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PRODUCTION OF BIOSURFACTANT FROM *RAOULTELLA ORNITHINOLYTICA* AND EVALUATION OF ITS DE-STAINING AND DE-GREASING PROPERTIES

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ABSTRACT

Biosurfactants are surface-active biomolecules that show large number of applications in pharmaceutical, food, cosmetic, pesticide and oil industries, and an immense potential as an environment protective agent. In the current study, various swabs were collected from grimy surfaces, and a sludge sample was obtained from Vashi creek, Navi Mumbai. They were screened for presence of biosurfactant producers based on visual foam production in growth media and oil spreading assay. Among the 23 isolates obtained, *Raoultella ornithinolytica* showed maximum oil spreading potential, and surface tension reduction to 67dynes/cm. Different hydrocarbon sources like petrol, used engine oil and liquid light paraffin were used as a nutrient source for biosurfactant production. The degraded hydrocarbons were detected using standard chemical assays and identified as aldehydes, ketones and carboxylic acids. The biosurfactant also showed effective bio-degreasing and bio-destaining properties which was evaluated using the plastic surface coated with paraffin, and cotton fabrics stained with sauces, gravy, mud, lipstick, grease and methyl red respectively.

Keywords: Biosurfactant, *Raoultella ornithinolytica*, Bio-degreasing, Bio-destaining

INTRODUCTION

Surfactants are amphiphilic molecules composed of hydrophobic tails and polar heads. Since the charges on hydrophobic tails are often similar, the surfactants are classified as anionic, cationic, non-ionic or zwitterionic, based on the charge on the polar heads **[1]**. They are surface-active agents that show characteristic properties like reduction of the surface or interfacial tension between a liquid-solid, gas-liquid or liquid-liquid interface. Hence they find widespread application as detergents, wetting agents, emulsifiers, foaming agents and dispersants **[2]**.

Biosurfactants are compounds with above properties and applications which are produced by a variety of microorganisms. However, as opposed to the synthetic nature of chemically produced surfactants that accumulate in the environment on inappropriate disposal, biosurfactants are biodegradable and non-toxic. Hence they are also known as "Green Surfactants" **[3]**. The production of biosurfactant is generally associated with the utilization of hydrocarbons and their metabolism by the microbial community. Microbial genera like *Acinetobacter, Arthrobacter, Pseudomonas, Halomonas, Bacillus, Rhodococcus, Enterobacter*, and some yeast species have been reported to produce biosurfactants **[4]**.

The influence of biosurfactants exerted on the interface of aqueous solutions and hydrocarbon mixtures allow formation of micro-emulsions. This occurs as a result of micelle formation that solubilizes (by entrapping) hydrocarbons in water, or water in hydrocarbon mixtures **[5]**. The biosurfactants show superior properties in terms of their Critical Micelle Concentration (CMC) when compared to chemical surfactant. The effectiveness of biosurfactants in small concentrations thus makes it more suitable for industrial applications **[6]**. It can effectively replace the chemical surfactants for processes like lubrication, wetting, softening, fixing dyes, making emulsions, foaming as well as its prevention in food, biomedical and pharmaceutical industries. It also shows immense potential for bioremediation of sites contaminated with organic and inorganic contaminants.

Different types of biosurfactants like glycolipids, lipopeptides and lipoproteins are produced by microorganisms. These molecules vary greatly in their chemical composition **[7]**. Glycolipids and lipopeptides have shown promising potential as bioremediation agent, and in medical applications. Hence there is an increased demand and scope for these compounds, especially in these fields. Organisms identified to be producers of rhamnolipids (sub-class of glycolipids) and suitable for bioremediation include *Pseudomonas aeruginosa*, *Pseudomonas putida* and *Renibacterium salmoninarum*. Biosurfactant producing *Pseudomonas chlororaphis* and *Bacillus subtilis* have been used as a bio-control and anti-fungal agent respectively. Another subclass of glycolipids i.e.,sophorolipids produced by fungal members like *Candida bombicola* and *Candidaapicola* have been employed in emulsification, microbial enhanced oil recovery and alkane dissimilation. Similarly, Trehalose lipids (sub-class of glycolipids) produced by *Tsukamurella* sp. and *Arthrobacter* sp. have shown anti-microbial properties, and *Rhodococcus* sp. has been utilized for bioremediation. Mannosylerythritol lipids (sub-class of glycolipids) produced by *Candida antartica* is identified as aneuroreceptor antagonist and an antimicrobial agent, whereas *Kurtzmanomyces* sp. hasshown variousbiomedical applications **[8]**. Among lipopeptides, Surfactin- produced by *Bacillus subtilis*show considerable antimicrobial and biomedical applications whereasLichenysin synthesized by *B. licheniformis*has been employed as a haemolytic and chelating agent **[9]**.

In the current study, *Raoultellaornithinolytica* was isolated from Vashi creek in Navi Mumbai, and evaluated for its biosurfactant producing ability. The main aim of our study was to determine the efficacy of the biosurfactant as a potential bio-degreaser and stain remover. A degreaser is a cleaning agent that effectively dissolves grease and oil from hard surfaces. They are widely used in industries, manufacturing setups, agriculture, hospitals, schools and households. The degreasers are either water-based or solvent-based **[10]**. Stain removers also have wide applications in textiles as they are an integral part of the detergents. The chemical degreasers and stain removers are potentially hazardous and have a strong impact on the environment. A green alternative of biosurfactant as degreaser and stain remover can hence become a sustainable solution.

MATERIALS AND METHODS

Screening for biosurfactant producers

Different samples were collected for screening of biosurfactant producers in our study. They included a sludge sample collected from Vashi creek and swabs from grimy surfaces like kitchen sink, utensil cleaning sponge, washbasin, oil cans, oil lamp (diya), chimney filter and microwave oven. Enrichment of biosurfactant producers from the collected sludge was carried out, in duplicates, by inoculating 10g sample in the Mineral Salts (MS) and Bushnell Haas medium **[8, 9]**. The organisms from the enriched sample were isolated on MS agar plates wherein, sterile liquid paraffin oil was provided as a hydrocarbon source, by soaking on sterile Whatman filter paper placed on the inner surface of the petri plate lids **[10]**. The plates were incubated in inverted position, allowing only the vapour phase hydrocarbons to reach the inoculated agar surface.

Identification of potential isolates

The preliminary identification of the

isolates was done based on morphology and gram nature. The most promising isolate was identified by automated microbial identification system [VITEK 2, Biomerieux].

Biosurfactant production and extraction

The production of biosurfactant was carried out in Nutrient Broth (NB) and MS medium, in duplicates, using 18h old cultures **[11]**. The hydrocarbon sources used in our study were 1% petrol, 2% engine oil, 2% sunflower food oil and 2% liquid light paraffin. The incubation was effectuated at Room Temperature (RT, ~28°C) for 7 days on the orbital shaker (150rpm) **[12]**. The culture broth was subjected to centrifugation at 10,000rpm for 30min at 4°C to obtain a crude biosurfactant. The crude biosurfactant was stored at 4°C until further use.

Evaluation of biosurfactant activity

The biosurfactant obtained in our study was evaluated based on the following methods.

Oil spreading assay

Oil spreading assay was performed in a petri dish filled with 10mL distilled water holding a drop of engine oil at the centre, on the surface of the plate. A drop of crude biosurfactant was carefully placed on the engine oil sample and allowed to stand until oil spreading activity could be recorded. The plates were placed on a standard graph paper to measure the diameter of the clear zone formed due to surface tension reduction. A control was also set up by replacing distilled water with biosurfactant. In addition, *P. aeruginosa* MTCC 2297 was used as a standard strain known to produce biosurfactant.

Surface tension reduction

The surface tension of the cell-free supernatant was measured using the Du Nouy Ring Principle with the help of atensiometer [Automatic surface Tensiometer- Model G.S.I. (WB) Sl. No. 182] using the method described by Rahman *et al*. **[13]**.

Hydrocarbon degradation analysis

On degradation, hydrocarbons form products like aldehydes, ketones, and carboxylic acids. Hence their presence in the production medium is an indication of the ability of the organism to metabolize the hydrocarbons. The formation of these products was analyzed using qualitative assays using standard methods. For these purpose, three sets of production medium containing paraffin oil, engine oil and petrol, respectively, were inoculated with the potential isolate and incubated at RT for 7 days. After incubation, the broths were tested for hydrocarbon breakdown products **[14]**. The tests were carried out sequentially to perform the preliminary tests before the confirmatory tests. Initially, the 2, 4-dinitrophenylhydrazine test was performed that indicates the presence of either aldehydes or ketones. Later, Tollen's reagent test and Sodium nitroprusside test was used to confirm the presence of aldehydes and ketones respectively.

Applications of biosurfactant

The biosurfactant produced was tested for its application as a bio-degreaser and stain remover.

Formulation and testing of biosurfactant as a bio-degreaser

Two bio-degreasers were formulated using the hydrocarbon-degrading microbial culture (1mL) and a biocatalyst solution **[15]**. The biocatalyst solution was designed to contain nitrogen source (0.05g), phosphate source (0.25g), chlorine absorbing salt (0.1g), biosurfactant (10mL) and distilled water (40mL). In the two formulations, urea and ammonium sulphate were used as nitrogen sources, and sodium hydrogen orthophosphate and calcium phosphate as phosphate sources. The chlorine absorbing salt used was sodium thiosulphate and the pH was adjusted to 7.

The prepared formulations were checked on the oiled plastic surface (10cmx 3cm). The selected plastic surfaces, after oiling, were placed in separate beakers to which, 50mL of the prepared formulations were added. Produced biosurfactant was added for oil removal from the plastic surface and the culture suspension was added for degradation of the removed oil. Chlorine absorbing salt, sodium thiosulphate was added in the formulation for absorbing free chlorine from the surrounding, as it interferes with microbial growth which is significant when applied in situ **[16]**. The formulation, after incubation for a week, was assessed for their de-greasing and degrading ability. For this purpose, the above mentioned tests, for detecting the presence of aldehydes, ketones and carboxylic acids, were performed.

Testing of biosurfactant as a stain remover

To check whether the biosurfactant has stain-removing property like detergents, cotton cloth pieces were stained with different stains and allowed to dry for 18h. The stain diameter was kept as 1cm. The stains were treated with 0.1mL of crude biosurfactant for 5min. The washing was carried out using 10mL of distilled water. The control set for every stain was set with an equal quantity of distilled water under the same incubation conditions. The cloth samples were stained with red chilli powder, turmeric, methyl red dye, mud, curry, permanent marker, ink, used engine oil and pasta sauce.

RESULTS

Screening and identification of biosurfactant producer

In our study, 23 isolates were obtained from enriched samples. All isolates were studied for their morphological characteristics including gram nature and colony characteristics. Also, they were checked for their ability to produce biosurfactant. Among these isolates, I-1 was found to be the most promising biosurfactant producer. Hence it was used for further studies. Preliminary tests identified this isolate as gram-negative short rod that produced a circular, white and opaque colony of 1mm size and exhibited a moist consistency. It was identified as *Raoultella ornithinolytica* by Automated Microbial Identification System [VITEK 2, bioMerieux].

Biosurfactant production and extraction

Biosurfactant production was examined by observing the formation of foam in the production medium in 7 days. Fourteen isolates demonstrated foam formation in the production medium. The crude biosurfactant was extracted by centrifugation at 10,000rpm for 30min at 4°C.

Evaluation of biosurfactant activity

The 14 bacterial isolates showing visual foam production **(Figure 1)** were chosen for performing the oil spreading assay. **Table 1** indicates the foaming properties of screened isolates and **Table 2** represents the reduction in surface tension observed.

Hydrocarbon degradation analysis

The production broth with 3 different types of hydrocarbons, in which the culture of *R.ornithinolytica* was grown, was verified for the tests for aldehydes/ketones and carboxylic acid. The results are shown in **Table 3**.

Biosurfactant as a bio-degreaser

The set-up for bio-degreasing analysis is illustrated in **Figure 2**. Both formulations were tested on paraffin greased plastic surfaces. The residual water was collected after a week. The degreased surfaces suggested the accumulation of oil in water. To test the efficacy of the formulation concerning degradation following degreasing, the tests mentioned above for aldehydes, ketones and carboxylic acid were performed. Both the formulations were found to contain aldehydes, ketones as well as carboxylic acids.

Biosurfactant as a stain remover

The stains were removed efficiently using the crude biosurfactant obtained from *R.ornithinolytica* **(Figure 3)**. Efficient removal of most of the stains was observed. Distilled water was used as a control. The results are reported in **Table 4**.

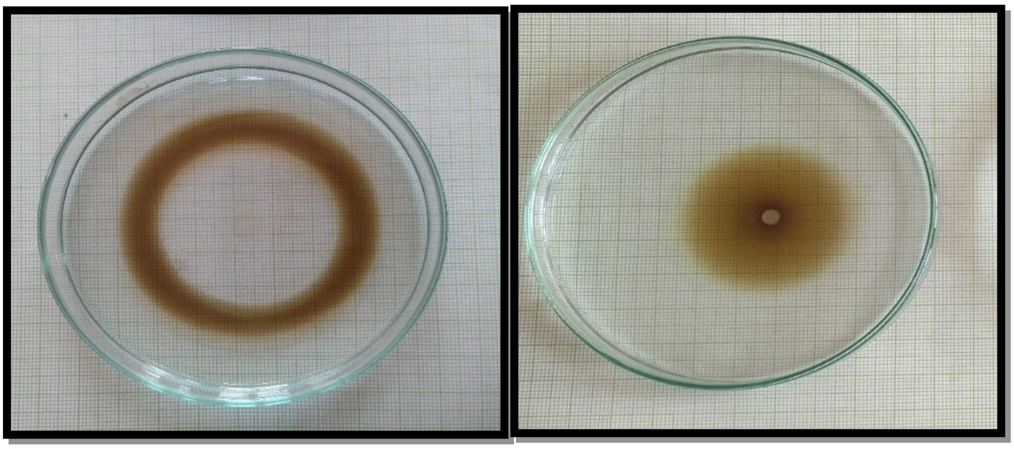

Figure 1: Oil spreading assay- Test (right) and Control (left)

Table 1: Result of oil spreading assay

Isolate No.	I-1	I-2	I-3	I-4	I-5	I-6	I-7	I-8	I-9	I-10	I-11	I-12	I-13	I-14	*Pseudomonas aeruginosa MTCC* 2297
Diameter of Oil Spreading (in mm)	27	22	19	13	14	07	03	12	18	05	21	15	14	05	14

Table 2: Result of surface tension analysis

Sr. No.	Particulars	Tensiometer reading [dynes/cm]
1	Control (Distilled water)	71
2	Crude biosurfactant of I-1	67

Table 3: Results of hydrocarbon degradation analysis

No.	Carbon source	Name of test	Observations	Inference
1	Paraffin oil	2, 4- Dinitrophenylhydrazine test	Orange precipitate	Aldehyde or ketone is present
		Tollen's test	Silver-gray precipitate	Aldehyde is present
		Sodium nitroprusside test	Colour change to black	ketone is absent
		Sodium bicarbonate test	Strong effervescence	Carboxylic acid may be present
2	Engine oil	2, 4- Dinitrophenylhydrazine test	Orange precipitate	Aldehyde or ketone is present
		Tollen's test	No precipitate	Aldehyde is absent
		Sodium nitroprusside test	Colour change to black	ketone is absent
		Sodium bicarbonate test	Strong effervescence + Solid formation	Carboxylic acid is present
3	Petrol	2, 4- Dinitrophenylhydrazine test	Orange precipitate	Aldehyde or ketone is present
		Tollen's test	Silver-gray precipitate	Aldehyde is present
		Sodium nitroprusside test	Colour change to orange red	Ketone is present
		Sodium bicarbonate test	Strong effervescence	Carboxylic acid may be present

Figure 2: Bio-degreasing analysis set-up

Table 4: Result of biosurfactant as stain remover

No.	Stain	Test	Control
1	Gravy	Efficient stain removal	Slight discoloration
2	Cosmetic Highlighter	Efficient stain removal	Slight discoloration
3	Grease	Efficient stain removal	No change
4	Lipstick	Slight discoloration	No change
5	Methyl red dye	Efficient stain removal	Slight discoloration
6	Pasta sauce	Efficient stain removal	No change
7	Mud	Efficient stain removal	No change
8	Permanent marker ink	No change	No change
9	Red chili powder	Efficient stain removal	Efficient stain removal
10	Turmeric	Slight discoloration	Slight discoloration

Figure 3: Observations of de-staining technique

DISCUSSION

In our study, a potential biosurfactant producer, *R. ornithinolytica* was isolated from Vashi creek, Navi Mumbai, and its activity was compared with *P. aeruginosa* MTCC 2297. The biosurfactant production was evident from visual froth formation, oil spreading assay and surface tension reduction.

The ability of the isolate to degrade hydrocarbons was confirmed by the presence of aldehydes, ketones and carboxylic acids by standard chemical tests after degradation. The comprehension of the degradation products is of significance since it provides an idea regarding the prevalence of by-products on environmental application of biosurfactants. A significant reduction in surface tension was observed in our study from to 67mN/m. A similar study indicated the reduction of surface tension of fermentation broth from 73 to 34.2mN/m by *P. aeruginosa* MTCC 2297 and from 76 to 29mN/m by *P. fluorescens* **[17]**.

The bio-degreaser formulations used in our study are also of environmental importance because they not only incorporate the use of produced biosurfactant but also the viable organisms. The primal function of the biosurfactant used in industries is the removal of oils from variable surfaces. This ensures cleaning although, it may lead to deposition of the residual water contaminated with all sorts of oils in the surrounding environment. The use of viable organisms in these formulations is for degradation of separated oil into much simpler compounds before their final release in the environment.

The degreasers were tested on plastic surfaces. *R. ornithinolytica* was also used for checking the crude biosurfactant's capability to remove different stains. The propitious biosurfactant, obtained in our study, not only removes most stains efficiently but also curbed the stain odour. Thus, it can be used in detergent formulations on a commercial scale. Smyth and Lambert **[15]** patented a biodegradable degreaser composition which included alkali metal hydroxide (~2%), corrosion inhibitor (~5%), biodegradable terpene solvent (~15%), biodegradable surfactant (D-limonine, ~30%), biodegradable co-solvent/co-surfactant (butoxyethanol, ~15%) and a balance consisting of water. Another patent of Guinn *et al*. **[15]** introduced the use of 'hydrocarbon-ingesting microbes' along with a biocatalyst solution. The microbial consortium for hydrocarbon ingestion included *Pseudomonas* sp. and *Bacillus* sp. whereas the biocatalyst solution comprised of a non-ionic chemical surfactant (nonylphenol PEG), chlorine absorbing salt ($Na_2S_2O_3.5H_2O$), microbial nutrition (urea and sodium hexaphosphate) and water.

CONCLUSION

Based on above results, it is evident that *R. ornithinolytica* is an efficient biosurfactant producer and hydrocarbon degrader. It can further be tested for a horde of applications in the fields like medicine, bioremediation [in situ], textile industries, industrial cleaning and degreasing as well as microbial enhanced oil recovery.

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REFERENCES

[1] Som I, Bhatia K, Yasir M. Status of surfactants as penetration enhancers in transdermal drug delivery. Journal of Pharma and Bioallied Sciences. 2012; 4(1): 2-9.

[2] Deleu M, Paquot M. From renewable vegetables resources to microorganisms: New trends in surfactants. Computers Rendus Chimie. 2004; 7(6-7): 641-646.

[3] Marchut- Mikolajczyk O, Drozdzyński P, Pietrzyk D, Antczak T. Biosurfactant production and hydrocarbon degradation activity of endophytic bacteria isolated from *Chelidonium majus* L. Microbial Cell Factories. 2018; 17(1): 1-9.

[4] Fenibo EO, Ijoma GN, Ramganesh S, Chikere CB. Microbial surfactants: the next generation multifunctional biomolecules for diverse applications- a review. Microorganisms. 2019; 7: 581.

[5] Banat IM. Characterization of biosurfactants and their use in pollution removal - state of the art (Review). Acta Biotechnology. 1995; 15(3): 251-267.

[6] Desai JD, Banat IM. Microbial production of surfactants and their commercial potential. Microbiology and Molecular Biology Reviews. 1997; 61(1): 47-64.

[7] Santos DKF, Rufino RD, Luna JM, Santos VA, Sarubbo LA. Biosurfactants: multifunctional bio-molecules of the 21st Century. International Journal of Molecular Sciences2016; 17(3): 401.

[8] Reis RS, Pacheco GJ, Pereira AG, Freire DMG. Biosurfactants: production and applications. Biodegradation - Life of Science, Rolando Chamy and Francisca Rosenkranz, Intech Open, 2013. DOI: 10.5772/56144. Available from: https://www.intechopen.com/books/biodegradation-life-of-science/biosurfactants-production-and-applications.

[9] Udgire M, Shah N, Jadhav M.Enrichment, isolation and identification of hydrocarbon degrading bacteria. International Journal of Current Microbiology and Applied Sciences.2015; 4(6): 708-713.

[10] IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Lyon (FR): International Agency for Research on Cancer; 2014.

[11] Obuekwe CO. Bioremediation of crude oil pollution in the Kuwaiti desert: the role of adherent microorganisms. Environmental International. 1998; 24(8): 823-834.

[12] Kannan A, Gautam P. A quantitative study on the formation of *Pseudomonas aeruginosa* biofilm. SpringerPlus2015; 4(1): 379.

[13] Rahman KSM, Banat IM, Thahira J, Thayumanavan T, Lakshmana-perumalsamy P. Bioremediation of gasoline contaminated soil by a bacterial consortium amended with poultry litter, coir pith and rhamnolipid biosurfactant. Bio-resource Technology. 2002; 81(1): 25-32.

[14] Tahzibi A, Kamal F, MazaheriAssadi M. Improved production of rhamnolipids by a *Pseudomonas aeruginosa* mutant. Iranian Biomedical Journal. 2004; 8(1): 25-31.

[15] Guinn LL, Russel PEE, William L. Microbial Cleaner. US Patent Number: 5,364,789. Date of Patent: Nov. 15, 1994.

[16] Smyth TA, Lambert DR. Biodegradable liquid degreaser and parts cleaner composition. US Patent Number: 5,965,512. Date of Patent: Oct. 12, 1999.

[17] Parthasarthi R, SivaKumar PK. Enhancement effects of biosurfactant produced by *Pseudomonas aeruginosa* MTCC 2297 and *Pseudomonas fluorescens* on sugar cane bagasse composting. Journal of Eco-biotechnology. 2010; 2(3): 33-39.