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Screening, stability and antibacterial potential of rhamnolipids from *Pseudomonas* sp., isolated from hydrocarbon contaminated soil

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ABSTRACT

Surfactant plays an important role in industrial application such as oil recovery, lubricants and emulsifier. But chemical surfactants are toxic to human and other small animals. In recent years, biological based surfactants have gained increasing attention due to their ecofriendly in nature. The present study was focused to isolate biosurfactant producing bacteria, their stability and antibacterial ability from hydrocarbon contaminated and uncontaminated soil collected from different locations in Kanchipuram, Tamil Nadu, India. Biosurfactant producing bacteria were screened by following the haemolytic activity, drop collapsing test, emulsion against kerosene and was further confirmed through surface activity. The stability of the biosurfactant was determined by different physic-chemical conditions like pH, temperature and salinity. A total of 37 strains were selected in three different samples based on cultural characters and finally only 7 strains were confirmed as positive for biosurfactant. Among these strain H11 was considered as potential based on emulsification index (44%), surface activity (34.45 x 10-3 nm-1) and surface tension (23.17 x 10-3 nm-1) and was identified as Pseudomonas sp. The emulsification activity was stable at broad range of pH (4-12), temperature (4-120°C) and salt concentration (0-10%). The biosurfactant was further characterized in HPLC and one major peak was observed at a retention time of 2.033. The antibacterial activity of biosurfactant was high against gram positive pathogenic bacteria than gram negative bacteria. The rhamnolipid produced Pseudomonas sp. may be used as a tool to manage the oil pollution and to control the disease causing bacteria.

INTRODUCTION

Surfactants are chemical compounds that lower the surface tension of a liquid. Such compounds have a predilection for interfaces of dissimilar polarities (liquid–air or liquid–liquid) and are soluble in both organic (non-polar) and aqueous (polar) solvents. Bacterial production of biosurfactant was first demonstrated in 1941 by Bushnell and Hass. According to Amiriyan *et al*. (2004) biosurfactant are classified based on chemical nature viz., glycolipids, lipopeptides, lipoproteins, fatty acids, neutral lipids, phospholipids, polymeric and particulate biosurfactant. These properties originated from amphipathic structures, which comprises both hydrophilic (head) and hydrophobic groups (tail) (Calvo *et al*., 2004), and render surfactants capable of reducing surface and interfacial tensions and forming micro-emulsion (Desai and Banat, 1997).

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Biosurfactant are heterogeneous groups of secondary metabolites from a variety of microorganism with surface active properties (Chandrasekaran and Bemiller, 1980).

In the past decades, much attention has been intended for biosurfactant owing to their advantages such as biodegra dability, low toxicity, lower critical micelle concentration, environmental compatibility, higher specificity and better activity at extreme conditions like high temperature, high pH and high salinity (Banat, 1995; Cameotra *et al*., 1998; Ron and Rosenberg, 2001; Van-Hamme *et al*., 2006; Singh *et al*., 2013).

Rhamnolipids was first isolated from *Pseudomonas aeruginosa* and this compound are predominantly constructed from the union of one or two rhamnose sugar molecules and one or two beta hydroxyl (3-hydroxy) fatty acids. Rhamnolipid with one sugar molecule are referred to as mono-rhamnolipid (L-rhamnosyl- β-hydroxydecanoyl-β-hydroxydecanoate), while those with two sugar molecules are referred to as di-rhamnolipids (L-rhamnosyl-L-rhamnosyl- β-hydroxydecanoyl-β-hydroxydecanoate).

The complete enzymatic synthesis of rhamnolipids, proceeds by sequential glycosyl transfer reaction, each reaction is catalyzed by specific rhamnosyl transferease and rhamnose acts as an efficient rhamnosyl donor in the synthesis of the rhamnolipid (Koch *et al*., 1991; Gunther *et al*., 2005). Biosurfactants having the surface properties make good candidate for enhanced oil recovery (EOR). The most effective biosurfactants reduce the surface tension (ST) of water from 72 dynes/cm to value of 25-30 dynes/cm (Georgiou *et al*., 1992; Rosenberg and Ron, 1999; Banat *et al*., 2000; Silva *et al*., 2014). Major functions of biosurfactants are foaming agents, solubilizers, wetting agents, emulsifiers, antimicrobial agents and mediators of enzyme action. On the other hand biosurfactants are also used in some cosmetic products like insect repellents, cleaners, lubricated condoms, baby products, shampoos etc., (Kosaric, 1992). The surfactin from *Bacillus subtilis* and rhamnolipid from *Pseudomonas* has a property of antimicrobial, antiadhesion, antibiofilm and detoxification of oil pollutant (Bechard *et al*., 1998; Mireles *et al*., 2001). Another most important application of biosurfactant is bioemlusifier, which stimulates oil production in marginal wells that have approached their economic limit (Banat, 1995; Ron and Rosenberg, 2001). Despite environmental application, biosurfactant have many biomedical application but only a very few work have been reported (Benincasa *et al*., 2004). Some biosurfactant are used as suitable alternative for synthetic medicines and antimicrobial agents and may be used as safe and effective therapeutic agents (Maier, 2003; Singh *et al*., 2007).

The increased need for the microbial biosurfactant has turned much attention among the researchers due to their versatile application. The main problem for the production of microbial surfactant in industry is high production cost. So, the demand of low cost raw material such as agro and industrial wastes are the best alternative sources for industrial production, these will compensates the right nutrient balance as an alternative for the substrate in the culture media (Makker and Cameotra, 1999). There are several renewable substrates from various sources, such as molasses (Makker and Cameotra, 1997), ground nut oil and corn steep liquor (Luna *et al*., 2013), potato processing effluents (Fox and Bala, 2000), olive and sunflower oil (Haba *et al*., 2000), cheese whey (Rodrigues *et al*., 2006), palm oil (Nawawi *et al*., 2010), motor lubricant oil and peanut oil (Thavasi *et al*., 2011) were used as a cheap sources for biosurfactant production. In this work, we evaluated the rhamnolipid producing *Pseudomonas sp*., from hydrocarbon contaminated soil, its stability and antibacterial activity.

MATERIAL AND METHODS

Isolation of bacteria from soil and oil sample

Soil and oil samples were collected from different locations in Kanchipuram town, Tamil Nadu, India. Soil samples were collected from hydrocarbon contaminated and agricultural fields, whereas oil samples like groundnut oil and 20W-40 oil were procured from extraction site and gasoline station respectively. All the samples were collected using new polyethylene container with utmost care to avoid contamination. The samples were transferred to the laboratory and microbiological analysis was carried out without any delay. 5 g of soil sample was added in a conical flask containing 50 ml of tap water and incubated in a rotary shaker (180 rpm) at room temperature for 21 days. Periodically 1 ml of sample was drawn at regular interval and serially diluted in sterile saline. 0.1 ml of diluted sample was spread plated onto nutrient agar medium and incubated for 7 days at room temperature (Bodour *et al*., 2003). For the isolation of bacteria from oil, 1 ml of oil sample was added into 9 ml of sterile saline and the mixture was placed on shaker for 1 h to produce well dispersed suspension (Amiriyan *et al*., 2004). Suspension was serially diluted in sterile saline, 0.1 ml of sample was spread plated onto nutrient agar medium and incubated at room temperature for 7 days. After incubation morphologically different colonies were selected from all the plates, purified and screened for biosurfactant.

Screening for biosurfactant producing strain

Hemolytic activity

In order to determine the hemolytic activity of pure isolates, 50 µl of broth culture was spot inoculated on blood agar plates incorporated with 5% human blood. Then the plates were incubated at room temperature (RT) for 24 h and clear zone was measured.

Drop collapse test

All the cultures that showed heamolysis were grown in mineral salt medium (MSM) supplemented with 0.1% of crude oil for 48 h at room temperature for drop collapse test. 96 well microtitre plate was rinsed with hot water (three times), 75% ethanol (one time), distilled water (two times) and then air dried (Hamed *et al*., 2012). 2 µl of crude oil was added to each well of a 96-well micro titer plate. The plate was equilibrated for 24 h at room temperature and then 5 µl of culture supernatant was added to the surface of the oil. The shape of the drop on the oil surface was observed within 60 seconds. 1% Sodium dodecyl sulfate (SDS) and deionized water were used as positive and negative control. If the drop of the culture was collapsed then it was considered as positive and rest of them was noted as negative.

Emulsification index

To determine the emulsification index (EI), the positive strains obtained from the drop collapse test were cultured in MSM incorporated with 0.1% of crude oil for 48 h at room temperature (Cooper and Goldenberg, 1987). 2 ml of kerosene was added with equal volume of cell free supernatant 8000 rpm for 20 min and vortex at high speed for 2 min, then the mixture was allowed to stand for 24 h. After 24 h, all the tubes were measured for the emulsification index by using the following formula

Emulsification index (E_{24}%) = height of the emulsion layer/total height of the mixture x 100

Blue agar method

This is the specific screening test used to detect extra cellular rhamnolipid production. The biosurfactant producing isolates confirmed from primary tests were inoculated onto previously prepared blue agar plate and incubated at room temperature for 24-48 h. After incubation, the plates were visually examined for the presence of dark blue halos around the colonies. If the colonies were surrounded by dark blue halo the result was scored as positive. The rhamnolipid concentration is proportional to halo diameter.

Quantitative test – drop weight method for the estimation of surface activity

The potential strain was tested for the estimation of surface activity quantitatively by the drop weighed method as described by Sabesan *et al*. (2002). The cell suspension in MSM was centrifuged at 8000 rpm for 20 min and the cell free supernatant was poured into a burette. The bottom of the burette was attached with glass tube via rubber tube. An empty preweighed beaker was placed under burette and the supernatant was released slowly drop by drop and 20 drops were collected into the beaker. This was weighed to determine the weight of 20 drops. The surface tension of the cell free supernatant was calculated by using the following formula

Mass of one drop = (Beaker + sample weight) – empty beaker weight/number of drops

Surface tension (T) = $Mg/\pi r \times 10^{-3} \times nm^{-1}$

Where, M – Mass of one drop, g – Gravity, r – Radius of glass tube

Surface activity of the each isolate was calculated by the following formula

Surface activity = surface tension of unionoculated medium – surface tension of supernatant.

Production and extraction of biosurfactant in MSM

1 ml of overnight nutrient broth grown culture was inoculated into 250 ml conical flask containing 100 ml of MSM (Cameotra and Makkar, 1998). Then the flask was incubated with 180 rpm for 48 h at 37°C. After 48 h of incubation the cell free supernatant was obtained by centrifugation at 8000 rpm for 20 min. The supernatant was then precipitated by means of acidification (pH 2) with 6N hydrochloric acid and left for overnight at 4°C. Afterwards, the precipitate was centrifuged at 10000 rpm for 10 min at 4°C. Then the supernatant was discarded and the precipitate was dissolved with 0.05M bicarbonate solution (pH 8.6) and centrifuged again at 15000 rpm for 20 min at 4°C. Following centrifugation, precipitate was suspended in chloroform: methanol (2:1) mixture and centrifuged at 10000 rpm for 5 min. After that three phases were formed, the upper and middle phase containing the solvent, the lower phase containing the biosurfactant and finally solvent was evaporated. The concentrated biosurfactant was extracted three times with pure methanol. The crude biosurfactant was collected and used for further chromatographic analysis.

Production and extraction of biosurfactant in MSM with molasses and glucose

The potential biosurfactant producing strain was inoculated into MSM supplemented with glucose (2%) and incubated at RT for 48 h at 180 rpm in 250 ml conical flask. A seed culture was prepared in nutrient broth, and the culture was inoculated in 250 ml conical flask containing 100 ml MSM supplemented with molasses (2%) as a carbon source and incubated at RT at 180 rpm for 48 h (Rodrigues *et al*., 2006).

Stability of biosurfactant

Stability studies were performed with crude biosurfactant (1000 mg/l). Each 10 ml of crude biosurfactant solution was maintained in a wide range of pH (4, 6, 8, 10 and 12) in 1 h 180 rpm in a rotary shaker and emulsification index was calculated. To evaluate the temperature stability of biosurfactant, the samples were kept at 4, RT, 40, 60, 80 and 120°C for 1 h and cooled to room temperature after the emulsification index was determined. The effect of salinity was investigated on biosurfactant by dissolving crude biosurfactant with various concentrations of sodium chloride (0, 2.5, 5, 7.5 and 10%).

Thin layer chromatography

Partial purification of crude biosurfactant was done by using thin layer chromatography on silica gel plate as described by Parra *et al*. (1989).

Column chromatography for the purification of the extract

The partially purified yellow colored rhamnolipid was dissolved in 1 ml of chloroform and subjected to column chromatography. For the purification of the mono-rhamnolipid, the glass column (Length – 20cm; internal diameter – 2cm) was dry packed with silica gel 60 and eluted with chloroform: methanol (10:1). After settling and equilibration, the extract dissolved in 1 ml chloroform, was gradually poured onto the column (Zhang and Miller, 1995). The eluent was collected as different fraction at one minute interval. Each fraction was further inspected for surface activity using drop collapse method to identify the fraction containing active compounds. The entire fractions were tested by drop collapse method to identify the active fraction.

Assessment of active column chromatographic fraction using High Performance Liquid Chromatography (HPLC)

The purity of biosurfactant in pooled fraction of column chromatography was assessed using high performance liquid chromatography (Lin *et al*., 1998). The active compound was further purified by using chloroform: methanol (9:1) by Waters Breeze HPLC equipped with C_{18} reverse phase column and UV wavelength detector set at 260 nm and λmax of the sample determined by UV-Vis spectral analysis.

Antimicrobial activity

The crude biosurfactant was dissolved in methanol (1 mg/ml), sterilized by syringe filter (0.22 µm) and kept in a sterile

glass tube. This stock was used for antimicrobial activity against various human bacterial pathogens like *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebseilla sp*., *Staphylococcus aureus* and *Bacillus subtilis*.

Disc diffusion test

The susceptibility of human pathogens was tested against biosurfactant in the Muller Hinton Agar (MHA) plate (Hi-Media, India) by adopting disc diffusion test. All the test strains were inoculated into nutrient broth (NB) medium, incubated at 37°C for overnight. The OD 480 nm was adjusted to 0.1 (10^8 CFU/ml) according to McFarland standard (CLSI, 2005). Using sterile cotton swab, strains were swabbed onto the pre-solidified MHA plates and allowed to dry. Sterile discs were impregnated with the purified biosurfactant stock solution and dried. One disc was impregnated with methanol as negative control. Disc was placed onto the MHA plates with 20 mm distance. All the plates were incubated at 37°C for 24 h and clearance zone was observed after 24 h.

Well diffusion method

MHA plates were prepared and swabbed with the above pathogens. Using a sterile cork borer, well was made and 50 µl of the purified biosurfactant stock solution was added to the wells and incubated at 37°C for 24 h. After incubation, the zone of clearance was measured.

Identification of the potent strain

The potential strain was characterized by standard bacteriological procedure through biochemical test and the results were interpreted with Bergey's manual for systematic bacteriology to identify the organism.

RESULTS

Isolation and screening of biosurfactant producing bacteria from soil and oil

For the screening of biosurfactant producers, soil and oil samples were collected from different locations in Kanchipuram town, Tamil Nadu, India. Total culturable bacteria were fluctuated from 4.4 to 6.6 x 10^6 CFU/g in soil sample and 2.5 to 4.3 x 10^3 CFU/ml in oil sample (Table 1). A total of 37 isolates were selected and isolated from all the samples to screen biosurfactant activity and it was found that only 22 strains were positive in primary screening through hemolytic activity in blood agar plate. All the strains positive for hemolytic activity were further screened for drop collapse test to confirm biosurfactant producers. The results revealed that only 7 (31.8%) strains confirmed as potent biosurfactant producers. (Table 1) Among the 7 strains, the strain H11 was found high potent strain for biosurfactant activity based on emulsification (44%), surface activity (34.45 x 10^{-3} nm^{-1}) and surface tension (23.17 x 10^{-3} nm^{-1}) (Table 2 & 3). Further, biosurfactant was characterized in blue agar plate and the dark blue halo was observed around the colonies. Hence, strain H11 was considered as positive for rhamnolipid.

Production and extraction

The strain H11 was grown in MSM medium containing 2% glucose and molasses for biosynthesis of biosurfactant. The amount of biosurfactant was noticed as 4.0 mg/100 ml and 5.8 mg/100 ml in glucose and molasses respectively. This biosurfactant was reconfirmed through drop collapse and surface tension test and the surface tension was 23.17 x 10^{-3} nm^{-1} in glucose 23.14 x 10^{-3} nm^{-1} in molasses.

Table 1: Enumeration of total viable counts and isolation of biosurfactant producing organism in different samples.

Sample	Total number of colonies (CFU/ml)	No. of isolates selected	Positive for Hemolysis	Positive for Drop collapse	Number of biosurfactant producers
Soil Hydro carbon contaminated	4.4 x 10^6	12	8 (66.6%)	3 (37.5%)	3 (25%)
Agricultural field **Oil**	6.6 x 10^6	10	6 (60%)	2 (33.3%)	2 (20%)
Oil extraction site	2.5 x 10^3	6	3 (50%)	1 (33.3%)	1 (16.6%)
Mechanic shed	4.3 x 10^3	9	5 (55.5%)	1 (20%)	1 (11.1%)

Table 2: Emulsification activity of the biosurfactant with kerosene.

Strain name	Emulsification index (E_{24})
O1	10%
O9	6.6%
H2	11%
H4	11%
H11	**44%**
H17	42%
H21	20%

Table 3: Quantitative measurement of surface activity and surface tension by drop collapse weight method

Strain name	Surface activity x 10^{-3} nm^{-1}	Surface tension x 10^{-3} nm^{-1}
O1	14.52	55.41
O9	15.46	52.78
H2	17.54	50.74
H4	22.48	45.72
H11	**34.45**	**23.17**
H17	29.68	33.49
H21	19.49	47.73

Stability of biosurfactant

The application of biosurfactant in several fields depends on their stability at different pH, temperature and salinity. In our observation, emulsification index of the biosurfactant was stable at neutral pH. In acidic and alkaline condition the stability was fluctuated between 4 and 12 (Fig 1-A). The stability of biosurfactant in different temperatures was shown in Fig 1-B and found to withstand even up to 120°C with optimum activity at 60°C. Similarly, in different salt concentration the stability of biosurfactant was constant at 2.5% NaCl and the stability was noticed up to 10% NaCl in lab condition (Fig 1-C).

Purification of compound by chromatography technique

The biosurfactant was partially purified from crude extract with chloroform:methanol:water by thin layer chromatography. Two spots were observed with spot 1 R_f value of 0.56 and spot 2 R_f value of 0.63. Spot 1 was confirmed by drop collapse and subjected to column and HPLC chromatographical techniques. The fraction was collected and tested for drop collapse assay (data not shown) and only one fraction corresponding to the peak retention time of 2.033 min in HPLC to be active and showed the high emulsification activity (Fig 2).

Antimicrobial activity

Antimicrobial activity of purified biosurfactant showed broad spectrum of activity against the pathogenic bacteria tested. The result showed that the highest activity against *Bacillus subtilis* (11 mm in disc and 12 mm in well) followed by *Staphylococcus aureus* and *Escherichia coli*. The least activity was observed against *Pseudomonas aeruginosa* (8 mm in disc and 9 mm in well) (Table 4).

Table 4: Antimicrobial activity by disc and well diffusion technique.

Test organism	Disc diffusion (mm)	Well diffusion (mm)
Staphylococcus aureus	10	11
Bacillus subtilis	11	12
Escherichia coli	10	9
Pseudomonas aeruginosa	8	9
Klebseilla sp.	9	10

The potential strain was identified as *Pseudomonas sp*., based on cultural characteristics and biochemical tests.

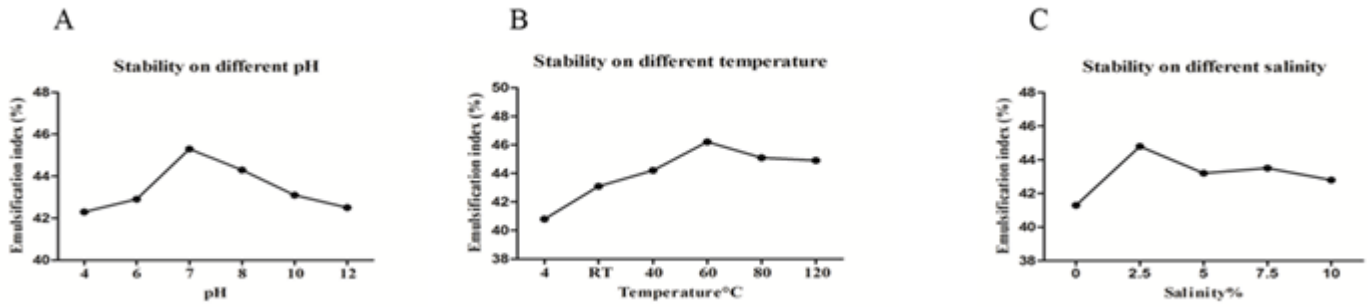

Fig. 1: Effect of pH (A), temperature (B) and salinity (C).

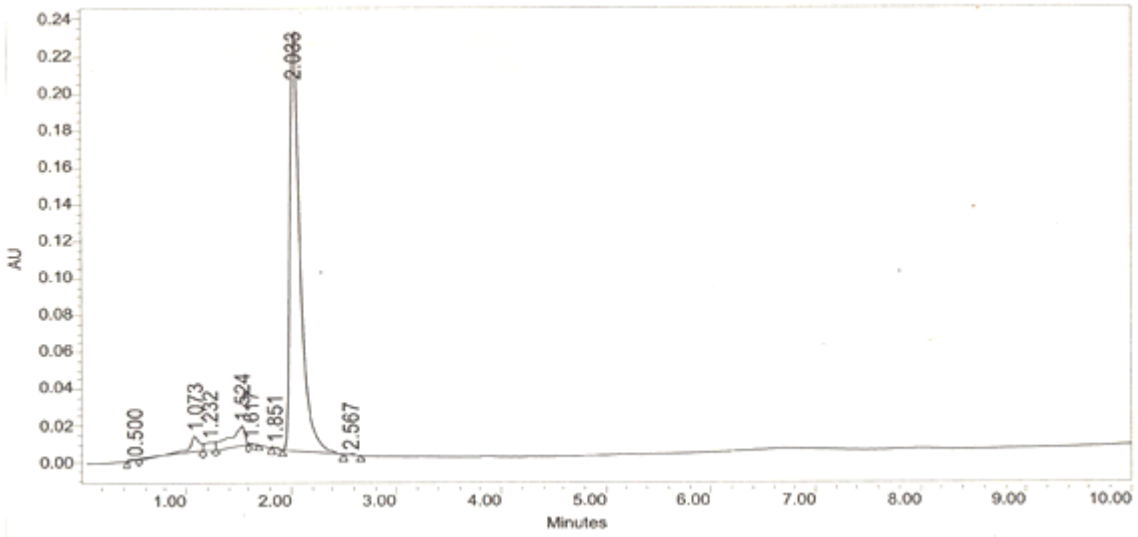

Fig. 2: RP-HPLC Chromatogram of rhamnolipid at 260nm.

DISCUSSION

In present days, surfactant plays a very significant role in food industry, bioremediation and medical application. So, researchers are paying attention on screening and isolation of biosurfactant from natural environment. A total of seven biosurfactant producing isolates were obtained from soil and oil samples. Bodour *et al*. (2003) obtained 45 biosurfactant producing isolates from hydrocarbon contaminated South Western soils of Arizona and most of the surfactant producing isolates was tending from contaminated and co-contaminated soil. Amiriyan *et al*. (2004) reported 11 biosurfactant producers from Iranian oil reservoirs. The present findings are in accordance with the findings of Bodour *et al*. (2003) and Amiriyan *et al*. (2004). However, findings of Ramesh *et al*. (2010) was contrary from this study who reported biosurfactant only in hydrocarbon contaminated soil. But in this study, biosurfactant produced organisms was isolated both in contaminated and uncontaminated soil. This might be due to the nature of the soil and nutrient content present in it. Out of 37 strains, only 22 strains were positive for hemolytic activity. The hemolysis was used widely to screen biosurfactant in microbes (Banat 1993; Carrillo *et al*., 1996; Yonebayashi *et al*., 2000). The hemolytic activity of biosurfactant was first studied by Bernheimer and Avigad (1970) in *B. subtilis*. Carrillo *et al*. (1996) found an association between hemolytic activity and surfactant production, and they recommended the use of blood agar lysis as a primary screening. Bodour *et al*. (2003) used drop collapse assay for screening surfactant producing isolates, they emulsified the coated oil in culture broth and made micro emulsion. Because of the formation of micro emulsions, the culture drops collapsed in coated oil. No other isolates except biosurfactant producing organisms, form micro emulsion in this specific screening test. In the present study, 22 strains were screened based on hemolysis, and only 7 strains were confirmed through drop collapse assay. Therefore, the results obtained in this study are more valuable in application point of view.

For the detection of potential strain, Amiriyan *et al*. (2004) measured the E_{24} value of isolates showed over 60% emulsification activity with kerosene. Aparna *et al*. (2012) used different hydrocarbon and vegetable oil for emulsification activity. They found vegetable oil give more emulsification when compare to hydrocarbons. Sunflower oil produces 84.0%, kerosene 67.5% and benzene produces 33.8%. Thavasi *et al*. (2011) isolated *P. aeruginosa* from sea water in Tuticorin harbor area used different hydrocarbon and Triton X-100. Maximum emulsification showed in Triton X-100 against waste motor lubricant oil. However, in the present study, the potential isolate showed only 44% emulsification. This might be due to poor emulsification ability with kerosene. The isolates may have the ability to emulsify more when using other suitable hydrocarbons or vegetable oil than kerosene. Siegmund and Wagner (1991) and Deziel *et al*. (1996) who recommended the blue agar plate technique for screening anionic glycolipid surfactant. Rhamnolipids are one of the members of anionic glycolipid biosurfactants. They form an insoluble ion pair with the cationic tenside cetyl trimethyl ammonium bromide and the basic dye methylene blue, which are included in mineral agar plates. In these study also dark blue halos was noticed in potential isolates around the colonies. This confirmed the surfactant produced by the potential strain as rhamnolipids. Production of biosurfactant is not economically impressive. To reduce the production cost of biosurfactants, various agro-industrial wastes are recommended by Maneerat (2005). Molasses have tremendous potential to support microbial growth and surfactant production. Patel and Desai (1997) used molasses for biosurfactant production from *P. aeruginosa* GS3 and yield was 0.25 g/l. Molasses is a cheap and easily available in India and it contains more than 50% carbon. Aparna *et al.* (2012) studied the production of biosurfactant by using different carbon sources like molasses (4.97 g/l), glycerol (4.14 g/l), coconut oil cake (4.38 g/l), orange peelings (3.24 g/l) and whey (4.09 g/l). They also attempted without carbon source and the yield was noted as 0.22 g/l. In the present study the amount of biosurfactant produced was 0.58 g/l and 0.40 g/l in molasses and glucose at a concentration of 20 g/l. This was higher when compared to Patel and Desai (1997) and lower to Aparna *et al*. (2012).

Sriram *et al*. (2011) who found the stability of biosurfactant from *B. cereus* NK1 (GU 167978) in alkaline pH. They also noticed the biosurfactant withstand at 120˚C for 1 h and resisted at 15% NaCl concentration. Luna *et al*. (2013) reported *Candida sphaerica* in the cell free broth stable at wide range of pH (2-12), stable 1 h at extreme temperature 120°C and withstand NaCl concentration of 10%. In the present study, the biosurfactant produced by *Pseudomonas sp*., had the capability of withstand at wide elevated pH ranges, and not in acidic condition, because in acidic condition biosurfactant can normally precipitated. However, alkaline condition is very useful to treat the oil spilling in marine environment extreme temperature and high salinity. This gives a better idea to use biosurfactant in an extreme condition prevailing in the environment. Crude compound of biosurfactant produced brown spot on the TLC plate and the R_f value was calculated as 0.59 (Parra *et al*., 1989). Same procedure was followed by Zhang and Miller in 1994, the R_f value was calculated as 0.70. R_f value noted as 0.52 and 0.69 in the crude extract biosurfactant visualized under UV-illuminator by Sriram *et al*. (2011). In the present study, two spots were observed and the R_f value was calculated as 0.56 and 0.68. This is in agreement with the findings of previous studies reported elsewhere. Further purification of compound was achieved by column chromatography. Stanghellini (1997) reported the purification of rhamnolipid in column chromatography by using the solvent system chloroform: methanol in the concentration of 9:1 and there was a contamination with di-rhamnolipid. In the present study, compound from the column chromatography was further purified by reverse phase HPLC. This was a better purified compound compared to the previous study. Zgola-Grzeskowiak and Kaczorek isolated rhamnolipid using different solvent system. They tested the mono-rhamnolipid Rha C10C10 and di-rhamnolipid RhaRhaC10C10. They identified different types of mono and di-rhamnolipid by reverse phase

HPLC, and had a retention time of 1.42 in mono-rhamnolipid and retention time of 1.02 in di-rhamnolipid. However, in our study only one major peak was observed with the retention time of 2.033 and this was confirmed as mon-orhamnolipid. Abalos *et al*. (2001) reported that mixture of rhamnolipid produced by strain M7 *P. aeruginosa* AT10 was active against wide range of microbes including gram positive and gram negative organisms. Das *et al*. (2008) found that surfactin produced by *B. circulans* had antimicrobial activity, it active against most gram positive bacteria than gram negative bacteria. Kiran *et al*. (2010) also found that column fraction having the high activity comparing to ethyl acetate extract and supernatant, however it has less activity against the gram positive bacteria *Bacillus sp*., yeast of *C. albicans* followed by *K. pneumonia*. Similar findings were also evident in the present study and it was found that antimicrobial activity was high against gram positive organisms compared to gram negative one.

CONCLUSION

Pseudomonas spp., isolated from the hydrocarbon contaminated soil a potent biosurfactant producer. Fascinatingly, biosurfactant was stable at wide pH ranges, temperature and NaCl concentration. At the same time it has antimicrobial activity against the various pathogens. Hence, we suggested that the biosurfactant produced by *Pseudomonas* sp., will be an immense useful both environmental as well as biomedical application.

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