Isolation and Screening of Biosurfactant producing Bacteria, their characterization and their potential applications

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Abstract

Biosurfactants, are amphiphilic substances generated on the cell membrane that have a tendency to lower surface tension, to lower the toxicity and to increase the biodegradability. This study focused on isolation, screening and identification of biosurfactant producing bacteria, their extraction and potential applications in enhancing plant growth and their antimicrobial activity. The bacteria were isolated from the soil polluted with old motor oil. The ability to produce biosurfactants was examined using series of tests including the emulsification index test, drop collapse assay, blood hemolysis, oil spread assay and microplate assay. The effective isolate was identified as Pseudomonas citronellolis by 16s rRNA sequencing. Further the extraction and characterization of rhamnolipid were carried out.

The characterization report from FTIR matches with the functional groups of rhamnolipid. Thus, the extracted rhamnolipid were explored for exhibiting their interest towards various biotechnological approaches. The antimicrobial activity of the extract was assessed against the selected pathogens and their applications in the plant growth showing enhanced growth compared to the control. This study can be further used to formulate rhamnolipid in large scale as plant growth promoter and oil cleanser in the future.

Keywords: Surfactant, *Pseudomonas citronellolis*, Rhamnolipid, Plant growth enhancer, Extraction.

Introduction

In current scenario, the petroleum hydrocarbon are found to be one of the major energy sources required in our daily life [20]. Despite their use, it has been reported that for various industrial requirements, almost 5 million tons of oil are transported and delivered by means of freights or maritime transport by abiding to the stringent safety standards to avoid oil spills. Still there is still potential threat involving accidental leakages[23]. Pollution arising due to hydrocarbon contaminants still remains a problem worldwide. There are different methods for the treatment of oil spillage including physical, chemical and biological methods. Although the conventional methods are involved in rapid removal of the oil spills, on the other hand, it poses severe harm including the production of toxic by-products[23]. Biological process which involves the use of living organism by its nature, plays a vital role in removal and degradation of toxic compounds including hydrocarbons and are considered to be environmental- friendly. Surface active molecules which are produced by micro-organisms are generally termed as biosurfactants (BS). The biosurfactant gains importance over synthetic surfactants because of their non- toxic nature[25]. They have established a place in the industry as a result of their environmental friendliness[26].

They have developed significantly since being initially discovered and labelled as "surfactin"[3]. Since then, several research institutions from all over the world have studied biosurfactants.

In recent times, biosurfactants have gained greater importance when compared to the chemically synthesized surfactants over several reasons which include lower toxicity, cheaper substrate utilization, high specificity[20]. Biosurfactants have been used in the biological, pharmacological industries, skin care, food industry, petroleum sectors due to their ability to degrade and to reduce toxicity[14,15,36]. In addition, the biosurfactants produced by *Pseudomonas aeruginosa* have also been used in oil recovery[5]. Biosurfactants are classified based on their surface charge and their molecular weight into different groups[13]. One among them is Rhamnolipid which is a type of glycolipid.

Lipid with rhamnose moiety and a carboxyl-terminated -hydroxy fatty acid is composition of Rhamnolipid(RLs)[26]. RLs, that are primarily generated by *Pseudomonas sp.* and have a structure matching glycolipids, are the most well researched biosurfactants. Based on the bacterial strain, the substrate used for production and their culture condition, there are different types of rhamnolipids. Rhamnolipids produced by *Pseudomonas* sp. are well known as they are easy to grow and have a high surface activity. Other bacteria like *Marinobacter* sp[34], *Burkholderia* sp[10] are also reported to synthesize rhamnolipid.

RLs are the glycosides that, according to their structural makeup, are made up of a rhamnose moiety, which is a glycon part and a lipid moiety, which is an aglycon part. The lipid moiety contains connected hydroxy fatty acid chains which can be saturated, mono-, or polyunsaturated and can be anywhere between C8 and C16 in length[12,33]. Rhamnolipids, microbial BS with advantageous properties, have a fast rate of biodegradation and display little aquatic toxicity[27].

Emulsification, wetting, dispersion, dissolution and disinfection are further processes that RLs are known to enhance and perform[38]. RL has 60 different chemically configured homologues. The modifications to their glycon and aglycon components are what cause the homologous distinctions to exist[1]. Although RLs have shown a number of benefits, their poor yield and difficult manufacturing process have prohibited RLs from being manufactured on a large scale. In 2016, Evonik industry was the first company to make RL on a large scale. They used butane and recombinant *Pseudomonas putida* to synthesise rhamnolipids[12]. Biosurfactants are widely explored for their potential in the production of various commercial products and their applications ranging from oil recovery to bioremediation in environmental sector to agriculture, bioprospecting to medical and pharmaceutical practices which include anti-microbial, anti-biofilm properties etc.[24,25] In the current study, biosurfactant producing strain *Pseudomonas citronellolis* was isolated and screened followed by the extraction and characterization of rhamnolipid. Further, extracted compound was explored towards various applications including antimicrobial property, oil degradation and its potential in plant growth promotion. The current study suggests their potential scope in medical, environmental and agricultural sectors.

Material and Methods

Sample collection: The soil sample was collected from Vidyanagar Gasoline station in Hubballi (15.3657 ^{o}N, 75.1229 ^{o}E) in a sterile container aseptically. The collected soil sample was transported to the laboratory and stored at 4°C for further studies.

Isolation of indigenous bacterial strains and preliminary screening: For selective and enhanced isolation of the effective strains, the samples were enriched in nutrient broth amended with 1% of engine oil and incubated in a shaking incubator for 48 hrs. The sample was serially diluted till 10^{-7} was inoculated on the nutrient agar using spread plate method and incubated at 37^{o}C for 24 hrs. Morphologically distinct colonies were selected and sub-cultured repeatedly to obtain pure culture. Preliminary screening was done using oil spread assay and the isolates were preserved in glycerol stock for further study.

Identification of the effective strain: The isolated bacteria obtained after screening were subjected to identification by microscopic and biochemical tests. Fermentation tests were performed and compared to Bergey's Manual of Systemic Bacteriology. Molecular identification was carried out by 16s rRNA sequencing using Sanger sequencing and the sequence data was aligned using ClustalW. The phylogenetic relationship with the related neighboring strains was determined and the phylogenetic tree was constructed using MEGA 11 software.

Secondary Screening of Isolate for biosurfactant production: The potent isolate obtained from preliminary screening was further subjected to secondary screening to study its ability for biosurfactant production. In a 500 ml conical flask,100 ml of MSM composed of 15g $NaNO_3$, 1.1g KCl, 1.1g NaCl, 0.00028g $FeSO_4$.$7H_2O$, 3.4g KH_2PO_4, 4.4g K_2HPO_4 and 0.5g $MgSO_4$.$7H_2O$.0.5g yeast extract was taken and 1% v/v of used engine oil was used as sole source of carbon. The flask was inoculated with the loopful of culture and kept for incubation in shaker incubator at 200rpm for 7 days at 30°C. After incubation for 7 days, the sample was withdrawn for centrifugation at 4°C at 6000rpm for 15min and the supernatent was collected and filtered using Whatmann filter paper. This filtrate was then run through screening assays like the drop collapse assay, oil spreading assay, emulsification assay, blood hemolysis and CTAB assay[21]to screen the production of biosurfactant.

Oil spread assay: The cell free culture supernatant (CFC) was tested for the biosurfactant activity by oil spread assay. Eloborately,20ml distilled water and 20µl of used engine oil were suspended on the surface of the water in a Petri plate. The next step involved spraying 10µl of CFC broth over the oil surface. The negative control was distilled water which did not contain surfactant[19].

Microplate Assay: Vaux and Cottingham developed and patented microplate assay which can be utilised to qualitatively assess the strains surface activity. A hydrophobic well's pure water has a surface that is smooth. Surfactants wet the well's edge a little, which makes the surface of the fluid concave and adopt the shape of diverging lens. In this test, 100µl sample of the supernatant from the CFC is taken and suspended in each well of a 96-well plate containing 100µl distilled water. The 96-well plate is visible on a grid back paper. If biosurfactant is present in the isolate, the concave surface of the grid image is distorted. The microplate test identifies surface-active compounds in real time and is easy, rapid and sensitive. The method may also be applied to automated high-throughput screening[37].

Drop collapse assay: In this assay, the well of a 96-well microplate was injected with 2µl used engine oil which was then given 24 hours to equilibrate. 5µl of the CFC broth were added to the engine oil and after one minute, the drop size was assessed using a microscope. When the drop was flat, the result was judged favourable for the generation of biosurfactants whereas cultures that produced rounder drops, were graded negatively[18].

Blood Hemolysis assay: Another screening assay is the blood hemolysis assay. In this method, the experiment was carried out on blood agar plates. A loopful of bacterial culture was streaked onto blood agar plates and the plates were kept for incubation at 37°C for 48 hours. Visual inspection of a clear zone (hemolysis) surrounding the colony confirms the positive result. The clear zone is characteristic feature of biosurfactant[4].

CTAB Agar assay: Another assay in line, for the identification of anionic surfactants is the CTAB agar plate

technique[29]. Briefly, 50ml of mineral salt agar media containing cetyltrimethylammonium bromide were prepared and 2g of agar-agar was added and sterilized. Then, 0.2g/l of filter sterilized methylene blue was added to the media and solidified. On the media that contains the basic pigment methylene blue and the cationic surfactant CTAB, the target bacteria are grown. To enhance the visual applicability of the test, wells are made to which the bacterial culture was added. An insoluble, dark blue ion pair is created when anionic surfactants, which are released by bacteria on the plate, interact with CTAB and methylene blue. As a result, prosperous colonies are encircled by dark blue zones[37].

Emulsification index calculation (E24): To evaluate the emulsification, the emulsification index of the supernatant was measured by adding 2ml of the culture supernatant to equal volume of engine. The mixture was subjected to 2mins of vortexing and the result was observed after allowing it to stand for 24hrs[21].

$$\text{Emulsification index} = \frac{\text{(Height of the emulsion layer)}}{\text{Total height}} * 10$$

Extraction of biosurfactant compound: The biosurfactant was produced in large scale for the extraction of rhamnolipid in the production medium mentioned by Nayarisseri et al [21] with slight modification. Briefly, MSM medium substituted with 1% (v/v) used engine oil as source of carbon was used for the production. After 7 days of incubation, the culture components were centrifuged at 10,000 rpm for 30 minutes at 4°C to remove the cells and the supernatant which contains rhamnolipid, was separated and used for the extraction process. The metabolite collected was then used to extract the compound by solvent extraction method[27]. The supernatant was treated with equal volume of chloroform: methanol: acetone at 1:1:1 (v/v) ratio and was kept into incubator shaker for 5hrs at 130rpm followed by its extraction, where the organic layer was collected. The product obtained after extraction was then dried by evaporation at 40°C. The extract was further subjected to characterization studies.

FT-IR analysis: To comprehend the presence of functional groups, the scrapings that had been obtained, were sent for FT-IR analysis. Rhamnolipid was analysed using FTIR in the mid-IR range of 400 – 4000 cm^{-1}.

Oil spread assay with extract: The extract obtained was tested for the biosurfactant activity by oil spread assay. The experiment of oil spread was carried out by methodology formulated by Morikawa et al[19]. Briefly, 20ml of distilled water and 20µl of crude oil were applied on the surface of Petri plate. The next step involved spraying 10µl of extracted compound over the oil surface. Distilled water without a surfactant served as the negative control.

Impact of Extract on Plant Growth: The extracted rhamnolipid was further applied to check the efficiency on plant growth contaminated with crude oil. The seeds of *Vigna radiata* were used for the study. In this experiment, the seeds were surface sterilized using 0.1% $HgCl_2$ for 10 mins prior to the treatment. The experiment was performed in 3 groups – sample A control using distilled water, sample B using engine oil and sample C to which engine oil and metabolite were added. All the samples were allowed to germinate while the circumstances (sunlight and water) remained constant and the data was recorded. This was done in order to track plant development in each sample and to determine how the extract improved plant growth in terms of stem length, leaf length and leaf breadth.

Antimicrobial assay: The extracted rhamnolipid was used for analysing it against microbes through well diffusion method [2, 6]. The antimicrobial assay was examined against 3 pathogenic bacteria *Bacillus cereus, Staphylococcus aureus, Escherichia coli* and1 fungal strain *Rhizopus sp.* Briefly, the bacterial strains were grown in nutrient broth and incubated at 37°C overnight. The culture was swabbed on Muller Hinton agar plates and approximately, 10 µL of the extract was added to the wells and the plates were incubated at 37°C. For control, the standard antibiotic discs like bactericidin B 10mcg and amphicillin 30mcg for bacterial cultures were used and amphotericin B was used for fungal strain. The zone of inhibition was further measured after incubation of 48hrs at 37°C.

Results and Discussion

Isolation and preliminary screening of the biosurfactant producing bacterial strain: Following enrichment, the sample was plated onto nutrient agar plates. After incubation period, 5 distinctive colonies were obtained and were designated as SDP1 – SDP 5. All the isolates were screened via oil spread assay in which only one strain SDP3 showed zone of clearance. This isolate was further used for secondary screening assays. The selected isolate was streak plated to produce pure culture. The colony morphology of the strain SDP3 is shown in fig. 1.

Identification of bacterial strain: The effective strain showed Gram negative, motile, non-spore formers under microscopic test. The colony morphology exhibited a bluish-green pigmentation which is in accordance with the typical characteristics of Pseudomonas. Furthermore, the Gram staining procedure confirmed the presence of Gram-negative, rod-shaped bacteria, consistent with the recognised attributes of this particular species (Fig. 2a)[37]. Additionally, the isolate showed positive result for catalase and oxidase but negative results for indole, methy red, Voges Praskauer, citrate utilization.

Moreover, it did not produce enzyme urease. But the isolate was able to ferment almost all sugars like glucose, sucrose, adonitol, arabinose, lactose, sorbitol and mannitol. Furthermore,16SrRNA sequencing revealed that the bacteria were found to be closely related to *Pseudomonas citronellolis* and were designated as *Pseudomonas*

citronellolis SSSP. The phylogenetic tree was constructed with the closest neighbour as shown in fig. 2b. The sequence was submitted to Genbank with the accession number OP120782.

Oil spread assay: The oil spread test of the potential strain showed a positive result. A clear zone forms when CFC is inoculated on the Petri dish. This demonstrates that the sample has a biosurfactant characteristic and that this causes the oil to deteriorate. The results are shown in fig. 3. The positive oil spread assay demonstrates the oil degradation capability of *P. citronellolis*, as evidenced by the formation of a distinct transparent region on the surface of the Petri dish.

The presence of concave surface distortion observed in the microplate assay provides additional evidence supporting the surfactant properties of *P. citronellolis strain SDP3*, thus demonstrating its ability to adapt to various experimental conditions. The results of the drop collapse assay demonstrate the formation of large supernatant droplets, suggesting the presence of biosurfactants and further supporting the potential usefulness of *P. citronellolis* in diverse applications[35].

Microplate assay: In the 96-well microplate test, a concave surface distortion formed when the sample was introduced to the water-filled wells; this may be seen using a grid paper. This demonstrates the surfactant nature of *Pseudomonas citronellolis*.

Drop collapse assay: The instability of liquid drops exhibited by surfactants is a pre-requisite for the drop collapse test.

Fig. 1: Pure culture of isolated *Pseudomonas citronellolis*

Fig. 2a: Gram staining result showing Gram negative rods of the isolate SDP3

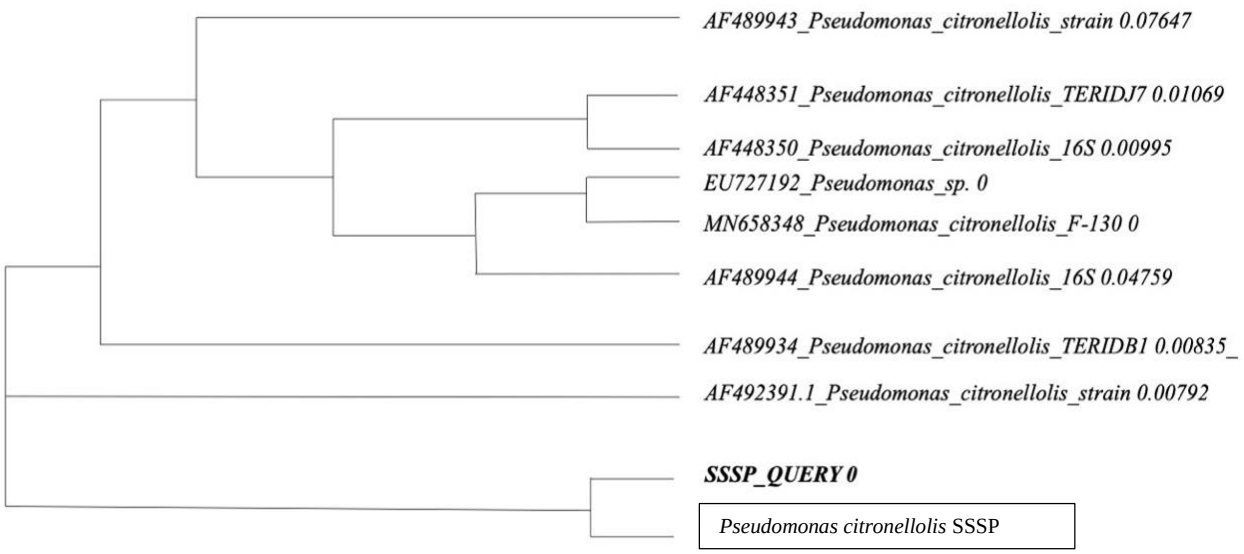

Fig. 2b: Phylogentic tree of P.citronellolis strain SSSP.

Fig. 3: Microplate assay with *P.citronellolis*

It offers a number of benefits including being quick and simple to do, requiring no specialist equipment and just a modest amount of sample. A reduction in interface tension and a hydrophobic surface are indicators that the supernatant droplets contain biosurfactants. The broad form of the supernatant droplets is the outcome. The drop was widely produced by *P.citronellolis*. Fig. 4a and fig. 4b showed the control containing engine oil and distilled water and the test containing the engine oil and the supernatant.

Blood haemolysis test: A distinct zone appeared on the blood agar after the samples containing *Pseudomonas citronellolis* was inoculated on it and kept for incubation for 48hr at 37°C, indicating partial heamolysis of the blood agar. The existence of organism that produced biosurfactant, is indicated by the partial hemolysis shown in fig. 5.

The blood hemolysis test exhibited evidence of partial hemolysis on blood agar, thereby providing support for the hypothesis that *P. citronellolis* is capable of biosurfactant production. This finding is consistent with existing literature that suggests hemolysis is a prevalent characteristic of bacteria that produce surfactants, providing additional evidence for the biosurfactant-producing ability of *P. citronellolis.*

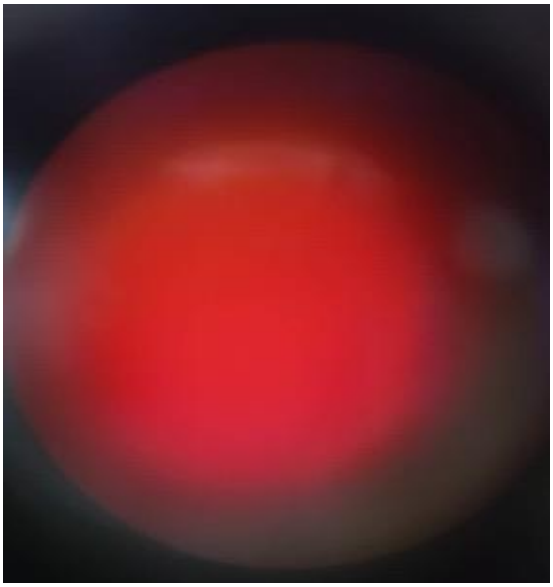

Fig. 4a: Drop collapse assay control with distilled water

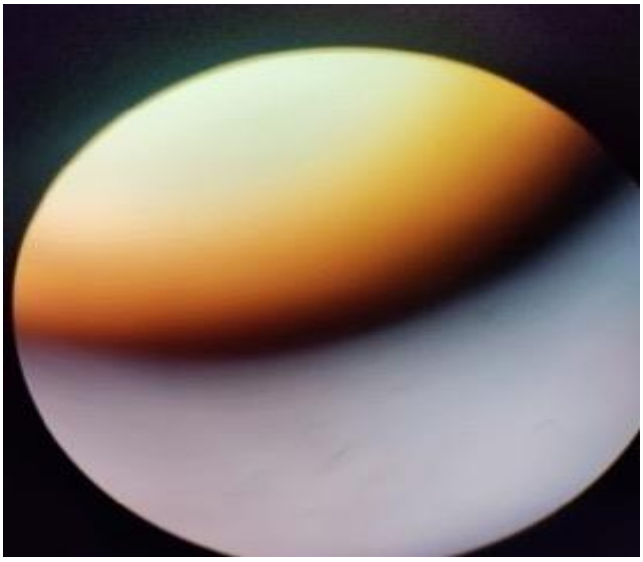

Fig. 4b: Drop collapse assay with engine oil and engine oil and *P.citronellolis*

Fig. 5: Blood hemolysis assay with *P.citronellolis* showing partial hemolysis

Fig. 6: Showing the CTAB assay with P.citronellolis SSSP showing a halo zone around the wells due to rhamnolipid biosurfactant production.

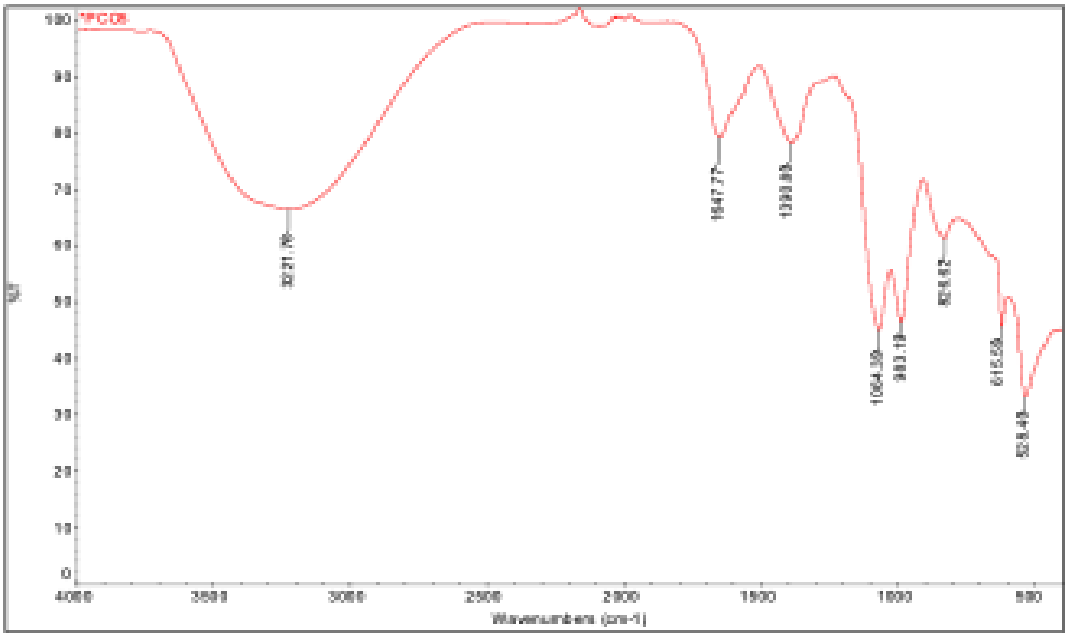

Fig. 7: FTIR spectrum of the extracted compound.

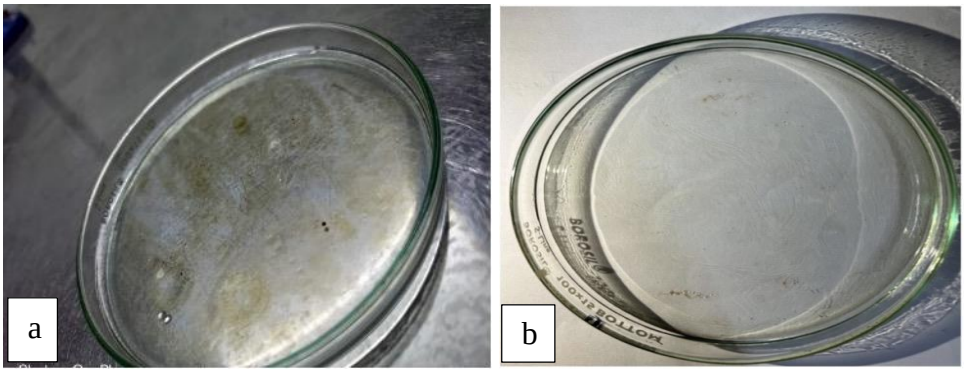

Fig. 8a and b: Showing the Oil spread assay with the extracted rhamnolipid before and after the addition of the extract.

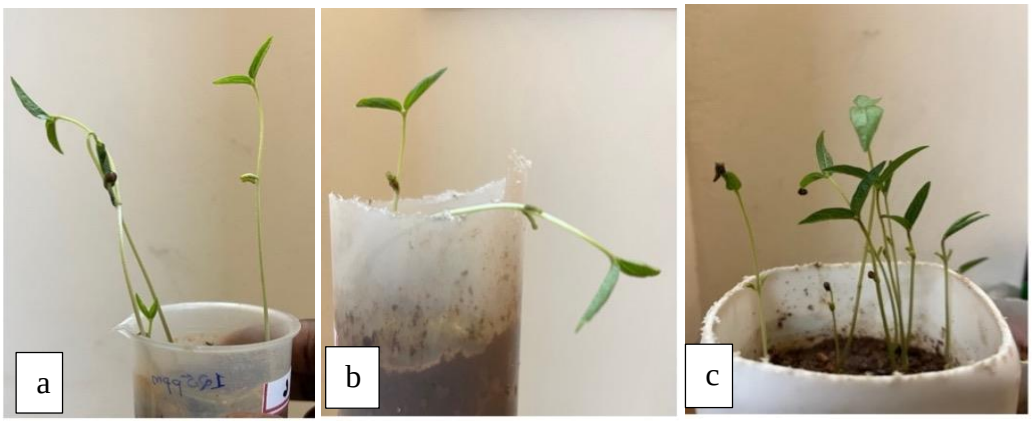

Fig. 9: Showing the growth observation of the seeds of *Vigna radiata* using 3 different sets of treatment as sample a, b, c respectively after 8 days of treatment. 9a containing the water, 9b containing the used engine oil and 9c with the engine oil and the extracted compound.

CTAB assay: A dark blue halo zone appeared around the wells when the CFC was added to the CTAB agar plate and kept for incubation at 37°C for 48hrs as in fig. 6. This shows the presence of the bacteria *Pseudomonas citronellolis* which produces biosurfactants.

Emulsification index: The emulsification test of soil sample revealed that the emulsification index in 62.5%. The soil sample has a significant emulsifying capacity, as evidenced by the emulsification index of 62.5%, which is frequently linked to the presence of biosurfactants. This provides more evidence that *Pseudomonas citronellolis* SSSP is capable of producing biosurfactants. In a study conducted previously, the emulsification index of *Pseudomonas sp.* was reported to be 45% for crude oil[28].

Extraction of rhamnolipid: By solvent extraction method 3.8g of compound was obtained for 1 litre of culture media. It has been reported that *Pseudomonas aeruginosa* produced a yield of 1g/L[11]. The biosynthesis of rhamnolipid is dependent on the carbon source provided and due to this reason, distinct rhamnolipids are produced by different strains of *Pseudomonas aeruginosa*[9]. Our strain used engine oil as the carbon source to produce the RLs. Comparing to the other methods of extraction i.e. zinc sulphate, ammonium sulphate and acid precipitation, it was shown that the solvent extraction approach is the most effective way for recovering the rhamnolipid, producing the greatest yield (7.5 g/L) of BS.[27] Hence solvent extraction method was used for RL extraction.

FT-IR: FT-IR was performed to identify the functional group present in the extracted biosurfactant produced by *Pseudomonas citronellolis* SSP. Fig. 7 represents the spectrum of the extracted biosurfactant. A band of absorption was observed at 3221cm^{-1} due to C-H groups along with O-H groups of carboxyl and N-H group of amide[8]. A band at 1647cm^{-1} correlated to proteins proved the presence of polypeptides. A band around 1390cm^{-1} occurred because of COO- antisymmetric anti symmetric stretching of COO-. C-H bending in CH_2 and CH_3 was interpreted. Absorption peak at 1064cm^{-1} is due to the -COC- group vibrations in carbohydrates which shows that bonds are present between carbon and hydroxyl functional groups in rings of rhamnose.

A comparison with earlier findings from Deepika et al[7] strengthens the study's credibility. The similarities in FTIR profiles serve as further evidence of the glycolipid composition of the biosurfactant and establish a strong connection to the body of existing literature. The presence of bonds between the carbon and hydroxyl functional groups in the rings of rhamnose is indicated by the absorption peak at 1064 cm^{-1} which is connected to vibrations of the -COC- group in carbohydrates. This particular discovery supports the compound's identification because it is consistent with the known structure of rhamnolipid biosurfactants.

Oil spread assay with extract: After 5 minutes, a clear zone was located on to the petri plate with oil and water. This demonstrates that the sample has the ability to behave as a biosurfactant, degrading the oil. With only a tiny amount of compound was required and no high-end equipment needed, the oil spreading method is quick and simple to use.[22]. The results are shown in figures 8a and 8b.

Impact of Extract on Plant Growth: After 8 days of time, the plant was assessed to check the length of stem and leaf and width of leaf. With respect to stem, the sample A i.e control was observed to have stem length of 9cm, sample B was having 5.5cm in length and the sample C was having 12cm length. When the leaf length was considered next, sample A showed growth of 1.3 in length, sample B showed growth of 1cm in length and sample C showed growth of 3.5cm in length. Lastly the leaf width was assessed, where the sample A width was 0.5cm, sample B width was 0.3cm and sample C width was 1cm. The results are shown in figures 9a, b and c. Due to its ability to degrade used engine oil, the extracted compound increased plant growth by increasing soil nutrient availability[31]. Similarly, rhamnolipid produced by *Pseudomonas sp.* PS17 has also been reported to increase the crop yield[17], thus confirming the extracted

compound to be used in agricultural sector to increase plant growth and yield.

Antimicrobial assay: In the current study, the rhamnolipid extracted from *Pseudomonas citronelolis* showed good antimicrobial activity against the test pathogens. culture plate with extracted substance showing 0.5cm of inhibition zone against *B. cereus*. 0.4cm zone of inhibition was observed against *E. coli* and no zone of inhibition was seen against *S. aureus* and against fungal strain *Rhizopus* while 0.3 cm inhibition zone was observed for control Bactericidin B. This data provides an insight of the extracted rhamnolipid to be used as antimicrobial agent. Further, purification of the compound would enhance the ability to inhibit the growth of pathogenic strains. In a study conducted by Deepika et al[7], the crude compound showed minimum antifungal property against fungus *Fusarium oxysporum* compared to the column purified which showed 95% of inhibition.

Conclusion

In the last few decades, extensive work has been carried out on biosurfactants. The applications of biosurfactants in dispersion and degradation forms to remediate oil spills are of rising interest in the oil and gas business. The successful application of the extracted compound from the biosurfactant demonstrated its ability to be applied in diverse fields. Testing on plants for a comparative examination of growth together with these applications provided a distinct perspective on the thought process. An agricultural revolution may result from the improvement in plant growth. Thus, it may also preserve the environment.

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