International Journal of Science and Research (IJSR)
ISSN: 2319-7064
SJIF (2022): 7.942

Isolation and Characterization of Un-Veiling Potential of Bio-Surfactant Isolated from Oil Contaminated Region near Petrol Pump

Shivam Rathaur, Nabya Nehal*

Department of Biotechnology, Faculty of Engineering and Technology, Rama University, Kanpur, Uttar Pradesh-209217, India

nabya.nehal[at]gmail.com

Abstract: ***Biosurfactant are surface active surfactant with wide spectrum of applications in varied industrial sectors such as cosmetics, pharmaceuticals, biomedical, food industries, microbial enhanced oil recovery and agriculture. Thirty-three distinct microbial colonies were isolated form petrol pump, collected from Kanpur region, for screening most potent biosurfactant strains. Primary screening methods such as emulsification index (E_{24}), drop-collapse assay, oil-spreading method, oil displacement method, CTAB (Cetyl Tri Ammonium Bromide) were conducted. Biosurfactant were further characterized by using Thin Layer Chromatography (TLC). The present study is concentrated on the utility of bio surfactants for its antibiotic potential against microbes capable of deteriorating crop production. Since the antibiotic character was driven from the environmentally safe metabolite of oil contamination causing microbes. The results showed that out of three, two isolates SL1 and SL3 showed potential of bio-surfactant production and their relative antagonistic property against Campylobacter jejuni, Listeria monocytogenes and Staphylococcus aureus.***

Keywords: Oil contamination, Bio-surfactants, Antagonism, Dual confrontation assay

1.Introduction

Chemically produced surfactants are used in a variety of industries. Chemically manufactured surfactants are expensive and responsible to pollute the environment. Bio-surfactants (BS) are microorganism-produced natural compounds. BS offer characteristics that chemically manufactured surfactants lack, such as high surface activity, environmental friendliness, biodegradability and strong antimicrobial action at severe environmental conditions (temperatures, pH and salinity). Hence, biologically active surfactants are more likely preferred as compare chemically manufactured surfactants [1].

Pseudomonas aeruginosa, *Bacillus subtilis*, *Corynebacterium diphtheriae*, *Klebsiella pneumoniae*, *Acinetobacter* spp., *Archromobacter* spp., *Flavobacterium* spp., *Virgibacillus salarius* and *Proteobacterium* spp. found to create effective bio-surfactants. The majority of biosurfactants are made from immiscible hydrocarbons, while some are made from miscible hydrocarbons [2, 3]. Hydrophilic and hydrophobic moieties make up biosurfactants. Rhamnolipids synthesized by *Pseudomonas aeruginosa* and surfactinsynthesized by *Bacillus* spp. are most frequent bacterial biosurfactants. Glycolipids, particularly rhamnolipids, have been investigated the most completely of all these classes [4].

There has been no evidence of the emergence of a substantial number of biosurfactant-producing strains. Surface tension can be reduced from 72 mN/m to 25-30 mN/m using the most effective biosurfactant. As a result, screening and evaluating efficient biosurfactant producing strains is important from both a biological and economic standpoint [5].

Biosurfactants can be employed in a variety of applications, including industry and environmental restoration. They used in the petroleum sector to enhance the recovery rate, increased oil recovery and crude oil transportation. MEOR (microbial enhanced oil recovery) is expected to be one of the most effective strategies for improving oil recovery in the future [6]. These biosurfactants have a strong potential for bioremediation of oil-contaminated areas, heavy metal pollution, insecticides, washing and cleaning of oil reservoirs, and other applications due to their hydrocarbon breakdown capability and bio-degradative nature. Biosurfactants have this ability, which can be used to manage wastewater contamination [7].

Biosurfactants emulsification and surface-active qualities have allowed them to be used as detergents, foaming and wetting agents, flocculants, and other applications. Biosurfactants are used in shampoos, dental pastes, and other cosmetic items because of their great compatibility with human skin. Biosurfactants are also used in the food industry because they improve the quality of fat-based goods [8]. Some biosurfactants have antibacterial and medicinal effects. Because of their microbicidal action, biosurfactants are utilized as a biocontrol for plant disease prevention. Metal toxicity is also reduced by rhamnolipids. Biosurfactants can be made from a variety of low-cost and renewable materials. Biosurfactants, which are metabolites produced by microorganisms, have the potential to emulsify crude oil and reduce its viscosity, which is one of the processes for microbial enhanced oil recovery (MEOR). This study aimed to isolate biosurfactant producing bacteria, primary screening assays clarifies the potential biosurfactant producers and characterization techniques confirmed the class the biosurfactant [9].

2.Materials and Methods

Procurement and biochemical analysis of Bacterial isolates

In order to isolate biosurfactant producing micro-organisms, soil was collected from oil contaminated area near petrol pump [10]. Specific bacterial strains were isolated using serial dilution methods [11]. Nutrient agar media supplemented with hydrocarbon, biosurfactant producing bacteria were grown on the surface of media. In order to identify the isolates, biochemical tests were done followed by gram staining, indole test, Methyl red (MR), Voges-proskauer (VP), Simmon's citrate test (SCA) and catalase, urease and gelatin hydrolysis tests were done.

Strains were collected and inoculated into the production media. Composition of production media (g/L) [12] $(NH_4)_2SO_2$ (10.5), KCl (1.04), NaCl (1.09), KH_2PO_4 (3.9), K_2HPO_4 (4.4), $MgSO_{4.7}H_2O$ (0.5), $FeSO_4$ (0.0025), EDTA (1), Yeast Extract (0.5), Liquefied paraffin (5) at constant pH 7.2. The Erlenmeyer flask contained 250 ml of production media and were inoculated with strains in separate flasks. Incubate flasks for 72 hours at 180 rpm at 37ºC.

Screening of bio-surfactant producing isolates

Bacterial isolates were subjected to primary screening methods and characterization was done followed by emulsification index (E_{24}), drop-collapse assay, oil displacement method, CTAB (Cetyl Tri Ammonium Bromide), oil-spreading method. Biosurfactant were further characterized by using Thin Layer Chromatography (TLC) and FTIR (Fourier Transform Infrared Spectroscopy).

Emulsification Index (E_{24}):

Bio-surfactant producing isolates were tested for their activity to emulsify the oil sample with slight modification in conventional method act was brought together. Measurement of emulsifying property (E_{24}) was computed by addition of about 3.5 ml of centrifuged content of without cells after 72-hour fermentation into 10 ml of kerosene in a tube which was cotton plugged and then continuous vortex for 5 minutes. The emulsification index was calculated by dividing height of elusion layer by total height of solution [13].

$$\textit{Emulsification index}\ (\%) = \frac{\textit{Height of the emulsion layer}}{\textit{Total height of solution}} \times 100$$

Drop collapse assay:

This method was one of the primary screening methods, used to check bacterial colonies capable for the coalescence of surfactants, when dropped on the oil film or surface. The observation hailed the idea that cellular components having lesser capabilities to coalesce BS remained in an undisturbed state [15, 16]. No change in the state of droplet is associated with the no synthesis of bio-surfactants or with very less production. Microbial agglomeration emerged in the presence of sugary components or bond groups of carbon and hydrogen were actively assessed with these methods [17].

CTAB (Cetyl Tri Ammonium Bromide):

Crude biosurfactant was placed onto the CTAB (Cetyl Tri Ammonium Bromide) media enriched with Methylene Blue Agar incubated at 37^0C for 9 days in static condition. CTAB media constituted (g/L): Glucose (20), Peptone (10), yeast extract (0.5), Beef extract (1), CTAB (0.78), Methylene Blue (0.0021), Agar (18) at pH 7.2. Colonies displayed halo-zone were confirming the presence of anionic biosurfactant [13].

Oil spreading Assay:

50 ml water was placed in a culture dish of φ 12 cm. Thereafter dropped 2-3 ml of Liquid paraffin was applied onto the center of the oil film. The efficacy was expressed in diameters [19].

Extraction, purification and recovery of Bio-surfactants

Viable cells and impurities were removed by the process of centrifugation (6000 g at 4^0C for 20 minutes). The remaining supernatant was brought to the pH of 2 by using HCl accompanied by the liquid solution having Chloroform and Methanol (2: 1 V/V). Supernatants were accompanied organic solvents and were kept in static condition and then vigorously shaken, again were brought to still static state [20, 21]. The phases of organic and inorganic materials were separated out under the condition of rotary evaporation further giving tacky yellowish product further dissolved in methanol and were further concentrated twice by evaporation. The remaining content was further verified for their bio-surfactant properties.

Characterization of Bio-surfactants:

Analysis of rhamnolipids by Thin Layer Chromatography:

Thin Layer Chromatography was performed with the objective of qualitative screening of Bio-surfactants synthesized. The obtained supernatant was centrifuged at 8000 rpm at 4^0C with acidic condition of 4N HCl. Crudebio-surfactant extricated out and was analyzed by chromatographic system [22]. The crude bio-surfactants were analyzed on chromatographic plate of Silica. The mobile phase implemented in this context contained Chloroform ($CHCl_3$), CH_3OH (Methanol), CH_3COOH (Acetic Acid) 81: 17: 2, the zones which sustained were imaged with H_2SO_4 (5%).

3.Results and Discussions

Isolation of bacterial strains from the oil contaminated region from Kanpur, Uttar Pradesh. Thirty-three morphologically distinct bacterial isolates were identified, twenty-one were gram positive and 12 were gram negative bacterial isolates. Isolates were screened for their bio-surfactant producing quality the first and foremost method to screened out the production of biosurfactant was emulsification index (E_{24}), oil spreading activity, drop collapse assay and CTAB method [23]. Out of 33, three of them showed maximum emulsification activity and isolates were named as SL1, SL2 and SL3. Emulsification activity were checked with different hydrocarbons viz kerosene, diesel and coconut. Out of different hydrocarbons, diesel showed maximum E_{24} activity in isolate SL2 (51%) followed by SL3 (39%) and SL1 (32%), whereas coconut oil showed maximum E_{24} in SL3 (43%) followed by SL2 (39%) and SL1 (35%) whereas kerosene showed maximum E_{24} in SL1 (39%) followed by SL2 (36%) and SL3 (34%), respectively. Apparently diesel showed maximum E_{24} then the procured strains *Pseudomonas aeruginosa* ATCC 9027 used as standard illustrated in figure 1.

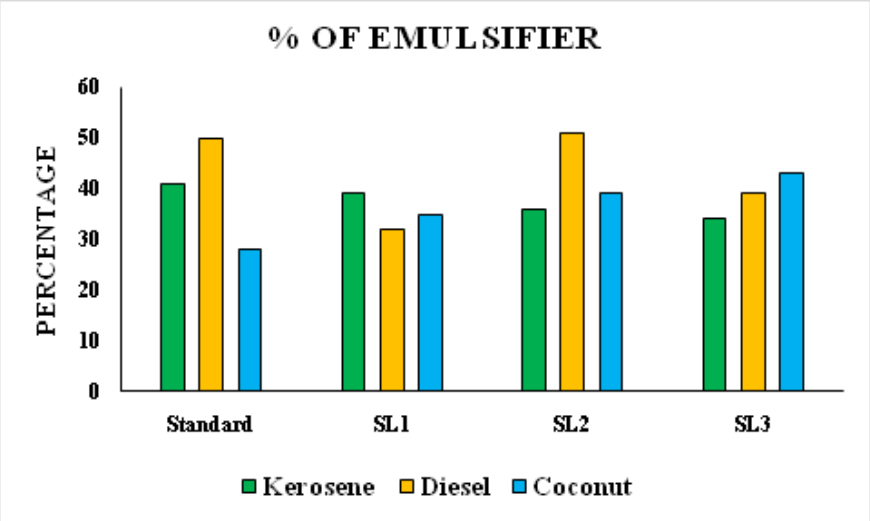

Figure 1: Comparative plot depicting % Emulsification

Drop Collapse assay of isolates was done at room temperature [24]. The assay that carries the non-impoverishment of liquid droplet by the produce. In accordance to carry out the next test, the droplet was placed over the oil droplets. Furthermore, drops of cell supernatant of SL1, SL2, and SL3 were dropped over the oil. Polar molecules (non-water) get reversed and hydrophobic molecules and intermediary actions were remains acceptable. Droplet of oil gets submissive that depicts relations between interfacial tension and stability. The drops collapsed after 20 seconds in all three cases as illustrated in figure 2 [25].

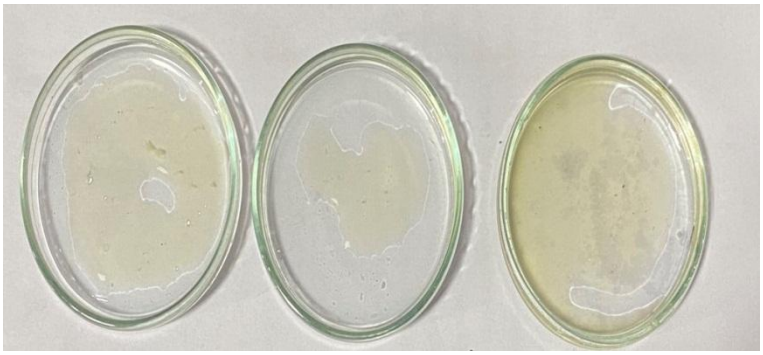

Figure 2: Drop collapse Assay

Drop collapse assay SL1, SL2 and SL3 from left to right depicts collapse of oil droplet because of the addition of surface active compounds showing inter-relation between the surface tension and collapsing of the oil droplet.

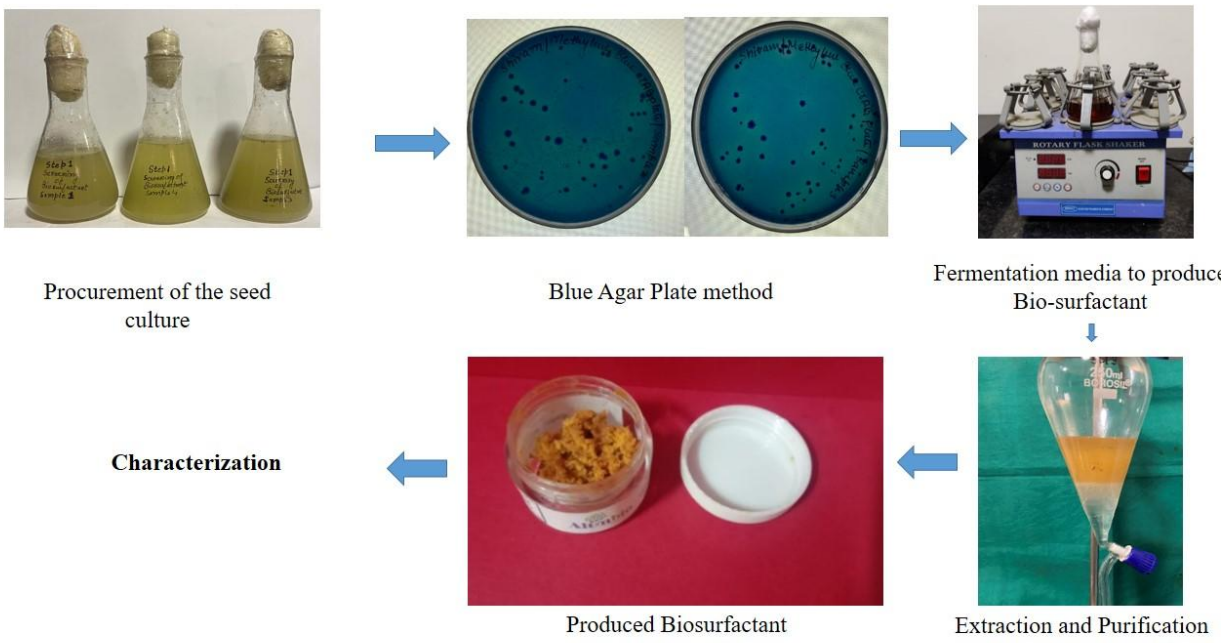

Figure 3: Work flow for the production of Bio-surfactants

Figure 3 illustrated the work flow of the work, most step was to provide seed culture in the fermentation media as it can be effectively observed that at first three seed cultures were inoculated in the nutrient mediums viz. SL1, SL2 and SL3. The Blue Agar plate was chosen for the screening of bio-surfactant produced by isolates. Colonies gave halo zone around the culture depicted the presence of BS. Out of three isolates, Sl1 and SL3 found to be potent. Fermentation media was then placed in incubator shaker, at180 RPM for 24 hours in aeration condition. Produced BS was filtered out through the separating funnel the upper layer was collected out and thereby heated at regulated temperature in order to collect crude BS from them. This work came out with some subtle modifications [26, 27, 28].

Oil Drop Assay

Figure 4: Oil Droplet Assay

From figure 4, it has been elucidated that the isolates were considered to have significant biosurfactant production if the they produced clear zone was at least ≥ 1.0 cm in diameter in the oil spreading assay and ≥ 3.0 mm in the drop collapse assay.

Thin Layer Chromatography

TLC detected all the biosurfactants to be rhamnolipid in nature (Figure 6). The various analyte which were run over the plate of Silica in giving it a stationary phase by the motion of mobile phase consisting polar and non-polar spots. The Rf values contained numbers of 0.78 depicting MRLs while the analyte having value of 0.88 depicted DRLs. This has considerate relation with the work of [29].

Figure 5: TLC plates for SL1 and SL3 showing spots

Sample name	Analyte of Interest	Distance moved by mobile phase	Rf Factor
SLI1	3.9	5	0.78
	4.2	5	0.84
	4.4	5	0.88
SLI3	3.9	5	0.78
	4.2	5	0.84

Dual Confrontation Assay

Dual confrontation assay was performed on the plates of agar medium. Isolates SL1 and SL3 were streaked onto the plate into the center and were streaked onto the edges of the plate. The difference in length of isolates was calculated using scale (mm) and the confrontation was measured (Antagonistic activity).

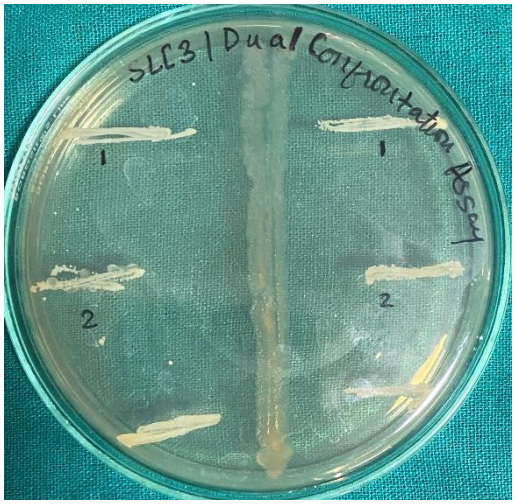

Figure 8: Antagonisms shown by isolate SLI1 and SLI3 on strain 1 (*Campylobacter jejuni*) 2 (*Listeria monocytogenes)*, 3 (*Staphylococcus aureus*)

The characteristics of the MBCA rely on the exploited mode of action. Attainable risks for humans or the atmosphere, risks for resistance development against the biocontrol agent, its infectious agent specificity and its dependency on environmental conditions and crop physiology could dissent between completely different modes of action. Preferences sure enough modes of action for associate envisaged application of a biocontrol agent will have impact on the screening strategies accustomed choose new antagonists [30].

4.Conclusion

In the present study, biosurfactant-producing organisms were enriched and isolated from an oil-contaminated site. The morphological and biochemical method analysis were performed to identify the organism. This research article describing the isolation and use of as a biosurfactant producer which possess actions against *Campylobacter jejuni, Listeria monocytogenes and Staphylococcus aureus*. During fermentation studies, the isolates was able to produce a rhamnolipid biosurfactant, using crude oil as the sole carbon source. The biosurfactant was extracted and partially characterized by using TLC to confirm its chemical nature. In order to examine the suitability of the biosurfactant in enhanced oil recovery processes, sand pack column studies were carried out. Analysis showed that materials required to exchange with chemically synthesized biocides presently used as anti-microbial (for agricultural harmful strains) agents by inexperienced solutions.

Acknowledgement

For this research work I would like to extend my sincere thanks to Department of Biotechnology, Faculty of Engineering and Technology, Rama University. I would like to extent my sincere thanks to Dr. Nabya Nehal who has been a great help in providing guidance for Lab and documentation.

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