants are a heterogeneous group of surface active molecules produced by a variety of microorganisms. These are amphiphatic molecules possessing both the hydrophilic and hydrophobic moieties that allow them to partition at the interfaces between fluid phases with the different degrees of polarity, such as oil-water or water-air interfaces[1]. Biosurfactant tends to decrease tension at the interfaces of hydrocarbon-water and results in pseudo-solubilization of hydrocarbons via micelle or vesicle formation. This leads to increase in mobility, wettability, bioavailability and subsequent degradation of hydrocarbons[2-3]. Low toxicity, functional under extreme conditionsspecificity, biocompatibility, biodegradability, production from renewable sources and ecological acceptibity are among the main advantages of biosurfactants when compared with synthetic surfactants[4]. Such unique properties make biosurfactant a potential alternate to the synthetic surfactants in a variety of applications[5]. Among the aerobic bacteria, *Pseudomonas aeruginosa* is best-known for its capability to produce biosurfactants, chemically related to glycolipid, when grown on a variety of carbon sources ranging from glucose to complex petroleum based hydrocarbons[6]. Rhamnolipid produced by *Pseudomonas aeruginosa* are well-characterized and basically composed of rhamnose and β-hydroxycarboxylic acid subunits[7]. Previous reports suggest that the surfactant properties of rhamnolipid depend on its chemical composition and distribution of homologs that vary according to the bacterial strain,

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culture conditions (including temperature and pH) and media composition[8] (especially the carbon and nitrogen sources). Among the media composition, carbon source is an important limiting factor affecting both the quality and quantity of biosurfactants[9]. Though rhamnolipids have many important biological activities, the main obstacles in their commercialization are their low productivity, cost-intensive recovery and involvement of high production cost. To overcome such hurdles, different strategies including development of genetically modified strains, development of economical scaling up processes, optimization of the fermentation process, use of cost-effective feedstock for microbial growth and biosurfactant production have been suggested towards making their production economically viable[10-11]. The selection of cost-effective culture media components which constitute 50% of the total production cost is the key bottleneck in the production of biosurfactant11-13. The rapid progress in the biodiesel production technology has led to the generation of tremendous quantities of glycerol wastes, as the main by-product of the process[14]. Stoichiometrically, it has been estimated that 10 kg of glycerol wastes are produced for every 100 kg of biodiesel[15]. On the basis of the technology used for the production of biodiesel, glycerol wastes may contain various types of impurities such as methanol, salts, soaps, heavy metals, and residual fatty acids[16]. Due to the presence of such heavy impurities, further purification of the biodiesel-derived glycerol for its industrial applications is reported to be unprofitable[15,17]. Therefore, the utilization of waste glycerol through biotechnological approaches represents a promising alternative for the effective management of such kind of industrial wastes[14]. Thus, use of such inexpensive by-products will be more economic for the production of biosurfactant having commercial value with waste minimization. The objective of this work was to assess the potentiality of biodiesel-derived waste crude glycerol for the production of biosurfactant by the indigenous bacterium and application of such biosurfactant in the bioremediation processes.

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2. Materials and methods

2.1 Enrichment and isolation of bacteria

Microorganisms were isolated from crude oil contaminated soil samples collected from Borhola oil fields of Jorhat district, Assam, India by enrichment culture technique[18]. The bacterial isolates were routinely sub cultured at an interval of 30 days. For long term preservation, the bacterial isolates were stored in 70% (v/v) glycerol at -70°C.

2.2 Screening for biosurfactant activity

For selecting the efficient biosurfactant producing bacteria, the isolates were grown on MSM agar plates containing cetyltrimethyl ammonium bromide (CTAB, 0.2 mg/ml) and methylene blue (5mmg/ml) and blood agar medium containing 2% (v/v) of goat blood[19]. Final screening was based on the rapid ability to collapse the water droplet on an oil coated surface[2].

Characterization of biosurfactant producing bacteria

Primary identification procedures including morphological features and standard physiological tests were carried out according to Bergey's Manual for Determination of Bacteriology[20]. The 16s rRNA gene sequence of *P. aeruginosa* strain JBK-1 was sequenced at NCCS, Pune, India. A BLAST search was performed for the 16s rRNA gene sequence of *P. aeruginosa* strain JBK-1 at the NCBI database using nucleotide BLAST. Subsequently, the 16s rRNA gene sequences of various strain of *P. aeruginosa* showing maximum similarity from the BLAST result were taken for phylogenetic analysis and nucleotide frequency count analysis using CLC Main work bench.

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Culture medium and fermentation conditions

The waste glycerol produced during converting *Jatropa* seed oil to biodiesel was collected from Department of Energy, Tezpur University, Assam. The pH of the waste product was adjusted to 7.0 by adding 5N NaOH. The glycerol fraction from the neutralized waste product was separated by using a glass separating funnel and dried at 50˚C in an oven to remove the residual moisture. For the fermentation, 100 ml of sterile mineral salt medium (MSM) in 250 ml Erlenmeyer flask was inoculated with 1%, of 16 h old inoculum culture and was incubated at 37˚C in an orbital incubator shaker at 180 rpm for 11 days[3]. The mineral salt medium was used for the fermentation process and supplemented with 3% (v/v) waste raw glycerol. All samples were withdrawn at regular interval from the culture broth to monitor the cell growth (g/l) using determining the OD at 600 nm[12], biosurfactant concentration (g/l) using determination of rhamnose content by orcinol assay and reduction in the surface tension (mN/m) using du Noüy ring method[2].

Biosurfactant recovery and purification.

The culture broth was centrifuged at 12,000 rpm for 20 min at 4˚C. The pH of the culture supernatant was adjusted to 2 by adding 6 N HCl and kept overnight at 4°C. The precipitate was extracted thrice with an equal volume of ethyl acetate. The organic phase was collected and dried in a rotary evaporator. The brownish oily residue left behind was dissolved in 3.0 ml of 0.1 M sodium phosphate buffer[21] (pH 7.2).

Total carbohydrate content in the biosurfactant was estimated by phenol-sulfuric acid method[22], protein by Lowry's method[23] using bovine serum albumin as the standard, and lipid by the standard protocol of Folch et al[24].

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Characterization of isolated biosurfactant

The thin layer chromatography technique (TLC) was used to detect the carbohydrate and lipid moieties using a mobile phase system consisting of chloroform: methanol: water (65:15:2, v/v/v), respectively. Anthrone, ninhydrin and iodine fumes were used as developing agents for carbohydrate, amino acids and lipid, respectively[13].

Fourier transform infrared spectroscopy (FTIR)

The IR spectrum of the isolated biosurfactant was recorded in potassium bromide pellet using a FTIR spectrophotometer (Nicolet Impact 410 spectrometer).

Mass spectroscopy (MS)

The mass spectroscopic (ESI-MS) study of the isolated biosurfactant was carried out using the standard protocol[5] on a Perkin Elmer system. The carrier gas was helium, maintained at the flow rate of 1.5 l/ml.

Surface tension and interfacial tension

The surface tension (ST), interfacial tension (IFT) and critical micelle concentration (CMC) of the isolated biosurfactant were measured at 25˚C using a digital tensiometer (Krűss Tensiometre K9 ET/25). The CMC value was determined from the plot of surface tension versus the biosurfactant concentration[25]. For determining critical micelle dilution (CMD), the biosurfactant solution at its CMC was diluted to 10 times (CMD^{-1}) and 100 times (CMD^{-2}).

Foaming index (F_{24}%)

20 ml of the biosurfactant solution (1g/l) was transferred to a measuring cylinder of 50 ml volume and then compressed N_2 gas was passed through the solution at a flow rate of 0.5 l/min for 2 min. The foaming index of the biosurfactant was calculated by the following equation

$F_{24}\% = (\text{height of the foam layer} \div \text{height of liquid + foam}) \times 100$

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Emulsification index (E_{24}%)

2.0 ml of test hydrocarbon was added to 2 ml of culture supernatant in a glass test tube and homogenized by vortexing at high speed for 2 min. The stability of the emulsion was determined after 24 h and 30 days. The E_{24}% was determined by the following equation:

E_{24}% = (height of the emulsion layer ÷ total height of the mixture) × 100

Bacteria Adhesion to Hydrocarbon (BATH) assay

The bacterial cells were washed and suspended in the PBS buffer (pH 7.2) to an initial absorbance of 1.2-1.4 at 400 nm. A mixture of 0.5 ml n-hexadecane and 2.0 ml of cell suspension was vortexed for 2 min and then allowed to settle down for 15 min at room temperature. The aqueous phase was removed and measured at OD_{400} nm. The hydrophobicity was expressed as the percentage decrease in OD_{400} of the lower aqueous phase by OD_{400} of the initial cell suspension multiplied by 100.

Application in bioremediation processes

Effect of environmental factors on biosurfactant stability

The culture supernatant was exposed separately to 4˚C, 15˚C, 25˚C, 37˚C, 45˚C, 75 ˚C, 90˚C, 100˚C and 121˚C for 60 min and the surface tension and the emulsification index were measured. The pH of the culture supernatant was adjusted to different pH values (2–11) and the surface tension and the emulsification activity were measured. Similarly, the effect of NaCl concentration (1–7%) on surface tension and the emulsification activity were determined[25].

Washing experiment

Crude oil contaminated sand samples each weighing 20g were transferred to 250 ml Erlenmeyer flasks containing 100 ml of aqueous solution of purified biosurfactant of different concentrations and kept at 200 rpm for 24h at room temperature. The contaminated sand samples

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were separated, dried and washed twice with dichloromethane. The solvent part was removed with the help of rotary evaporator and the residual oil was determined gravimetrically[4]. The percentage of oil removed was calculated using the following equation

Crude oil removed (%) = $[(O_i – O_r) \div O_i] \times 100\%$, where Oi is the initial oil in the sand sample(g) before washing and Or is the oil remaining in the sand sample (g) after washing.

Sand pack column experiment

The sand pack column experiment was carried out as described by Suthar et al[3]. A vertical glass column with a dimension of 25 cm × 3cm (internal diameter) was packed with 150 g of acid washed sand. The column was initially saturated with brine (5% NaCl) solution, followed by flooding with crude oil supplied by ONGCL, Assam, India until no water is present in the effluent. The column was further flooded with brine until no further oil appeared in the effluent. Finally, cell free culture supernatant of stationary phase of growth was pumped into the column and was kept undisturbed for 24h at a constant temperature. The oil released from the column after exposure to various temperatures (room temperature, 50, 70 and 90˚C) was then measured.

Antimicrobial test

The purified biosurfactant was tested for its antimicrobial property using well diffusion method. Wells with 6 mm internal diameter were made and 50µl (20mg/ml) of biosurfactant was added into the wells and incubated at 37˚C for 24h. For fungi, the plates were incubated at 25±2˚C for 36-48 h. After incubation, the clear zone was measured and calculated with the help of an antibiotic zone scale-C (PW 297-3NO Himedia).

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3. Results and Discussion

Isolation and screening for biosurfactant producing bacteria

From the enrichment culture technique, 21 different bacteria were isolated from the crude oil contaminated soil samples, collected from the oil drilling sites of Borhola oil fields of Titabor, Jorhat, Assam, India. Bacterial isolates were selected based on standard morphological and biochemical methods up to the genus level. Only 8 bacterial isolates showed positive results in CTAB and blood agar plate tests. On the basis of drop collapse test, only bacterial strain JBK1 was selected as a potent biosurfactant producer and selected for the present investigation. The bacterial strain reduces the surface tension of the culture media from 68.4 to 33.7 mN/m and able to grow on all the tested carbon sources. Further, both the biomass and biosurfactant yield per carbon (glycerol, n-hexane, dodecane, n-hexadecane, nonadecane, paraffin, crude oil and vegetable oils) was determined. Finally on the basis of biosurfactant yield per liter of carbon source, availability and the cost involved with the carbon source, waste raw glycerol was selected as a suitable candidate.

Characterization and identification of bacterial strain

The bacterial strain is morphologically large, light yellow in color, flat with irregular boundaries, translucent and has non-shiny surface when grown on nutrient agar plates. The bacterial strain produces yellow colored pigments when cultivated in Pseudomonas isolation agar medium. Scanning electron microscopic study shows that the bacterial strain is rod shaped with smooth surface (shown in supplementary Figure S1). Biochemical and physiological experiments revealed that the bacterial strain is aerobic, motile, non-spore forming, gram negative and able to grow between 30 and 40°C temperature. Based on the results of above experiments, the bacterial strain was identified as *Pseudomonas sp*. The 16S rRNA gene sequencing as well as BLAST search

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revealed that the bacterial strain is closely related to the species of *Pseudomonas aeruginosa* with 99% similarity (Supplementary Table 1). The sequence was deposited to the NCBI GenBank Database and assign the accession number JX843422. The BLAST result of *Pseudomonas aeruginosa* strain JBK1 showed maximum similarity with 13 different strains of *Pseudomonas aeruginosa* as shown in Supplementary Table 1 along with their score, query coverage, E-value and maximum identity. The phylogenetic tree generated from the sequence alignment is shown in Supplementary Figure S2. As presented in Supplementary Figure S3, the ATGC composition and C+G and A+T composition of *Pseudomonas aeruginosa* strain JBK1 possessed the same frequency distribution as compared to other strains of *Pseudomonas aeruginosa*.

Production of biosurfactant using waste bio-glycerol

The increase in the cost involved with the waste treatment/disposal in relation to the environmental safety, the use of biodiesel-derived crude glycerol as an alternative substrate for the production of biosurfactant is highly desirable and beneficial. Results clearly indicated that the biodiesel-derived crude glycerol has a high potentiality for the production of biosurfactant. The profiles of biomass, biosurfactant production and changes in surface tension are determined with respect to time (Fig 1). For initiation of the fermentation process, 3% biodiesel derived glycerol was used as the sole carbon substrate. The bacterium reached the exponential phase of growth within 24h of incubation indicating the suitability of the biodiesel-derived crude glycerol as the carbon substrate. At the end of 132h of fermentation, a maximum of 1.9 g/l of biomass was reached. The bacterial strain began to produce biosurfactant significantly after 12h of incubation and reached a yield of 3.9 g/l of biosurfactant at the end of 132h. With the production of biosurfactant, the surface

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tension of the culture medium started reducing after 24h of incubation indicating the accumulation of biosurfactant at the interfaces. Results indicated that the production of biosurfactant by the bacterium occurred during the exponential phase of growth suggesting the production of biosurfactant as a primary metabolite along with the formation of cellular biomass[12, 26-27].

Chemical characterization

Biochemical analyses showed that the biosurfactant contains 22.5% carbohydrate, 71.7% lipids and 1.3% proteins. However, no amino acids has been detected in thin layer chromatography (TLC) after spraying with ninhydrin solution, which indicated that the isolated biosurfactant could be glycolipid. Three distinct peaks with the R_f value of 0.17, 0.34 and 0.53 were observed. After spraying with anthrone solution, two dark spots appeared in the TLC plate indicating the presence of carbohydrate moiety in the separated compounds. Dark brown color developed following exposure of the TLC plate to the iodine fume, confirmed the presence of lipid moieties in the separated compounds. The results obtained are found to be consistent with the previous reports [8, 13, 28]. This suggested the production of glycolipid in the culture medium supplemented with biodiesel derived waste raw glycerol as the carbon substrate. The glycolipidic nature of the isolated biosurfactant was further confirmed by Fourier transform infra red spectroscopy (FTIR) spectra as shown in Fig 2a. The important characteristic band at 3396.98 cm^{-1} indicates the presence O-H stretching vibrations of hydroxyl groups. Adsorption at 2955.99 cm^{-1} is due to the C-H stretching vibration of $-CH_2$ and $-CH_3$ groups. The adsorption peak at 1719.34 and 1639.16 cm^{-1} point towards the C=O stretching vibrations of the carbonyl groups in -COOH. The presence of ester carbonyl group was also confirmed from the band at 1215.89 cm^{-1} which corresponds to –C=O deformation vibrations, although other groups are also absorbed in this region. Another peak around 1300-1000

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cm^{-1} is due to the C-O deformation vibrations of the bond between the carbon atoms and the hydroxyl groups in the rhamnose ring. Weak bands of –NH/-C=O combination of the amide II bands observed at 1542.26 cm^{-1}, indicates the presence of protein-related compounds in the isolated biosurfactant. The appearance of such additional bands could be the result from the contamination of polypeptides from cell debris during the extraction process. These results confirmed the glycolipid nature of the biosurfactant as reported by other workers[11, 23]. The study of the mass spectrum of the isolated biosurfactant was carried out with the confirmation of the pseudo-molecular ions and their fragmentation pattern (Fig 2b). These data clearly show the synthesis of the rhamnolipids by *P. aeruginosa* JBK1 growing in waste raw glycerol. The pseudo-molecular ions with m/z 505 and was found to be the most abundant in the analyzed samples, indicating a higher degree of its biosynthesis. The variation in the production of different congers of rhamnolipid such as Rha-C_{10}-C_{10}, Rha-$C_{8:2}$, Rha-Rha-C_8-$C_{12:1}$ and Rha-Rha-C_{10}-$C_{14:1}$, m/z 505, 304, 644, 710 respectively, as compared with the other reported strains of *Pseudomonas aeruginosa* may be due to the culture conditions, carbon and nitrogen sources as well as their ratio[28].

Physical characterization

The surface tension of the culture medium got reduced to 33.7 from 68.4 mN/m within 132h of incubation period (Fig. 3) and it became constant after 60h, indicating attainment of critical micelle concentration (CMC) by the biosurfactant thereafter the biosurfactant released by the bacterial cells starts accumulating in the culture medium in the micellear form[26, 29]. Reduction in the interfacial tension (IFT) between two different media is an important character of a biosurfactant molecule. The minimum IFT of the cell free culture supernatant against diesel and kerosene was found to be 4.7 and 3.4, respectively. The reduction in surface tension is directly proportional to the biosurfactant concentration of the solution until it attains CMC[25]. Changes in the surface tension

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with the increase in biosurfactant concentration are presented in Fig.3. The CMC of the isolated biosurfactant was approximately 540 mg/l. Previous reports indicate that the intrinsic variability of rhamnolipid accumulation, difference in number, composition and proportion of homologues, length and branching of the aliphatic chain, presence of unsaturated bonds and number of alkyl groups effect the CMC values of a biosurfactant[8]. After 10 times dilution (CMD^{-1}), the surface tension of water increased upto 34.3 mN/m. However, at 100 times dilution (CMD^{-2}) the surface tension was 39.8 mN/m indicating an increase in the surface tension of the water suggesting the presence of lesser quantity of biosurfactant molecules in the aqueous system. The cell free culture supernatant produced stable foam showing a foaming index (F_{24}%) of 39%. The foam produced during the experiment was relatively stable upto 24 h, indicating its possible application in coal and mineral froth-flotation processes as a frothing and co-frothing agent. The cell free culture supernatant shows appreciable emulsification indices (E_{24}%) with all the hydrocarbons tested especially against kerosene (Fig. 4a). The emulsion formed in the presence of surfactant present in the aqueous system gets stabilized by reducing the interfacial tension and coalescence rate[30]. The emulsions were significantly stable upto 24 h and even upto 30 days indicating the formation of stable O-W (water-oil) type emulsion which might delay the coalescence of oil droplets. Lesser emulsification of some hydrocarbons might be due to the inability of the biosurfactant to stabilize the microscopic droplets of the hydrocarbon in the aqueous phase. The percentage hydrophobicity of bacterial cell was much higher when grown in waste residual glycerol than that of glucose and pure glycerol as shown in Fig.4b. As compared to the exponential phase, the cell surface hydrophobicity was much higher towards the stationary phase of growth suggesting that the accumulated biosurfactant in the culture medium alters the bacterial outer membrane components. Biosurfactant at higher concentration causes a reduction in the total bacterial lipopolysaccharide

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(LPS) content. The alterations further enhance the hydrophobicity of the bacterial cell surfaces towards the hydrophobic substrates like n-hexadecane[4]. Increase in the cell surface hydrophobicity enhances the attachment of cell to the hydrocarbon thereby enhancing degradation process[31]. During the bioremediation of crude oil, bacteria has to come in close physical contact with the pollutant as because during the initial stage of hydrocarbon degradation, the molecular oxygen is introduced into the degradable molecules by a membrane bound oxygenase enzyme[32].

Suitability in bioremediation process

Environmental factors such as pH, salinity and temperature affect biosurfactant's activity and stability. Hence, these are necessary parameters to be studied when considering specific applications of biosurfactants[26]. Studies on the reduction of surface tension of the culture supernatant containing the biosurfactant showed its significant stability over a wide pH range between 5 to 10 (Fig.5 A). The E_{24}% of the biosurfactant in the culture supernatant was found to be stable over a wide pH range of 4–10, also a considerable stability in its activity was observed beyond pH 11. However, maximum surface activity was observed at pH 7 to 8. Increase in the pH from 5 to 8 caused an increased in the negative charge on the polar head of the rhamnolipid molecule (pK_a 5.6) that enhancing its solubility in water. However, below pH 3 surface activity decreases due to the protonation of rhamnolipid molecules which causes their precipitation. The biosurfactant showed stability in its surface activity after incubation for 1h at temperatures ranging from 4-100˚C. The biosurfactant remained effective even after autoclaving at 121˚C for 30 min. Such thermal stability of biosurfactant indicates its utility in those industries where wet sterilization is of principal importance[8]. Generally, increase in temperature decreases the CMC of some non-ionic surfactants, but increases solubility of ionic surfactants. The E_{24}% against kerosene was quite stable at all the tested temperatures (Fig. 5 B) indicating the thermal stability of culture supernatant

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that broadens the scope of its applicability in microbial enhanced oil recovery (MEOR). The biosurfactant retained its surface activity by reducing the surface tension up to a concentration of 7% NaCl (Fig.5 C), and its E_{24}% against kerosene remained unchanged over the tested salt concentrations. On the other hand chemical surfactants are deactivated by 2-3% salt concentrations[33]. It has been reported that the presence of electrolyte in the solution directly affects the carboxlyate groups of rhamnolipid. At alkaline pH, the interface between the solution/air has a net negative charge due to the accumulation of ionized carboxylic acid groups of rhamnolipid molecules that have strong intermolecular repulsive electrostatic forces. When NaCl is added, the negative charge of carboxylic acid groups of rhamnolipid molecules is shielded by the Na^+ ions in the electrical double layer that leads to the formation of a close-packed monolayer. Thus, the formation of Na^+-rhamnolipid complex decreases surface tension values[8]. The stability of culture supernatant against higher pH and salinity suggests its applicability in bioremediation of marine environment and in industries (concerned with emulsions) where high salinities and pH prevail. Findings suggest that environmental factors play a vital role in the bioremediation of soils contaminated with the petroleum hydrocarbons.

Oil removal process

The released crude oil from the sand pack column was quantified and the data are shown in Table 1a. At the room temperature (RT) the culture supernatant could recover 8.2 % oil from the crude oil saturated sand pack column, 9.7 % at 50˚C, 10.2 % at 70˚C and 10.8 % at 90˚C. In the control having distilled water only, lesser amount of oil was recovered (1.1 %). Biosurfactant lowers both the surface tension and interfacial tension between the crude oil-water-sand and make them mobile in the sand pack column during the passage of the biosurfactant solution at higher temperatures. Results indicate that exposure of biosurfactant to higher temperatures between 70-

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90˚C causes a higher recovery of crude oil from the saturated sand pack column that shows the stability of biosurfactant in the recovery process at higher temperatures. These findings are in agreement with the previous reports[5, 4]. Further, addition of fresh culture supernatant could not increase the crude oil recovery from the saturated sand pack column specifying that biosurfactant is mainly responsible for the mobilization of crude oil from the column which indicated its significant role in surfactant enhanced oil recovery. The capability of the aqueous biosurfactant solution to remove crude oil from contaminated sand was investigated and the results are shown in Table 1b. The maximum removal of crude oil by the biosurfactants was attained within their critical micelle concentration (CMC), showing a total removal upto 83.7%. However, increase in the biosurfactant concentration beyond the CMC could not enhance further removal of crude oil from the contaminated sand. Concentration of the surfactant below the CMC level reduces the surface and interfacial tension between air/water, oil/water and sand/water systems. With the reduction in the interfacial tension between the crude oil and the sand particles, the capillary force that holds them together in the sand-oil mixture gets reduced. Such reduction in the tension further increases the contact angle between the oil and soil particles and changes the wettability nature of the system. This results in the mobilization of the crude oil from the sand-oil mixture into the aqueous solution. Such effect is directly related to the biosurfactant concentration in the solution until it reaches the CMC, the concentration at which the surfactant molecules start to form micelles and show the lowest tensional force. The control with only distilled water was able to remove only 24.8 % of crude oil from the contaminated sand. Efficient washing off of crude oil from the contaminated sand could be achieved even with synthetic surfactant (SDS); however it has been reported that synthetic surfactants are more recalcitrant than the petroleum hydrocarbons and potentially toxic to

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most of the environmental components[31]. Hence use of biosurfactant seems to be more advantageous.

Antimicrobial properties

The antimicrobial activity of the isolated biosurfactant was determined against a variety of bacterial and fungal strains and results are shown in Table 2. The zone of inhibition ranges from 7 to 17 mm indicating the potential antimicrobial property of the biosurfactant. The purified concentrated extract of ethyl acetate fraction of the biosurfactant was much effective as compared to the culture broth. The antimicrobial property was much pronounced towards the tested bacterial strain in comparison to the fungal strain. Results clearly indicated the effectiveness of biosurfactant towards both Gram positive and Gram negative bacterial strains. To produce biosurfactant with an antimicrobial property could be a survival strategy allowing the bacteria to flourish ahead of other organisms in the environment[25]. It has been previously reported that cytoplasmic membranes are the primary target sites of cellular damage by the surfactant. Another possibility of their action is that, the shorter acyl tails could get inserted into the cell membrane and cause a disruption between the cytoskeletal element and plasma membrane, leading to the shifting of the membrane from the cytoplasmic contents. Biosurfactant also causes inhibition of microbial growth by affecting the release of intracellular materials or by inhibiting respiration suggesting their effective application in the bioaugmentation process in hydrocarbon contaminated sites[25]. Such type of biosurfactant could also be used in combination with the other antimicrobial compounds to improve their antimicrobial activity against several harmful bacteria.

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4. Conclusion

The objective of the present study is the conversion of biodiesel-derived waste crude glycerol into an important valuable product through a biotechnological process. Based on results obtained, it is confirmed that biodiesel-derived waste glycerol could be used as a renewable low-cost substrate and acceptable from the perspective of biotransformation in converting waste byproduct into valuable product. The biosurfactant showed appreciable stability under extreme conditions of temperature, pH and stability. Due to such desirable physicochemical properties and significant antimicrobial activity, the biosurfactant produced by the bacterial strain could find their place in bioremediation technologies and also in many other industrial applications.

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Conflict of interest

The authors declare no conflict of interest exits.

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Research highlights

- *Biodiesel plant waste glycerol as low-cost substrate for biosurfactant production*
- *Highly stable oil-water emulsion with complex hydrocarbons (kerosene and xylene)*
- *Surface and emulsifying activity unaffected with high temperature, pH and salinity*
- *Culture supernatant is potential in bioremediation and MEOR processes*
- *Antimicrobial activity against a variety of bacteria and fungus*

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Captions for tables

Table 1a. Recovery of crude oil (%) from the sand pack column at different temperatures (room temperature, 50, 70 and 90˚C) after treatment with cell free culture broth

Table 1b. Removal of crude oil (%) from contaminated sand after washing with aqueous biosurfactant solutions

Table 2. Antimicrobial activity of biosurfactant produced by *Pseudomonas aeruginosa* JBK1 during growth in mineral salt medium supplemented with 3% waste raw glycerol

Captions for figures

Fig. 1. Growth, biosurfactant production, and surface activity profiles of *Pseudomonas aeruginosa* JBK1in mineral salt medium supplemented with 3% waste raw glycerol.

Fig. 2 (A) FTIR of the biosurfactant produced by *Pseudomonas aeruginosa* JBK1and (B) ESI mass spectrum of isolated biosurfactant produced by *Pseudomonas aeruginosa* JBK1 using 3% waste raw glycerol.

Fig. 3. Surface tension versus the concentration of biosurfactant produced by *Pseudomonas aeruginosa* JBK1 during growth in mineral salt medium supplemented with 3% waste raw glycerol.

Fig. 4. (A) Emulsifying activity (E_{24}%) of cell free culture supernatant of *Pseudomonas aeruginosa* JBK1 against different hydrocarbon and (B) Changes in cell surface hydrophobicity of *Pseudomonas aeruginosa* JBK1 during growth in mineral salt medium supplemented with different carbon substrates (glucose/pure glycerol/ waste raw glycerol).

Fig. 5. Effect of (A) pH, (B) temperature and (C) sodium chloride concentration on the surface activity and emulsifying property (E_{24}%) of cell free culture supernatant of *Pseudomonas aeruginosa* JBK1.

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Captions for supplementary materials

Supplementary figure S1: SEM micrographs of *Pseudomonas aeruginosa* strain JBK1.

Supplementary figure S2: Phylogenetic tree generated using NJ method showing the position of *Pseudomonas aeruginosa* strain JBK1 compared to other highly similar 16S rRNA sequence of various *Pseudomonas aeruginosa* strains.

Supplementary figure S3: Frequency of nucleotide distribution for *Pseudomonas aeruginosa* strain JBK1 and various strains of *Pseudomonas aeruginosa* strain having maximum similarity with *Pseudomonas aeruginosa* strain JBK1.

Supplementary Table 1. BLAST result of 16s rRNA of *Pseudomonas aeruginosa* strain JBK-1

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List of Tables

Table 1a. Recovery of crude oil (%) from the sand pack column at different temperatures (room temperature, 50, 70 and 90˚C) after treatment with cell free culture broth

S. No.	Temperature (˚C)	Crude oil released after		Enhanced oil recovery (%)
		First water flooding (%)	Second water flooding (%)	
1.	Room temperature	56.37±0.4	64.57±0.1	8.2
2.	50	57.35±0.7	67.05±0.9	9.7
3.	70	56.43±0.2	66.73±0.3	10.2
4.	90	56.31±0.5	67.11±0.6	10.8
5.	Control	56.28±0.7	57.38±0.8	1.1

Table 1b. Removal of crude oil (%) from contaminated sand after washing with aqueous biosurfactant solutions

S. No.	Treatment	Crude oil removed from sand (%)
1.	0.25% of biosurfactant solution	67.8±0.5
2.	0.5% of biosurfactant solution	80.7±0.5
3.	1.0% of biosurfactant solution	82.8±0.8
4.	2.0% of biosurfactant solution	81.4±0.4
5.	At critical micelle concentration (CMC)	83.7±0.9
6.	Cell free culture broth	77.4±0.5
7.	0.01% of sodium dodecyl sulphate (SDS)	65.7±0.3
8.	Distilled water	24.8± 0.6

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Table 2. Antimicrobial activity of biosurfactant produced by *Pseudomonas aeruginosa* JBK1 during growth in mineral salt medium supplemented with 3% waste raw glycerol

S. No.	Microorganism	Zone of inhibition produced (mm) by	
		Culture supernatant	Ethyl acetate extract
1.	*Escherichia coli* (MG 1655)	10±0.3	14±0.4
2.	*Escherichia coli* (MTCC 40)	n.d	09±0.7
3.	*Bacillus subtilis* (MTCC 441)	08±0.5	12±0.5
4.	*Bacillus subtilis* (MTCC 121)	08±0.2	11±0.9
5.	*Staphylococcus aureus* (MTCC 737)	09±0.4	17±0.3
6.	*Staphylococcus aureus* (MTCC 3160)	n.d	10±0.7
7.	*Klebsilla pneumoniae* (MTCC 618)	07±0.3	12±0.4
8.	*Pseudomonas aeruginosa* (MTCC 7815)	n.d	09±0.1
9.	*Pseudomonas diminuta* (from AAU)	08±0.3	10±0.5
10.	*Candida ablicans* (MTCC 227)	10±0.8	12±0.8
11.	*Fusarium oxysporium*(MTCC 284)	n.d	09±0.5
12.	*Aspergillus niger* (from AAU)	n.d	07±0.7
13.	*Colleototricum capaci* (from AAU)	n.d	08±0.3
14.	*Alternaria solani* (from AAU)	09±0.5	11±0.3

NB: from AAU, Assam Agricultural University, Jorhat, Assam, India

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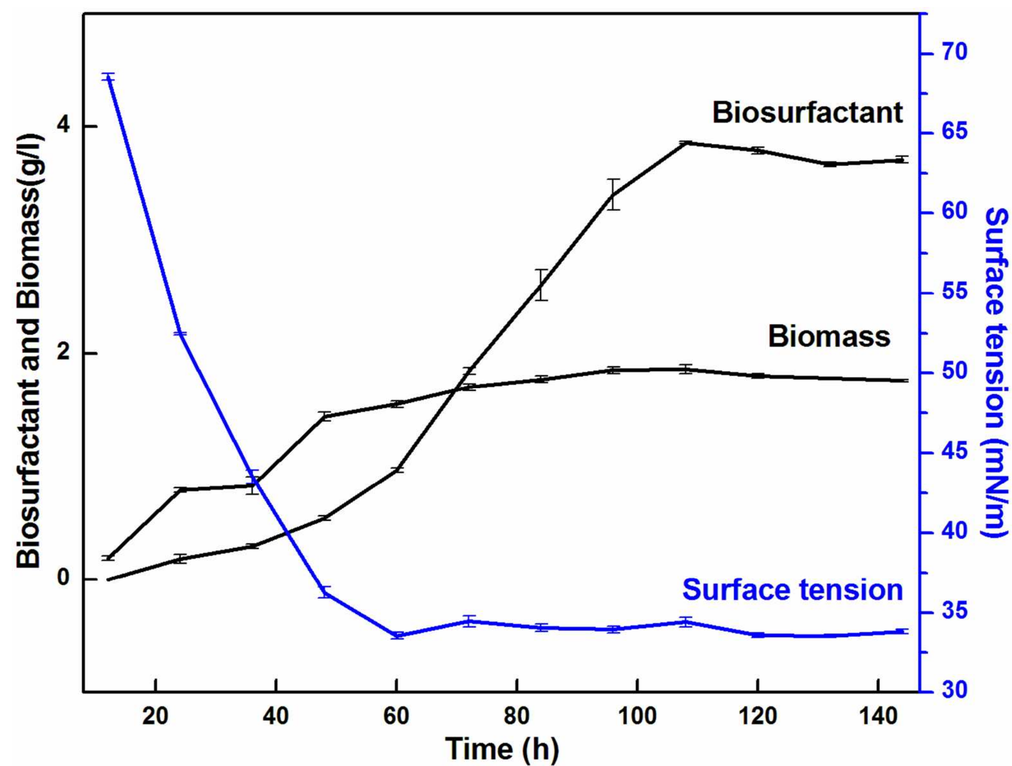

Fig. 1. Growth, biosurfactant production, and surface activity profiles of Pseudomonas aeruginosa JBK1in mineral salt medium supplemented with 3% waste raw glycerol.
227x173mm (150 x 150 DPI)

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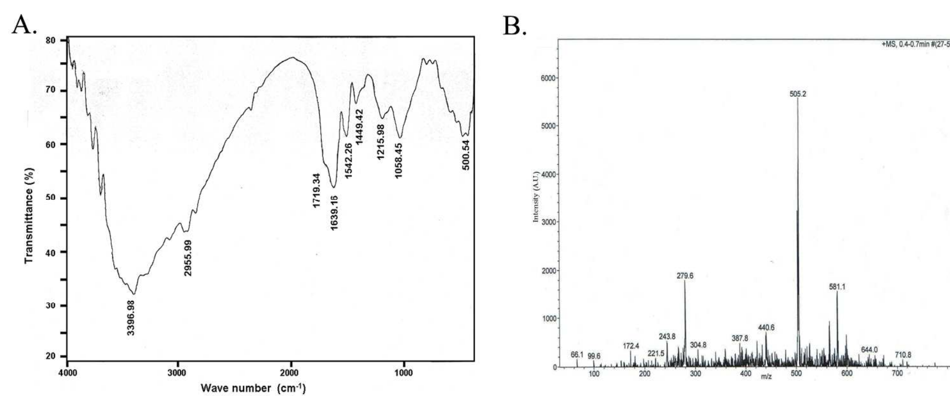

Fig. 2 (A) FTIR of the biosurfactant produced by Pseudomonas aeruginosa JBK1and (B) ESI mass spectrum of isolated biosurfactant produced by Pseudomonas aeruginosa JBK1 using 3% waste raw glycerol.
203x83mm (300 x 300 DPI)

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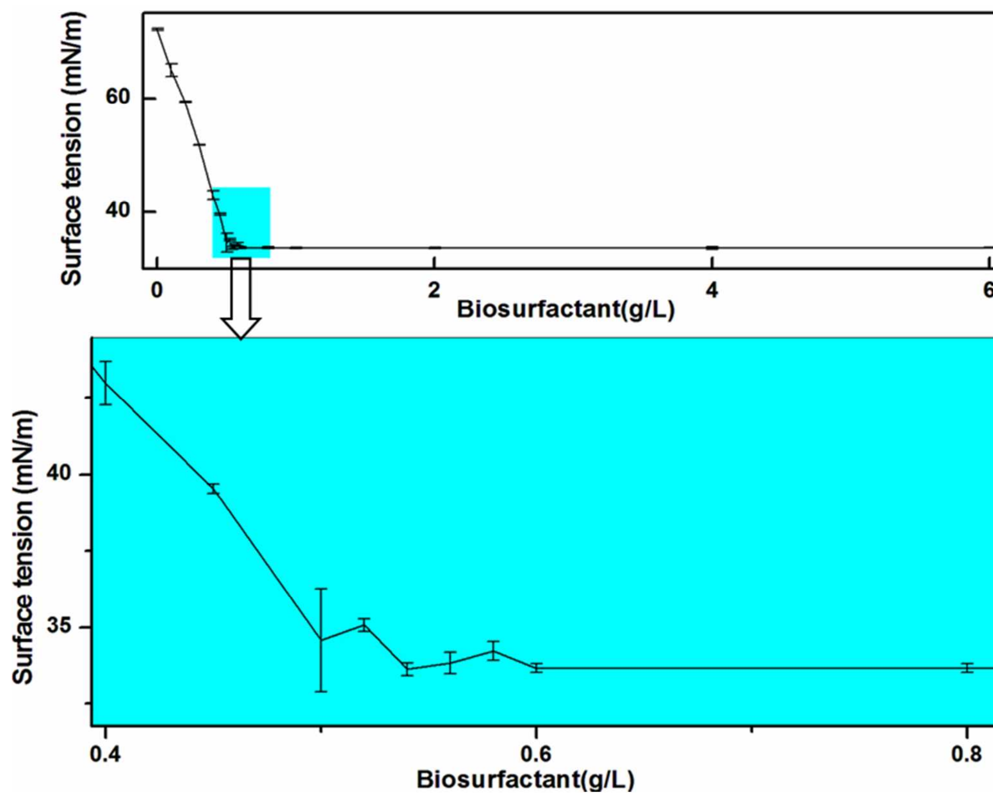

Fig. 3. Surface tension versus the concentration of biosurfactant produced by Pseudomonas aeruginosa JBK1 during growth in mineral salt medium supplemented with 3% waste raw glycerol.
216x170mm (96 x 96 DPI)

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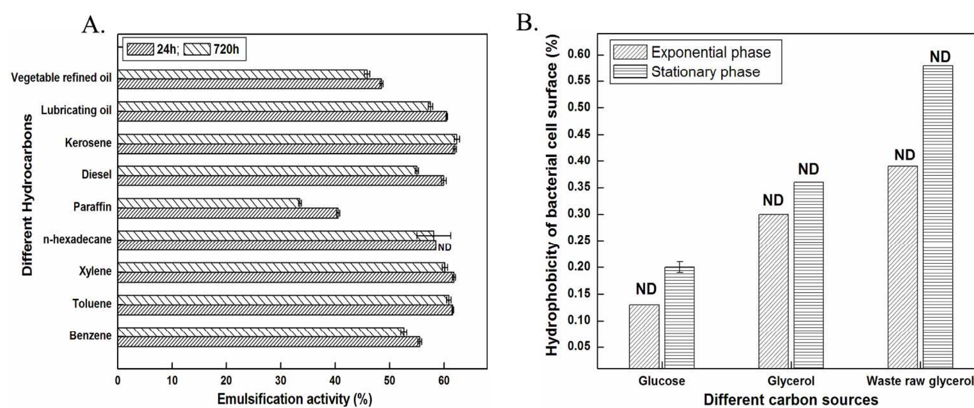

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222x96mm (300 x 300 DPI)

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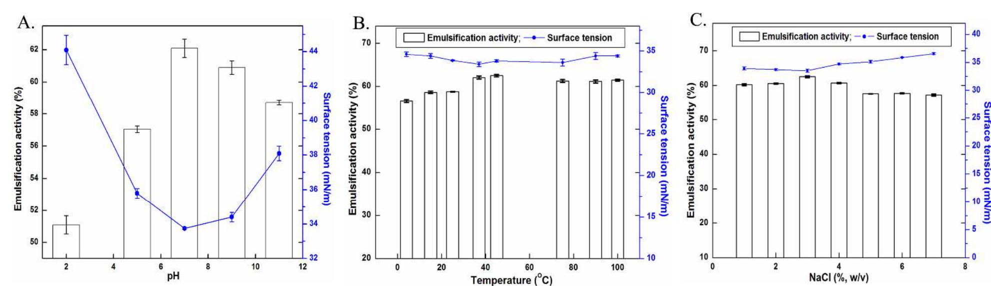

Fig. 5. Effect of (A) pH, (B) temperature and (C) sodium chloride concentration on the surface activity and emulsifying property (E24%) of cell free culture supernatant of Pseudomonas aeruginosa JBK1.
300x92mm (300 x 300 DPI)

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