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Exploration on production of rhamnolipid biosurfactants using native *Pseudomonas aeruginosa* strains

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ABSTRACT

Biosurfactants are structurally diverse surface-active molecules, produced on living surfaces, mostly microbial cell surfaces or excreted extracellularly. Rhamnolipid biosurfactants have wide spectrum use and are predominantly produced by the bacteria *Pseudomonas aeruginosa*. In this study, 75 *Pseudomonas* strains isolated from distinct native habitats were screened following oil spreading technique, methylene blue agar method, hemolytic blood agar method and surface tension measurement of the cell free culture. Ten selected isolates were tested for their ability to produce rhamnolipid biosurfactants in glycerol mediated broth. The best among them, Pa24, was confirmed as *Pseudomonas aeruginosa* through 16S rRNA sequence analysis. Experiments carried out on the ability of *P. aeruginosa* strain Pa24 revealed its potential to utilize range of vegetable oils such as coconut oil, palm oil, jatropha oil, neem oil and mineral glycerol as sole source of carbon and produce rhamnolipid biosurfactant. The extracted biosurfactant was characterized by thin layer chromatography and high performance thin layer chromatography as mixture of di-rhamnolipid and mono-rhamnolipid biosurfactants. The crude extract of rhamnolipid was tested *in-vitro* for antifungal activity against *Phytophthora capsici* and *Phytophthora infestans* and the MIC₅₀ were found to be 815.8 ppm and 373.9 ppm, respectively. Further exploration on different renewable carbon sources including agriculture industrial wastes to produce rhamnolipid biosurfactants can improve the efficiency and reduce the environmental pollution through waste discharge from these industries.

Key words: rhamnolipid biosurfactants, *Pseudomonas aeruginosa*, *Phytophthora capsici*, *Phytophthora infestans*

Introduction

Biosurfactants are surface-active molecules having both hydrophilic and lipophilic properties. A section of microorganisms (bacteria, yeasts and filamentous fungi) can produce biosurfactants, which have diverse industrial applications such as plant protection against fungal pathogens, enhanced oil recovery, crude oil drilling, lubrication, bioremediation, healthcare, pharmaceuticals and food (Cameotra & Makkar, 2004). The research attempts on biosurfactants, particularly on development of rhamnolipid

biosurfactants, because of their biodegradability, low toxicity, potentially high activities and their stability at extreme temperature, pH and salinity. Growing environmental awareness, the bio-accumulation and eco-toxicity of synthetic surfactants has become an issue of major concern (Banat et al., 2010; Pacwa-Płociniczak et al., 2011) and biosurfactants are practical alternatives. Optimization of biosurfactant production is one of the main fields of research owing to the low yields, high cost of raw materials and need for potential microorganism. The biosurfactant production is an important survival strategy by different microorganisms as it helps in

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uptake of hydrophobic substrates for surface-associated modes of motility (Chrzanowski et al., 2012). *Pseudomonas aeruginosa* is the preferred microorganism for the production of rhamnolipid type of biosurfactant utilizing glycerol, mannitol, fructose, glucose, and vegetable oils (Koch et al., 1991; Santos et al., 2002). Rhamnolipid biosurfactants have been approved for applications in various fields like food, cosmetics, agriculture and pharmaceutical industry (Nitschke & Costa, 2007; Campos et al., 2013). Rhamnolipid biosurfactants have very good fungicidal properties and can be effective molecules to manage dreaded crop diseases such as downy mildews, wilts and rots caused by *Pythium* spp., *Phytophthora* spp. and other fungal and bacterial pathogens (Sachdev & Cameotra, 2013). While pesticides have a long history of being extremely toxic, persistent, and harmful to the environment, rhamnolipids are natural “green” pesticides (Vatsa et al., 2010; Sha et al., 2012). Exploration is still on to search for novel and potential microorganisms from native habitat and for an efficient process to produce the bioactive metabolites of desirable properties. The present study is aimed at the exploration of the potential biosurfactant producing *P. aeruginosa* strains in regions of Karnataka and Kerala where zoosporic fungal plant diseases like *Phytophthora* wilt is very prevalent on many cultivated crops and to check the ability of the bacteria to utilize different vegetable oils and mineral glycerol as nutrient source to produce rhamnolipid biosurfactant to use as natural origin fungicide against zoosporic plant pathogens.

Materials and Methods

Isolation of microorganisms

The soil samples were collected from distant regions of different rhizosphere soils (plantation crops, vegetables and cereals) in Karnataka and Kerala. The places and crops selected for collection was based on the fair idea of severity of incidence of zoosporic plant pathogens like *Phytophthora* spp and *Pythium* spp. About 50 g of the soil was collected from each site in sterile bags. The isolation of bacteria was carried out by mixing 1g of each soil sample with 10 ml of sterile water and serially diluted, plated on *Pseudomonas* agar (HiMedia) for selective screening. Inoculated plates were incubated at 37°C for 24 hours and the plates were observed for bacterial colonies. Each of the isolates were inoculated in 100 ml of Luria Bertani (LB) broth (HiMedia) and incubated at 37°C, 120 rpm, for 24 hours. The bacterial isolates were examined for Gram reaction and cell morphology. The select

bacterial strains were identified based on the morphological, biochemical and physiological characteristics according to Bergey’s Manual of Systematic Bacteriology.

Screening and identification of efficient strains for rhamnolipid production

Oil displacement: 15 µL of crude oil is placed on the surface of 40 ml of distilled water placed in a petri dish, and 10 µl supernatant of each culture was slightly put on the surface of the oil film. The strains which caused oil spreading under visible light were considered positive (Satpute et al., 2008).

Hemolytic activity: The pure culture of bacterial isolates were streaked on the freshly prepared blood agar and incubated at 37°C for 48-72 h. Hemolytic activity was detected with the presence of a clear zone around bacterial colonies (Satpute et al., 2008).

Blue agar plate (Bap) method: Mineral salt agar medium supplemented with glucose (2%), cetyltrimethylammonium bromide (0.05%) and methylene blue (0.02%) was used for the detection of anionic (rhamnolipid) biosurfactant (Satpute et al., 2008). Wells of 4 mm diameter were made using cork borer on methylene blue agar plates and loaded with 30 µl of fresh culture of individual isolate. The plates were incubated at 37°C for 48-72 h. A dark blue halo zone around the culture was considered positive for anionic biosurfactant production.

Surface tension method: Surface tension of cell free supernatant was determined by using Du Nouy ring tensiometer (K6, Kruss, Hamburg, Germany) at room temperature (25±2°C). Instrument was standardized with distilled water and methanol. All the measurements were made in triplicates and the isolates exhibited the surface tension below 45 mN/m were considered as potential biosurfactant-producing organisms.

Culture medium and growth conditions for rhamnolipid production

The culture medium used for rhamnolipid production from select isolates is the mineral salt medium (MSM) containing following composition (g/l): KH₂PO₄ (1.0), K₂HPO₄ (0.5), MgSO₄·7H₂O (0.5), NaCl (2.0), (NH₄)₂SO₄ (1.0), NaNO₃ (1.0), Glycine (1.5), Glycerol (30.0). The media pH was adjusted to 7.35 ± 0.05 and sterilized at 121°C, 15 psi for 20 mins. To this medium 1% (v/v) of sterile trace elements solution (Composition: 0.02% MnSO₄·2H₂O, 0.05% FeCl₃, 0.001% FeSO₄, 0.1% CaCl₂·2H₂O, 0.02% CoCl₂·2H₂O, 0.001% H₃BO₄, 0.01% ZnSO₄, 0.02% CuCl₂

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and 0.001% NaMoO₄) was added prior to inoculation. The pre culture of individual isolates were inoculated and incubated at 37°C, 120 rpm for 120 hours. The fermented broth was subjected to centrifugation at 8000 x g for 20 min and the cell free supernatant was used for all the analysis.

Rhamnolipid estimation

Rhamnolipid concentration in the cell-free culture broth was estimated by a colorimetric method for the determination of rhamnose concentration by anthrone-sulphuric acid method explained by (Smyth *et al.*, 2010) with slight modification. One ml of the cell-free culture broth was acidified (pH: 2.0) with 1N HCl and extracted with di-ethyl ether for two times. The organic phase was collected carefully, evaporated to dryness and the contents were dissolved in 1ml of 1% NaHCO₃ solution. These samples were used for the estimation of rhamnose content by colorimetric method using anthrone reagent. The reaction mixture constitutes 0.5 ml of prediluted sample, 1 ml of 75% sulphuric acid and 1 ml of anthrone reagent (200 mg of anthrone in 100 ml of 75% sulphuric acid). These contents in tube were mixed well and incubated in water bath at 100°C for 10 mins. It was cooled to room temperature, absorbance was measured at 620 nm, and the rhamnolipid concentration was calculated using a standard curve prepared using different concentrations of L-rhamnose as described by (George & Jayachandran, 2013).

Molecular characterization of a selected isolate

The selected bacterial isolate Pa24 exhibited high yield of rhamnolipid and shown positive in qualitative tests was chosen for molecular identification. The molecular identification was carried out by amplification, sequencing and analysis of conserved 16S rRNA region. The total genomic DNA was extracted from overnight culture of the isolate as described by (Sambrook *et al.*, 1989) and the quality of DNA was checked by gel electrophoresis on a 1% agarose gel. The PCRs were performed using universal 16S primers to amplify the 16S region, the PCR products were sequenced (Chromous Biotech, Bangalore, India) and analysed using BLASTn. The sequence was submitted to the GenBank.

Evaluation of different carbon sources for rhamnolipid production

The selected isolate (Pa24) was used to evaluate different carbon sources such as coconut oil, palm oil, jatropha oil, neem oil and mineral glycerol for the production of

rhamnolipid biosurfactant. The experiments were carried out in 500 ml Erlenmeyer flasks containing 200 ml of mineral salts medium (MSM) with a composition as described earlier. The media was supplemented (w/v) with each of the following components as carbon source (30 g/l); coconut oil, palm oil, jatropha oil, neem oil and glycerol. The pH was adjusted to 7.35 ± 0.05 and the flasks containing media were autoclaved at 121°C, 15 psi for 20 min. The flasks were supplemented with 1% (v/v) trace elements solution (compositions as described earlier) and inoculated with 10% (v/v) of overnight culture grown in LB broth. These were incubated at 37°C, 120 rpm for 120 hours. The fermented broth was subjected to centrifugation at 8000 x g for 20 min and the cell free supernatant was used for yield and quality analysis.

Analysis of rhamnolipid mixture by Thin-Layer Chromatography

The cell free supernatant obtained from centrifugation at 8000 x g, 4°C, for 30 mins was acidified to pH 2.0 with 5N HCl and kept at 4°C for precipitation. The resulting solution was extracted with the equal volume of ethyl acetate. The organic phase was separated, dried with anhydrous sodium sulfide, and the solvent was evaporated under vacuum in rotavapour. The contents in round bottom flask were redissolved in methanol. The rhamnolipids extracted were analyzed by thin-layer chromatography (TLC) according to (Schenk *et al.*, 1995) with few modifications. The TLC was carried out on silica 60 gel aluminum sheets (Merck, Darmstadt, Germany) using the solvent system CHCl₃/CH₃OH/CH₃COOH (81:17:2). The developed zones were stained with 5% sulfuric acid in methanol followed by drying at 100°C for 15 min. The rhamnolipid supply by Jeneil Biotech Inc (JBR 425) was used as standard (1 mg/ml) in respective experiments.

Structural characterization of rhamnolipid

High performance thin layer chromatography (HPTLC) was carried out using a CAMAG thin layer chromatography system composed of an automatic TLC sampler (CAMAG Linomat 5), automatic development chamber (CAMAG ADC2), detector (CAMAG TLC Scanner 3), and an electronic integrator (winCATS software). The crude rhamnolipid extracts were diluted in methanol to reach the final concentration of 1 mg/ml of rhamnolipid concentration based on quantification assay done by colorimetric method. Four aliquots (5, 10, 15 and 20 µl) of the standard rhamnolipid solution (Jeneil Biosurfactant JBR 425) and 20

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μl of the respective crude rhamnolipid samples were band applied (~ 15 mm) on to an HPTLC precoated silica gel 60F 254 plate (20 × 10 cm). The sample was loaded at a dosage speed 150 nl/s under nitrogen stream. The sample was developed (ascending) using 10 ml of the mobile phase of $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{H}_2\text{O}$ (81:17:2, v/v/v), in plates preconditioned for 3 min, to a migration distance of 85 mm. The plate was dried, sprayed with solution of 5% H_2SO_4 in Methanol, and then put in a hot-air oven at 120°C for 20 min. The developed chromatogram was scanned in remission type, absorbance mode at 429 nm. The signals recovered from the scanner were integrated into absorbance chromatograms from which peak area was automatically calculated using the winCATS software. Based on the R_f value of the separated spot of standard rhamnolipid the mono rhamnolipid and di rhamnolipid were detected in sample.

Bioassays against zoosporic plant pathogens

Sterile supernatant containing rhamnolipid produced using select isolate in was evaluated *in-vitro* with three replications for efficacy against *Phytophthora infestans* and *Phytophthora capsici* using poison plate method in Carrot media agar (Medina & Platt, 1999). Based on the colorimetric assay, the rhamnolipid content in the supernatant was estimated and considered for working out test concentrations (0-1000 ppm). The plates were incubated at 27°C for 5 days and the average colony diameter was observed by measuring the diameter 3 times across the colony. The growth inhibition at different concentrations of rhamnolipid biosurfactant was worked out vis-à-vis untreated control to determine minimum inhibitory concentration (MIC_{50}).

Statistical analysis

The average values presented for rhamnolipid concentration with different isolates, evaluation of different carbon sources, surface tension measurement and the antifungal activity were determined by triplicate experiment and expressed as mean \pm standard deviation.

Results and Discussion

Screening for potential *Pseudomonas aeruginosa* strain for rhamnolipid production

The rhamnolipid biosurfactants are predominantly produced by *P. aeruginosa* utilizing varied sources of nutrition available in nature. The quantity and quality of rhamnolipid produced is reported to vary a lot depending on the nutrition source and on the microbial strain evolved under

particular environment. In the present study, also, the new native strain of the bacteria is targeted from area where the incidence of zoosporic plant pathogens are prevalent on crops like pepper, arecanut, cocoa, zinger, chilli, tomato, etc., with an expectation that the strain would have obtained specific fungicidal characteristics naturally to suppress the pathogens.

The soil samples collected from rhizospheres of different crop production systems were used to obtain 75 isolates of *Pseudomonas* spp. on selective medium and were serially numbered from Pa1 to Pa75. More than one screening methods were adopted (Satpute et al., 2008) in the primary screening of isolates for their ability to produce rhamnolipids. The bacterial colony characteristics and Gram's staining confirmed all of them as *Pseudomonas* spp. The isolates exhibited varied results in rhamnolipid biosurfactant specific parameters such as oil displacement, hemolytic test and blue agar test (Table 1). All isolates cleared the oil displacement test and confirmed the production of biosurfactant. However, the extent of oil displacement differed considerably among strains suggesting direct correlation to biosurfactant presence in the supernatant. Similar observations were made by Rodrigues et al. (2006). All the strains showed haemolytic activity on blood agar media as observed by Mulligan et al. (1984) and Mulligan et al. (1989). The dark blue halo zone in the methylene blue agar plate supplemented with CTAB confirmed the production of anionic (rhamnolipid) biosurfactant (Figure 1) by certain isolates. The blue agar method was an unconventional method to screen *P. aeruginosa* for rhamnolipid biosurfactant production (Siegmond & Wagner, 1991). The native isolates were further screened based on the surface tension of cell free supernatant keeping the standard surface tension target at 45mN/m. Among 72 isolates, 10 isolates (Table 1) were consistently better for all the 4 screening methods *viz.*, oil displacement test, hemolytic test, blue agar test and surface tension of cell free supernatant.

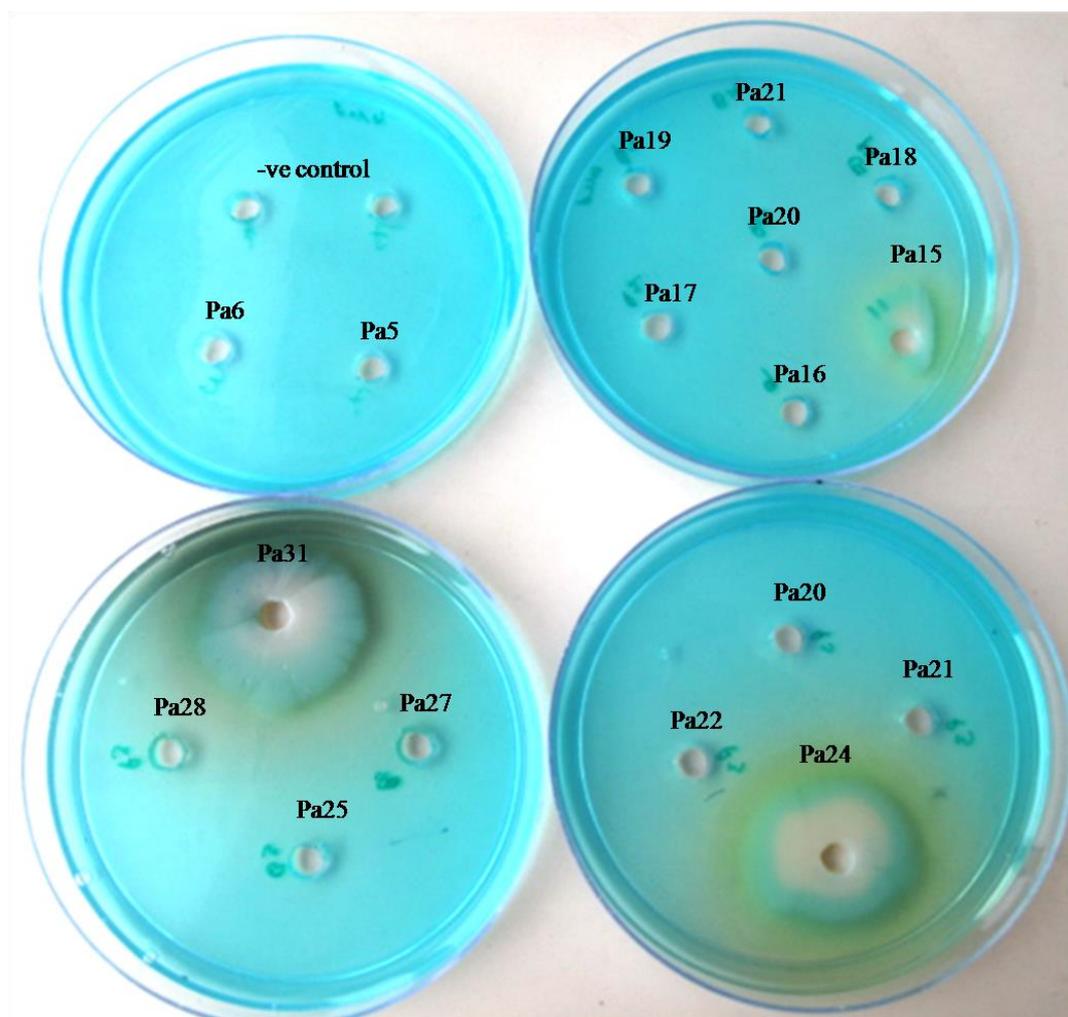
The selected isolates were cultivated singularly on culture medium containing MSM with glycerol (3%) as carbon source to evaluate their ability to produce rhamnolipid biosurfactant. Among them, the isolate Pa24 has shown considerably maximum rhamnolipid (2.36 g/l) production (Table 1). The 16S rRNA region of the isolate Pa24 was sequenced and analyzed using BLASTn. The identity of the isolate Pa24 was confirmed as *Pseudomonas aeruginosa* and all the details were submitted to Genbank (accession id: HG738847).

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Table 1. Parameters used to screen bacterial isolates for the production of rhamnolipid biosurfactants

Test isolate	CTAB- methylene blue agar test	Hemolytic activity	Oil spread method	Surface tension average in mN/m	Rhamnolipid concentration (g/l)
Pa 7	++	+	+	40.5 ± 0.87	0.85 ± 0.03
Pa 14	++	+	+	42.16 ± 0.76	0.72 ± 0.03
Pa 15	++	+	+	43.26 ± 0.87	0.85 ± 0.06
Pa 24	+++	+	++	30.03 ± 1.05	2.36 ± 0.08
Pa 31	+++	+	++	34.33 ± 0.58	0.98 ± 0.06
Pa 43	+	+	+	41.16 ± 1.26	0.15 ± 0.03
Pa 45	++	+	+	43.16 ± 1.04	0.23 ± 0.02
Pa 55	+	+	+	42.3 ± 1.08	0.10 ± 0.02
Pa 65	+	+	+	44.00 ± 0.87	0.13 ± 0.04
Pa 70	++	+	+	44.66 ± 0.76	0.12 ± 0.02

*Values expressed in mean ± SD

**Figure 1.** The methylene blue agar method to screen *Pseudomonas* isolates for rhamnolipid biosurfactants production.

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Evaluation of different carbon sources for rhamnolipid production

The efficiency of rhamnolipid biosurfactant in terms of batch yield and quality becomes very important if it has to excel as a good natural origin fungicide. It is believed that depending on the nutrition the bacteria gets, the efficiency of production vary. In this experiment we tried to compare different sources of carbon to the *P. aeruginosa* strain Pa24 through the fermentation in culture media. The isolate was able to grow by utilizing various carbon sources viz., coconut oil, palm oil, jatropha oil, neem oil, and mineral glycerol supplemented with MSM. However, the rhamnolipid production varied considerably (Table 2) across carbon sources used. Mineral glycerol as source of carbon to the bacteria could produce significantly high rhamnolipid production (2.44 g/l) followed by palm oil and coconut oil as source of carbon (0.53 g/l and 0.52 g/l).

Table 2. Rhamnolipid production from *Pseudomonas aeruginosa* Pa24 with different substrates as the sole carbon source

Carbon source	Rhamnolipid yield in g/l
Coconut oil	0.52 ± 0.04
Palm oil	0.53 ± 0.06
Jatropha oil	0.44 ± 0.05
Neem oil	0.47 ± 0.05
Glycerol	2.44 ± 0.18

*Values expressed is mean ± SD

Thin-Layer Chromatography

The partial characterization of the biosurfactant products obtained from fermentation media with different nutrition sources to the bacterial strain *P. aeruginosa* Pa24, through thin layer chromatography technique revealed the presence of two distinct areas with *R_f* (retention factor) values equal to 0.73 and 0.35 and matched to the spots developed for standard rhamnolipid (Figure 2). According to Schenk et al. (1995) and Arino et al. (1996), the *R_f* values of 0.74 and 0.36 are consistent and relate to monorhamnolipid and dirhamnolipid, respectively. Though the spot intensity varied, the presence of both mono and dirhamnolipids is confirmed with all sources nutrition tested for the bacterial strain.

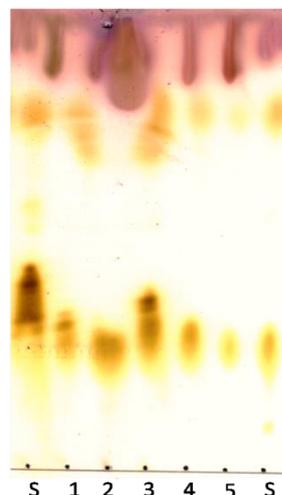


Figure 2. Thin layer chromatography of rhamnolipid mixture obtained with different sources of carbon for *P. aeruginosa* strain Pa24, S – Standard; 1- Palm oil; 2 – Mineral glycerol; 3 – Coconut oil; 4 – Jatropha oil; 5- Neem oil.

High performance thin layer chromatography

High performance thin layer chromatography (HPTLC) analysis of rhamnolipid mixture obtained after fermentation with varied sources of carbon for the bacteria showed the presence of two major peaks having the *R_f* value of 0.55 and 1.28 indicating the presence of two major homologues in the samples (Figure 3).

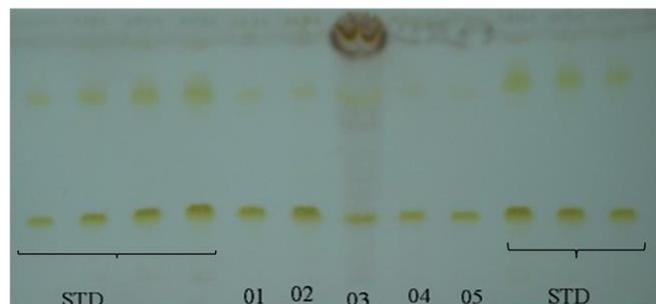


Figure 3. HPTLC of the rhamnolipid produced by *P. aeruginosa* Pa24 utilizing different carbon sources. STD – Rhamnolipid Standard with aliquots of 5, 10, 15, 20µl; test samples of 20µl aliquot rhamnolipid obtained from 01- Palm oil; 02 – Mineral glycerol; 03 – Coconut oil; 04 – Jatropha oil; 05- Neem oil.

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The 3-D absorbance chromatogram (Figure 4) plotted after scanning through different spots on the HPTLC chromatogram (Figure 3) for different carbon sources (lanes E, F, G, H, I) showed five peaks suggesting the presence of five homologues similar to the peaks observed in the rhamnolipid standard (lanes A, B, C, D, J, K, L). The five homologues of the standard recorded the R_f values as 0.55, 0.82, 1.28, 1.71 and 1.80. The first, third and fifth peaks related to major homologues, whereas the second and fourth peaks corresponded to minor homologues. The structural congeners with R_f value of 0.55 and 0.82 were identified as di-rhamnolipids (RRL) and while mono-rhamnolipid (RLL) had R_f value of 1.28, 1.71, and 1.80. The homologues of

different congeners of mono rhamnolipid and di-rhamnolipid were well established by HPTLC densitometry (Hazra *et al.*, 2011).

The rhamnolipid biosurfactants (Figure 4; tracks E, F, G, H and I) produced through bacterial fermentation using varied carbon sources explicitly showed three major homologues with R_f value 0.55 and 1.28 and 1.80. The strong similarity between the R_f values of the three rhamnolipid homologues in the test samples and the di-rhamnolipid (RRL) and mono-rhamnolipid (RLL) homologues of the rhamnolipid standard confirm the identity of these two test homologues as RRL and RLL.

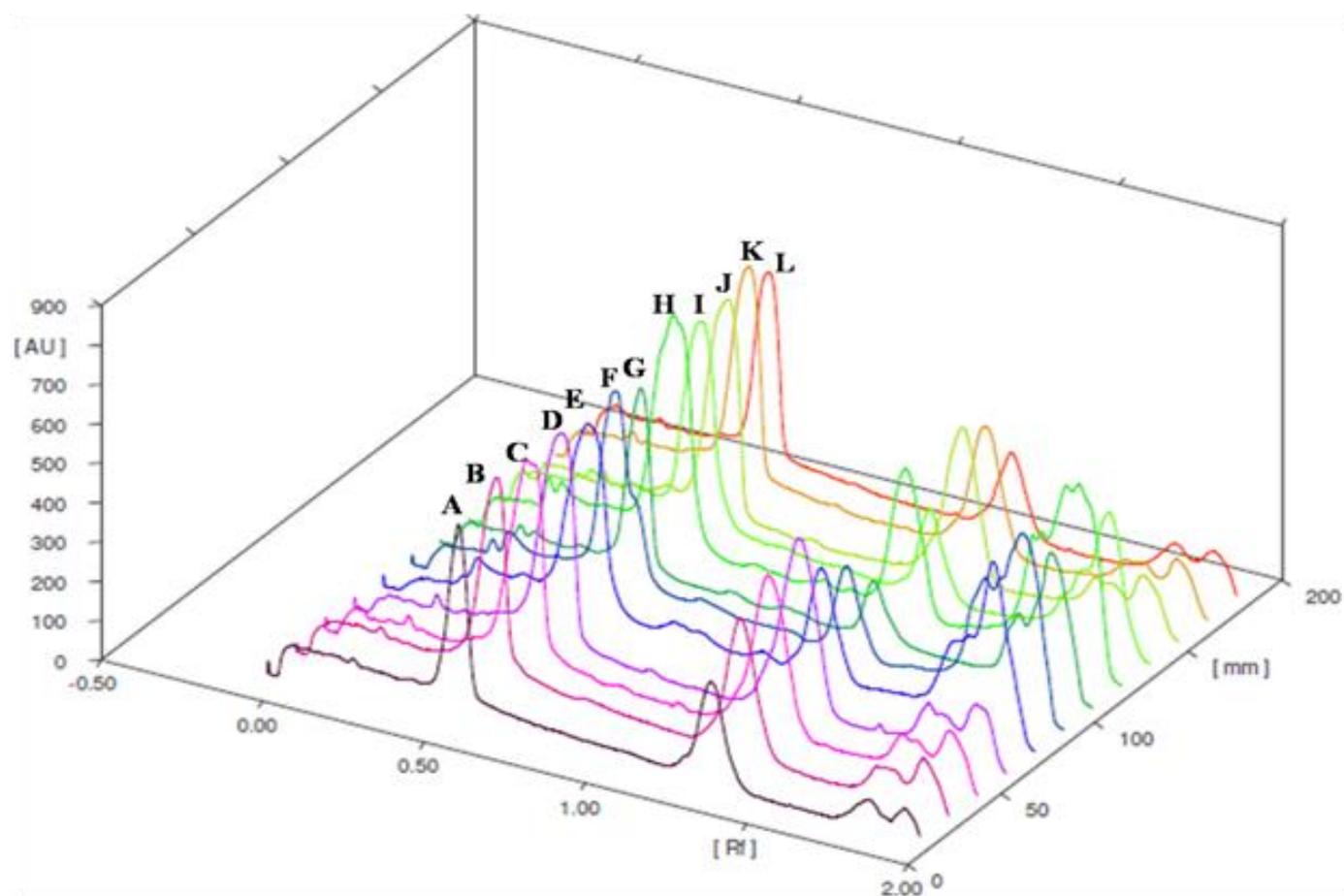


Figure 4. 3-D absorbance chromatogram for rhamnolipid standard and test samples measured at 429 nm. Lane A & J - 5 μ L, B & K - 10 μ L, C & L - 15 μ L and D - 20 μ L of 1 mg/ml standard rhamnolipid solution; Lane E, F, G, H and I; as test rhamnolipid solutions of 20 μ L aliquot (1 mg l⁻¹) of rhamnolipid biosurfactant produced using different C-sources viz., E- Palm oil, F - Mineral glycerol, G - Coconut oil, H - Jatropha oil, I- Neem oil.

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Efficacy against plant pathogenic fungi

The effectiveness of rhamnolipid biosurfactant against plant pathogenic fungi, particularly zoosporic plant pathogens is well documented. The inhibitory effect of rhamnolipid is mostly due to the lytic effect on zoospore, inhibiting spore germination and hyphal growth of fungi. In the present study, the extent of growth inhibition of the zoosporic plant pathogens *P. capsici* and *P. infestans* was measured *in-vitro* through poison plate technique on potato dextrose agar medium using the rhamnolipid biosurfactant produced with mineral glycerol as source of carbon. The dose response study showed a maximum inhibition of 60% and 72% against *P. capsici* and *P. infestans*, respectively (Figure 5). The

MIC₅₀ of rhamnolipid biosurfactant were determined as 815.8 ppm and 373.9 ppm for *P. capsici* and *P. infestans*, respectively. However, the MIC₅₀ values recorded in our study were much higher compared to earlier reports. For instance, Kim *et al.* (2000) reported an MIC of 50 ppm rhamnolipids against *P. capsici*. The only difference between the experiments is that we have used the aqueous extract containing rhamnolipid as obtained from culture broth while in many of the earlier reports; the purified rhamnolipid was used for the study. Nevertheless, the bacterial metabolites were effective against both the plant pathogens tested as reported in many studies earlier.

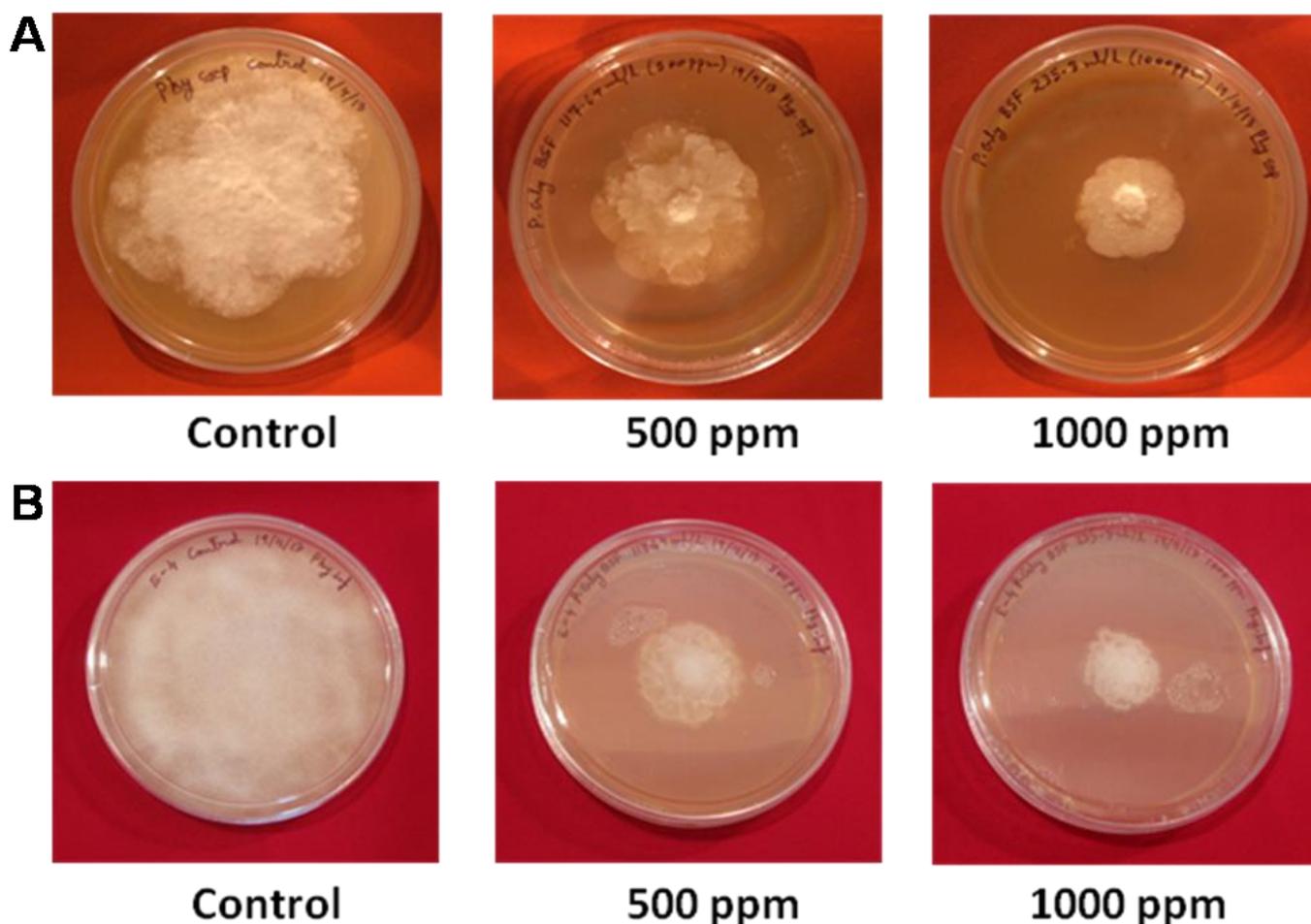


Figure 5. Growth inhibition of plant pathogenic fungi (A) *P. capsici* and (B) *P. infestans* by rhamnolipid biosurfactant.

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Conclusion

The present study was intended to explore on rhamnolipid biosurfactant production using a native strain. The regions surveyed for the strain collection were the most diverse habitats, high rainfall tracts, and more particularly the zoosporic plant pathogens are very prevalent. Among several microbial isolates/strains, the superior strain of *Pseudomonas aeruginosa*, Pa24, was found to produce maximum rhamnolipid biosurfactant utilizing mineral glycerol and other vegetable oils as carbon source. The analysis through TLC and HPTLC clearly showed the presence of both dirhamnolipid and monorhamnolipid in the extracted product. The *in-vitro* studies against *P. capsici* and *P. infestans* with the rhamnolipid produced offer further prospect on the chances of developing natural origin rhamnolipid biosurfactants to manage zoosporic and other plant pathogenic fungi on crop plants. More targeted studies, however, to develop methods to scale up production of rhamnolipid biosurfactant and also exploration of renewable resources including agricultural industrial wastes as substrate for the bacterial strain for higher and efficient rhamnolipid production, developing effective formulations, field efficacy and animal toxicity studies, should be the way forward.

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