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Extraction and antimicrobial activity of rhamnolipid biosurfactant produced by *Pseudomonas aeruginosa* UKMP14T

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ABSTRACT

Aims: Rhamnolipids are seeking utmost attention as a new class of biosurfactants having promising potential in diverse fields as they offer a wide range of advantages over chemically synthesised surfactants. However, the high extraction costs make large scale production face difficulty. In present study, hydrocarbon degrading bacteria *Pseudomonas aeruginosa* UKMP14T was exploited for its biosurfactant producing ability including a comparative study between different extraction procedures for its recovery. In addition to this, the recovered biosurfactant was explored for its potential application as an antimicrobial agent.
Methodology and results: The production of rhamnolipid biosurfactant was confirmed through various detection methods which are drop-collapse test, oil spreading assay, emulsification index, cetyltrimethylammonium bromide (CTAB) assay and hemolytic assay. The test strain *P. aeruginosa* UKMP14T showed positive results for all the detection assays. Following this, shake flask cultivation was carried out for several time intervals (1, 3, 5, 7 and 9 days) to discover the optimum time for rhamnolipid biosurfactant production. The results were evaluated by quantifying the rhamnolipid yield using Anthrone method and maximum yield was obtained on day 7. Then, three commonly employed rhamnolipid biosurfactant extraction methods (acid precipitation, solvent extraction and zinc sulphate precipitation) were incorporated for the extraction of rhamnolipid biosurfactant. Among these methods, organic solvent extraction (using methanol, chloroform and acetone in 1:1:1 ratio) gave the highest yield (7.37 ± 0.81 g/L) of biosurfactant, followed by zinc sulphate precipitation (5.83 ± 0.02 g/L), whereas acid precipitation gave the lowest yield (2.8 ± 0.12 g/L) and required longer time (30 days). Finally, the antimicrobial activity of several concentrations of rhamnolipid was tested using modified microdilution method and highest antibacterial activity (in the form of percent reduction in growth) of 95.05% and 91.89% was recorded for *Escherichia coli* ATCC 10536 and *Staphylococcus aureus* ATCC 11632, respectively, at 100 µg/mL concentration of rhamnolipid biosurfactant.
Conclusion, significance and impact of study: The ability of *P. aeruginosa* UKMP14T in producing rhamnolipid biosurfactant was confirmed. Despite the higher yield obtained by organic solvent extraction method, the recovery technique (involving the separation of solvent system) caused some loss in product. In addition, the transfer and storage of rhamnolipid was challenging using solvent extraction in comparison to acid precipitation and zinc sulphate precipitation. On the other hand, recovery using acid precipitation suffered from lowest yield of rhamnolipid. Therefore, zinc sulphate precipitation is prioritised over the other two methods. Furthermore, the antimicrobial potential of rhamnolipid biosurfactant was tested successfully for as low as 10 µg/mL concentration against *E. coli* ATCC 10536 and *S. aureus* ATCC 11632. Therefore, the recovery cost of a high value product like rhamnolipid can be reduced by incorporating the results of this study in the downstream processing and promote rhamnolipid biosurfactant as a potential antimicrobial agent.

Keywords: Rhamnolipid, biosurfactant, antimicrobial, *Pseudomonas aeruginosa*, extraction

INTRODUCTION

Biosurfactants are the surfactants of low molecular weight produced by microorganisms. They are heterogeneous group of surface active molecules which are amphiphilic in nature produced by microbes on cell surface or secreted extracellularly. They consist of a hydrophilic (polar or nonpolar) and a hydrophobic moiety (lipid or fatty acid) in their structure. Due to this unique property, they possess the ability to solubilise insoluble compounds like

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hydrocarbons (Shah *et al*., 2016). The ability of biosurfactants to reduce the surface and interfacial tension afford them exceptional detergency, emulsification, foaming, and dispersing characteristics (Shekhar *et al*., 2015). They offer several advantages over synthetic surfactants as they are diverse, biodegradable and have minimal toxicity. In addition, biosurfactants are also effective at extreme levels of temperature, pH and wide range of salt concentrations (Muthusamy *et al*., 2008). Microbial biosurfactants have made their mark with respect to their applicability in environmental protection, which majorly includes the enhancement of oil recovery, oil spills control, biodegradation and detoxification of industrial effluents and soils polluted with oil. Apart from this, biosurfactants are also well known for their application in pharmaceutical, medical, food, cosmetic and pesticides industries (Nitschke and Costa, 2007; Banat *et al*., 2010; Soberon-Chavez and Maier, 2011; Fracchia *et al*., 2012; Gudina *et al*., 2013).

Rhamnolipids are one of the prominent categories of biosurfactants produced by a variety of microorganisms like *Pseudomonas aeruginosa*, *P. fluorescens* and *Serratia rubidaea* (El-Amine *et al*., 2012; Nalini and Parthasarathi, 2014). Among these, *P. aeruginosa* is said to be the best strain for rhamnolipid production, and is studied extensively (Rikalovic *et al*., 2012). Applications of rhamnolipids are noteworthy in various industries such as agriculture, microbial enhanced oil recovery and bioremediation of oil-contaminated sites (Banat *et al*., 2000). Although considerable amount of work has been dedicated in improvisation of fermentation technology, the applicability of biosurfactants still remains limited. The main reason for this is the high cost incurred in product recovery due to the infeasibility of methods at a large scale (Shah *et al*., 2016).

The potential applications of biosurfactants from the genus *Pseudomonas* as a successful antimicrobial agent have been demonstrated by several researchers. For example, the biosurfactant produced by *P. aeruginosa* PB3A showed wide range of antimicrobial activity against *Staphylococcus aureus* ATCC 29736, *S. epidermidis* ATCC 12228*, Escherichia coli* ATCC 8739 and *P. aeruginosa* ATCC 27853 strains used in the study conducted by Vijayakumar and Saravanan in 2015. In addition, Rodrigues *et al*. (2004) demonstrated that rhamnolipids have the antimicrobial activity against pathogenic bacteria isolated from explanted voice prostheses to silicone rubber. Furthermore, rhamnolipid applications as antimicrobial agent in preventing food spoilage and for sanitization in food manufacturing industries have been pointed out by several researchers (Irie *et al*., 2005; Vatsa *et al*., 2010). The prevention of biofilm formation owing to the anti-adhesive nature of rhamnolipid has also been highlighted against the foodborne pathogenic bacteria *Listeria monocytogenes* and *Bacillus subtilis* (Magalhaes and Nitschke, 2013).

Despite the numerous applications afforded by rhamnolipid biosurfactants, they still lack a proper recognition in market because of the high production costs involved coupled with minimal yield obtained. Therefore, a comparative analysis between the extraction methods can contribute to knowledge that can be incorporated as a better extraction method that can be feasible even at industrial level/ for large scale production. Additionally, the potential of rhamnolipids as antimicrobials has already been described by several researchers. However, this paper provides a preliminary analysis of a rhamnolipid biosurfactant as a potential antibacterial agent being extracted from a native bacterial species (*P. aeruginosa* UKMP14T) sampled from Malaysia.

MATERIALS AND METHODS

Inoculum and media preparation

The bacterial strain *P. aeruginosa* UKMP14T previously isolated from oil contaminated soil was obtained from Culture Collections Laboratory, Department of Biological Sciences and Biotechnology, Universiti Kebangsaan Malaysia. Firstly, the culture was subjected for revival in order to prepare the inoculum. The mother culture was revived following the standard microbiological protocols. Briefly, a loop full of culture from the previous stock was inoculated into a 100 mL shake flask containing 50 mL nutrient broth and was kept in incubator shaker at a speed of 180 rpm for 24 h. After 24 h, a loop full of inoculum was taken from the fresh broth and streaked over several nutrient agar plates using quadrant streaking technique. The plates were further incubated and kept at 4 °C for later use. Modified mineral salt medium (MSM) was used throughout the study for production of rhamnolipid biosurfactant. This media contained 0.1% (v/v) vitamins and trace elements and for carbon source it was supplemented with 1% (v/v) glycerol (Hamzah *et al*., 2010). The pH of the medium was adjusted to 7.0 with 1 M sodium hydroxide (NaOH) or 1 M hydrochloric acid (HCl). The medium was autoclaved at 121 °C, 15 psi for 15 min before use.

Screening for biosurfactant production

A standard inoculum of *P. aeruginosa* UKMP14T was prepared in nutrient agar according to the method of Hamzah *et al.* (2010). The density of cells was standardised by adjusting the OD reading to 0.5 at 590 nm which approximates to 10^5 CFU/mL. About 10% (v/v) of this inoculum was inoculated into 50 mL of MSM contained in a 250 mL conical flask and incubated as previously described. After a period of 24 h, the culture medium was centrifuged at 8022 *g* (RC5C Sorvall Centrifuge Instrument, USA) at 4 °C for 30 min. The supernatant was collected and used for preliminary screening of biosurfactant presence.

Qualitative screening for biosurfactant detection drop-collapse test

The drop-collapse test was carried out as described by

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Bodour and Miller-Maier (1998). Briefly, 2 µL of Tapis crude oil (supplied by Petronas Research Scientific Berhad, Malaysia) was applied to the wells of a 96 well plate (12.7 × 8.6 cm^2) (Corning Incorporated, USA) and left to equilibrate for a period of 24 h (Hamzah *et al*., 2013). Then, a drop of supernatant (after removing cells by centrifuging at 8022 *g* for 30 mins) was placed on the oil-coated wells. The drop was observed after 1 min using a magnifying glass. Sodium dodecyl sulphate (SDS) and distilled water were used as positive and negative controls respectively. The results were recorded as negative if the drop remained beaded even after 1 min and, scored positive if the drop collapsed.

Oil spreading assay

The oil spreading technique was performed based on the method suggested by Youssef *et al*. (2004). In this method, 20 mL of distilled water was added to a Petri dish (90 mm × 15 mm) followed by addition of 20 µL of Tapis crude oil to the surface of water. Then, 10 µL of supernatant was dropped onto the Tapis crude oil surface. The Petri dish was observed for appearance of oil free clear zone on the surface of water which is an indication of the surfactant activity. SDS and distilled water were used as positive and negative controls, respectively.

Emulsification index (*%EI_{24}*)

The %EI_{24} was determined using the method based on Cooper and Goldenberg (1987). Equal volume of Tapis crude oil was added to the supernatant (1:1) and was mixed using a glass test tube (125 mm × 15 mm). Then, the mixture was vortexed for 2 min and left to stand for 24 h. The %EI_{24} was calculated using the formula below. A higher value indicates a higher emulsification activity of the tested surfactant. SDS was used as the positive control, whereas distilled water was used as the negative control for this assay.

$$\% EI_{24} = \frac{\text{Height of the emulsion layer (mm)}}{\text{Total height (mm)}} \times 100$$

Hemolytic assay

Fresh blood agar plates were prepared and pure culture of *P. aeruginosa* UKMP14T was streaked on the agar plate and incubated at 37 °C for 24-48 h. The presence of clear zone around the colonies indicated the presence of biosurfactant producing organisms. Results were recorded based on the type of clear zone observed i.e., when the colony was surrounded by greenish zone — α-hemolysis, clear white zone — β-hemolysis and no change in the medium surrounding the colony — γ-hemolysis (Carrillo *et al*., 1996).

Cetyltrimethylammonium bromide (CTAB) assay

This method was performed as described by Vijayakumar and Saravanan (2015). Briefly, mineral salt agar medium supplemented with glucose as carbon source (2%) and cetyltrimethylammonium bromide (CTAB: 0.5 mg/mL) and methylene blue (MB: 0.2 mg/mL) was autoclaved at 121 °C for 15 min. After autoclaving, the media was poured into Petri dishes and allowed to solidify. Then, the wells were punctured into the agar using borer (4 mm). Following this, 30 µL of culture broth supernatant was added into the wells using micropipette. Similarly, SDS and sterile distilled water were added to the methylene blue agar plate wells as positive and negative controls, respectively, and the plates were incubated at 37 °C for 48-72 h in an incubator (Yihder Technology, China). A dark blue halo zone around the well was taken as a positive result for anionic biosurfactant production.

Quantitative screening for biosurfactant detection

Rhamnolipid was quantified using Anthrone method as described below (Bailey, 1958). The quantification of rhamnolipid was carried out in an indirect way, by measuring the amount of rhamnose. Rhamnose is a byproduct obtained on acid hydrolysis of rhamnolipids and this was used as a reference in this assay. Therefore, the extracellularly produced rhamnolipid was quantified by measuring the concentration of rhamnose. At first, Anthrone reagent was prepared by dissolving about 20 mg of Anthrone (Sigma Aldrich, USA) in 70% sulphuric acid (H_2SO_4) and it was allowed to stand for 2 h at 4 °C. Following this, 0.2–1 mL rhamnose solutions were pipetted into clean and dry test tubes (20 cm × 2 cm). In addition to this, 0.5 mL of culture supernatant was pipetted into separate test tubes labelled as 'Test'. The volume in each tube was made up to 1 mL with distilled water. The tubes were kept in ice and agitated whilst the Anthrone reagent (4 mL) was being added slowly to each tube. The tubes were stoppered, and the contents were vortexed for a minute. Then, the tubes were kept in a boiling water bath at 90 °C for 10 min and then transferred to ice box for cooling. Following this, the tubes were left in dark for 30 min. The greenish-blue color developed was measured against blank at an absorbance of 630 nm in a spectrophotometer (Biochrom Libra, UK). A standard curve of L-rhamnose was plotted and the rhamnolipid biosurfactant concentration was calculated by extrapolating the OD values obtained.

Shake flask cultivation

To optimise the best time interval required for the production of biosurfactant, a standardised inoculum of the isolate was prepared as described earlier. Then, 10% (v/v) of this standardized inoculum was inoculated into five separate flasks (500 mL capacity) containing 200 mL of MSM. Further, 1% (v/v) glycerol was added to all flasks and incubated at 37 °C on an orbital shaker with agitation speed 150 rpm. Later, the contents from respectively

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labelled flasks were withdrawn on day 1, 3, 5, 7 and 9 for quantifying the rhamnolipid using Anthrone assay as discussed in the previous section.

Biosurfactant extraction

After incubating for optimum number of days required for biosurfactant production, the culture medium was centrifuged at 8022 *g* (RC5C Sorvall Centrifuge Instrument, USA) at 4 °C for 30 min. The supernatant was collected and used for extraction of the rhamnolipid biosurfactant using three separate approaches as mentioned below.

Acid precipitation

The supernatant containing biosurfactant was acidified to pH 2.0 using 1 M HCl. This was left up to 4 weeks at 4 °C until the biosurfactant precipitated completely. At 1st, 2nd, 3rd and 4th week interval, the solution containing the precipitate was centrifuged at 8022 *g* at 4 °C for 30 min. Then, the precipitate was dissolved in 0.1 M sodium bicarbonate ($NaHCO_3$), (pH 8.0) and then re-acidified and re-centrifuged. Later, chloroform-methanol (2:1, v/v) was used to extract the precipitate. The organic phase was evaporated on a rotary evaporator (EYELA NH001S-W, Tokyo Rikakikai, Japan) to remove the solvent. Finally, a viscous dark-brown product was obtained which was the biosurfactant and this was weighed after drying to quantify the yield (El-Sheshtawy *et al*., 2015).

Solvent extraction

The supernatant from previous step of centrifugation was treated with a mixture of extraction solvent (methanol/ chloroform/acetone) in the ratio of 1:1:1 by volume. The mixture was transferred to an incubator shaker and continuously shaken at 200 rpm, 30 °C for 5 h. At the end of 5 h, two layers of precipitate were obtained. The upper layer was discarded and the bottom layer was left to dry overnight. Finally, the dry powder obtained at the end was weighed on a weighing scale (Mukherjee *et al*., 2006).

Zinc sulphate precipitation method

To the supernatant containing biosurfactant, 40% (w/v) zinc sulphate was gradually added in order to precipitate the biosurfactant. This mixture was kept for incubation at 4 °C for 24 h. Then, the contents were centrifuged at 8022 *g*, 4 °C for 30 min to obtain the biosurfactant, which was then dried by placing the tubes inside a pre-sterilised box lined with filter papers and allowed to stand for 24-48 h at 27 °C inside a laminar flow unit until the biosurfactant was dry. Finally, the product was weighed to quantify the yield (Shah *et al*., 2016).

Antimicrobial activity of biosurfactant

The antimicrobial activity of the extracted biosurfactant was tested against *E. coli* ATCC 10536 and *S. aureus* ATCC 11632 obtained from UKM culture collection and determined in 96-well plates using a modified microdilution approach (Sambanthamoorthy *et al*., 2014). Briefly, 100 µL of sterile Muller Hinton broth (MHB) was pipetted into the first column of the 96 wells microplate which served as the bacterial control. The second column had 100 µL of biosurfactant solution in PBS (200 µg/mL) which was mixed with the medium; this resulted in a biosurfactant concentration of 100 µg/mL and 100 µL was transferred serially to two subsequent rows resulting in two-fold dilution of 50 and 25 µg/mL, whereas the last column had 10 µg/mL concentration of biosurfactant (prepared separately). Wells without biosurfactant served as negative controls and wells with ampicillin (50 µg/mL) served as positive controls of the assay. All wells were inoculated with 100 µL of 10^8 CFU from a log phase culture of each test strain. The plates were then covered and incubated at 37 °C for 24 h. All the experiments were done in triplicates. The contents of each well were plated onto LB agar plates with appropriate dilutions and CFU were enumerated the next day. Percent reduction in growth was calculated using the formula below:

$$\text{Percent reduction} = \frac{\text{Control-Treatment}}{\text{Control}} \times 100$$

Where, Control= number of CFU for untreated samples; Treatment= number of CFU for samples treated with rhamnolipid biosurfactant

Biosurfactant efficiency

The extracted biosurfactant which was stored at –20 °C in air-tight tubes was used for these experiments. After a period of 1, 3, 6 and 9 months, the tubes were picked from the cold storage and left to stand until they reached room temperature. Then, the agar well diffusion assay was performed to learn the effect of storage on the effectiveness of biosurfactant activity (Joshi *et al*., 2009). Briefly, an overnight nutrient broth culture of test pathogens was prepared, and this was swabbed to make a lawn culture over the Muller-Hinton Agar (MHA) plates using sterile hockey stick. After lawn preparation, the plates were kept aside until the cultures had dried over the agar surface, then using a sterile borer; wells (8 mm) were punched in agar before loading 100 µL biosurfactant solution of 100 µg/mL concentration. Distilled water served as a negative control, whereas ampicillin (50 µg/mL) served as the positive control. The plates were incubated for 18-24 h and results were recorded as the diameter of the zone of inhibition (ZOI).

Statistical analysis

The data represented for all the experiments is the arithmetic mean of at least three replicates. The variability in mean values has been performed by introduction of standard deviation standard error and variance in the mean values to each experimental output. For the

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comparison of means, T-test was done using IBM SPSS statistics software version 25.

RESULTS AND DISCUSSION

Biosurfactant detection

It is highly recommended by Sekhon *et al*. (2011) to apply more than one detection method to confirm the production of biosurfactant. Therefore, five highly recommended methods were applied. The strain *P. aeruginosa* UKMP14T showed positive results for all the screening methods: drop collapse method, oil spreading assay, emulsification assay, hemolytic assay and CTAB assay.

The biosurfactant solution collapsed in less than a minute on the oil for drop collapse test indicating the presence of biosurfactant in higher levels (Supplementary Figure 1). However, this method is not sensitive enough to detect lower levels of biosurfactant (Youssef *et al*., 2004). The zone of clearance was observed distinctly, for the oil spreading assay as well, which according to the same researcher is a far more superior assay for testing the presence of biosurfactant. A value of 48.28 ± 1.67 was obtained for the $\%EI_{24}$ (Supplementary Figure 2). The value obtained in our study is lower in comparison to the $\%EI_{24}$ values obtained by other researchers (Hamzah *et al*., 2013; Vijayakumar and Saravanan, 2015; Ferhat *et al*., 2017). However, the emulsion obtained was highly stable, as it was maintained as it is even after 24 h of emulsification. Therefore, these results are suggestive of biosurfactant being produced in the culture medium according to previous literature (Willumsen and Karlson 1996).

The results for hemolytic assay correlated well with findings from other researchers (Vijayakumar and Saravanan, 2015). The said researchers found an association between the productions of biosurfactant with that of the hemolytic activity. *Pseudomonas aeruginosa* UKMP14T showed clear hemolytic zone (β-hemolysis; white clearance zone around colony) in the blood agar plates confirming the presence of biosurfactant production (Supplementary Figure 3).

CTAB assay is a semi-quantitative assay for detecting biosurfactant presence, especially the anionic class of biosurfactants such as rhamnolipids (Wu *et al*., 2008). In our results, a blue color halo was observed surrounding the wells supplemented with supernatant from *P. aeruginosa* culture (Supplementary Figure 4). The principle behind dark blue halo formation is a complexation reaction between the anionic surfactant and cationic methylene blue dye. Similar results were obtained by Vijayakumar and Saravanan (2015) for strain *P. aeruginosa* PB3A used in their study. All the above five detection methods with their respective results are summarized in Table 1.

Table 1: Summary of biosurfactant detection methods.

Detection method	Observation	Inference
Drop collapse test	Drop collapsed/ flattened	Presence of biosurfactant
Oil spreading assay	Appearance of clear zone on the surface of water	Presence of biosurfactant
Emulsification assay	Formation of emulsion layer with an *%EI₂₄* value of 48.28 ± 1.67	Indirect indication of biosurfactant presence
Haemolytic assay	β-haemolysis around the colony	Presence of biosurfactant
CTAB assay	Dark blue halo formation around wells	Presence of anionic biosurfactant

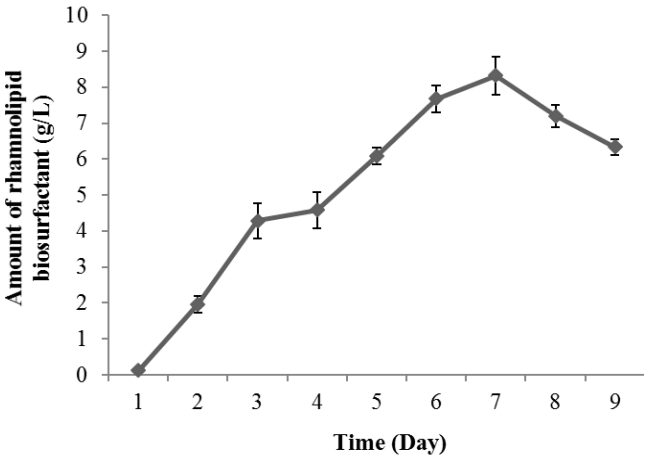

Figure 1: Amount of rhamnolipid biosurfactant produced by *P. aeruginosa* UKMP14T from day 1 to day 9 based on quantification using Anthrone assay.

Optimisation of time interval for biosurfactant production

The amount of rhamnolipid biosurfactant produced was quantified from the culture medium set up for shake flask experiments after following an incubation period of 1, 3, 5, 7 and 9 days using Anthrone method. Rhamnolipid was detected in the culture medium in small quantities within 12 h of incubation and was found to be about 0.12 ± 0.04 g/L on day 1. Then, it was found to increase to 1.96 ± 0.23 g/L on day 2 and kept on increasing thereafter. A peak was reached on day 7 and was recorded as the highest value (8.32 ± 0.52 g/L), this was followed by a decrease that was noticed day 8 onwards, falling to 6.33 ± 0.20 g/L on day 9 (Figure 1). Distinctly, the color of the medium also started to change and appeared to become reddish brown which intensified as the biosurfactant

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production increased in the medium as shown in supplementary figure 5. Similar results in relation to the appearance of brownish color in the medium have been reported by Patowary *et al.* (2017).

Biosurfactant recovery

Various methods have been used till date to recover the biosurfactant from the culture medium (Bodour *et al*., 2003; Lee *et al*., 2008; Satpute *et al*., 2010). Among the three recovery methods that were employed for the biosurfactant extraction in this study, it was found that solvent extraction was superior in comparison to acid precipitation and zinc sulphate precipitation in terms of product yield (Figure 2). Solvent extraction using methanol/chloroform/acetone (1:1:1) gave a white coloured powder having yield of 7.37 ± 0.81 g/L of the culture medium. This can be explained owing to the amphipathic nature of the lipid moiety in the rhamnolipid. The hydrophobic end of this moiety solubilises in the organic solvent and thus can be extracted almost completely. However, this method seemed a bit challenging as compared to zinc sulphate precipitation in terms of separation of the solvent system from the product which resulted in some loss of the product during the procedure. The results from this study were in good agreement with the findings from other researchers both in terms of yield as well as properties (Johny and Saravanakumari, 2013; Sharma *et al.*, 2015).

On the other hand, the second highest value for the amount of biosurfactant recovered was recorded for zinc sulphate precipitation (5.38 ± 0.02 g/L). The biosurfactant was obtained as a white colour powder that was easy to handle and store until further use. A good percent of biosurfactant was extracted using this method as the recovery depends mainly on the ionic charge, and solubility in the desired solvent (Desai and Banat, 1997; Shah *et al*., 2016).

Furthermore, acid precipitation gave the lowest yield (2.8 ± 0.12 g/L) and also took the longest recovery period of 30 days for complete precipitation of rhamnolipid (Figure 3). In addition, the biosurfactant obtained by this method was in the form of a dark brown sticky paste which was difficult to handle and transfer (Supplementary Figure 6). The transfer process of product for storage and further use from the Schott bottle (used for precipitating the rhamnolipid) was a difficult task as the product was in the form of a sticky paste, which led to loss of product. The yield obtained in present study using this method is way higher than the yield obtained by Sabturani *et al*. (2016) using solvent extraction in combination with acid precipitation (dual recovery method). Table 2 summarises a comparison between these recovery methods and in view of the results obtained in our study. It can be said that zinc sulphate precipitation is a better method in comparison to acid precipitation and organic solvent extraction. This is because long duration is required to recover the rhamnolipid biosurfactant using acid precipitation and two-fold decrease in yield of product compared to what is obtained by zinc sulphate

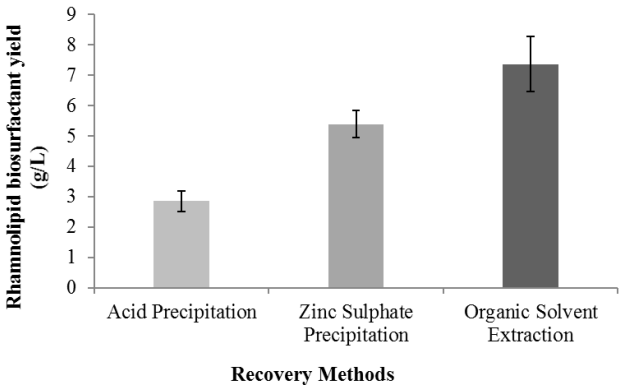

Figure 2: A comparison of methods for rhamnolipid biosurfactant recovery following 7 days fermentation.

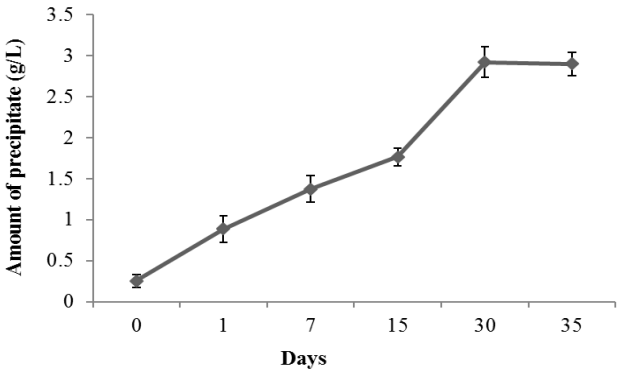

Figure 3: Amount of precipitate of rhamnolipid biosurfactant produced by *P. aeruginosa* UKMP14T using acid precipitation method from day 0 to day 35, measured by weighing dry weight of product.

precipitation. On the other hand, although the yield obtained using organic solvent extraction is higher than that achieved by zinc sulphate precipitation; the problem arises when there was a loss in product yield as explained above. In an industrial set up, it is always prefer the method that consume less time, give higher yields and easy to carry out. Therefore, with all the facts considered, we would prefer zinc sulphate precipitation over the two other methods mentioned herein.

Antimicrobial assay with rhamnolipid biosurfactant

Biosurfactants have been increasingly suggested for use in a number of industrial sectors such as hydrocarbon emulsification, solubilisation of contaminants in complex hydrocarbons, microbial enhanced oil recovery (MEOR) and so on. Apart from this, biosurfactants have also been proven to be useful as good antimicrobial agents even at lower concentrations which can find applications as biocontrol agents in healthcare sectors. This has been correlated to the incredible surface active properties they possess (Raza *et al*., 2007).

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Table 2: Comparison of rhamnolipid biosurfactant recovery methods.

Recovery method	Recovery time	Product yield (g/L)	Solvents used	Nature of recovered product	Handling
Acid precipitation	30 days	2.8 ± 0.12	1 M HCl	Blackish-brown sticky paste	Difficult
Organic solvent extraction	24 h	7.37 ± 0.81	Methanol, chloroform, acetone (1:1:1)	White coloured powder	Difficult
Zinc sulphate precipitation	24 h	5.38 ± 0.02	Zinc sulphate	Light brown colour powder	Easy

The antimicrobial activities of biosurfactant are shown by many researchers using various environmental and clinical isolates for testing. Vijaykumar and Saravanan (2015) have shown significant antimicrobial activity of biosurfactant produced from *P. aeruginosa* strain PB3A against pathogens *S. aureus*, *Staphylococcus epidermidis*, *E. coli* and *P. aeruginosa*. The antimicrobial activity of lipopeptide surfactants produced from *B. subtilis* was successfully demonstrated by Tsuge *et al*. (1996). Another researcher, Kiran *et al*. (2010) showed similar results using a biosurfactant produced from *Candida antartica.*

Therefore, in our study, the extracted rhamnolipid biosurfactant was tested against two *E. coli* ATCC 10536 and *S. aureus* ATCC 11632 in order to study its antimicrobial potential. Interestingly, it was found to be effective on both the test bacteria starting from as low as 10 µg/mL concentration in comparison to results obtained by Sambanthamoorthy *et al*. (2014). The said researcher used biosurfactant produced from *Lactobacillus jensenii* and *L. rhamnosus*. In the mentioned study, biosurfactant concentration of 50 mg/mL was found to be effective on the test bacteria; *E. coli*, and methicillin-resistant *S. aureus* (MRSA), which is very high compared to the concentrations we have used in our study (highest being 100 µg/mL). Activity accounting to 100% was obtained in their study with the biosurfactant from *L. jensenii* and between 72-85% for biosurfactant from *L. rahmnosus* against *E. coli* and 80% and 93% for *S. aureus* UAMS-1 and MRSA, respectively. However, in our study, highest antibacterial activity of 95.05% and 91.89% cell death was recorded for *E. coli* ATCC 10536 and *S. aureus* ATCC 11632, respectively at 100 µg/mL concentration of rhamnolipid biosurfactant. The values represented are the percent reduction in cell numbers when treated with rhamnolipid biosurfactant. Percentage reduction in growth has been presented in Table 3 and the results are from three independent preparations of rhamnolipid biosurfactant.

Table 3: Antimicrobial activity of rhamnolipid biosurfactant extracted using acid precipitation and measured using modified microdilution method**.**

Concentration of rhamnolipid (µg/mL)	Percentage reduction in growth (%)	
	E. coli ATCC 10536	*S. aureus* ATCC 11632
10	37.30 ± 7.63	29.10 ± 8.18
25	51.80 ± 10.26	49.46 ± 5.29
50	76.64 ± 7.23	70.22 ± 0.57
100	95.05 ± 1.15	91.89 ± 1.52
Ampicillin (50 µg/mL)*	96.45 ± 1.52	95.29 ± 2.08

*Ampicillin (50 µg/mL) was used as positive control for this experiment.

Table 4: Zone of inhibition produced by rhamnolipid biosurfactants at different intervals of storage period at –20 °C.

Test organism	Zone of inhibition (mm)			
		Storage period		
	Positive control	1 month	3 months	6 months
E. coli ATCC 10536	18.7 ± 1.21	12.33 ± 0.58	12.5 ± 0.28	11.8 ± 0.41
S. aureus ATCC 11632	10.7 ± 1.63	7.9 ± 0.80	8.2 ± 0.77	8.0 ± 0.26

Rhamnolipid biosurfactant (100 µg/mL) was used for testing. Cephalosporin (50 µg/mL) was used as positive control against *E. coli* ATCC 10536 and Tetracycline (50 µg/mL) was used as positive control against *S. aureus* ATCC 11632.

Effect of aging on rhamnolipid biosurfactant efficiency

Diameter of zone of inhibition (ZOI) recorded at different time intervals (1, 3 and 6 months) showed no significant difference in values. This shows that the rhamnolipid biosurfactant is good to use even up to a storage period of 6 months when stored at -20 °C. The results of this assay are provided in Table 4.

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CONCLUSION

The ability of *P. aeruginosa* UKMP14T to produce rhamnolipid biosurfactant was successfully detected. The biosurfactant production period was optimised, so that the future scale-up becomes easy and practically applicable even for large scale production. Although rhamnolipid biosurfactant possess unique properties making them advantageous over the synthetic surfactants, their usage is not fully flourished. This is due to the enormous financial input and industrially infeasible techniques demanded for the recovery of final product. Therefore, through our comparative analysis, we have pointed out a better method among the three common methods employed for rhamnolipid biosurfactant recovery. Although organic solvent extraction gave the highest yield, product recovery using zinc sulphate precipitation was much easier and feasible method in terms of recovery technique used and time consumption. In addition, the rhamnolipid biosurfactant recovered by zinc sulphate precipitation was easy to store for further usage. Lastly, rhamnolipid biosurfactant showed antimicrobial activity against clinically important bacteria like *E. coli* ATCC 10536 and *S. aureus* ATCC 11632. The findings from this research are surely a key factor in promoting rhamnolipid biosurfactant application in diverse fields, especially in combating the clinically prominent bacteria like *E. coli* and *S. aureus*.

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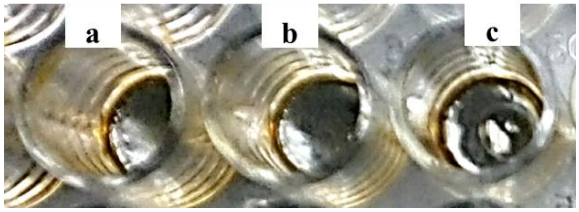
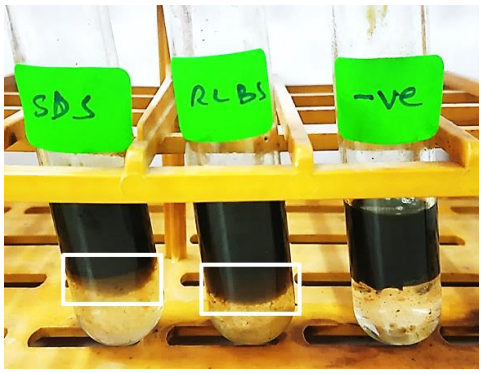
SUPPLEMENTARY INFORMATION



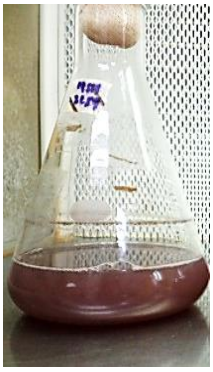
Supplementary Figure 1: Drop collapse assay with a) supernatant from *P. aeruginosa* UKMP14T culture (test), b) 1% SDS (positive control) and c) distilled water (negative control). Drop has collapsed in well (a) and (b), whereas remained beaded in (c).



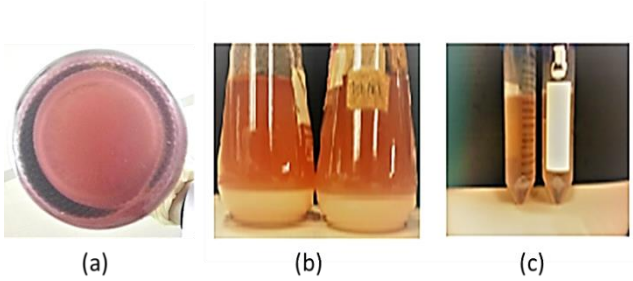
Supplementary Figure 2: Emulsion layer formed upon mixing of crude oil with (a) SDS-positive control, (b) supernatant from *P. aeruginosa* UKMP14T - test and (c) sterile distilled water - negative control. Emulsification layer has been highlighted in the figure using a white box.

Supplementary Figure 3: Beta-hemolysis on blood agar produced by culture supernatant of *P. aeruginosa* UKMP14T.

Supplementary Figure 4: Methylene Blue agar plate with (a) SDS (positive control), (b) sterile distilled water (negative control) and (c) supernatant from *P. aeruginosa* UKMP14T. Dark blue halo around the wells is indication of anionic surfactant presence.



Supplementary Figure 5: Flasks containing *P. aeruginosa* UKMP14T on day 7 showing reddish-brown colour due to production of rhamnolipid biosurfactant.



Supplementary Figure 6: Recovery of rhamnolipid biosurfactant from *P. aeruginosa* UKMP14T using three approaches (a) Blackish- brown precipitate obtained at the bottom of a Schott bottle on 30th day of precipitation using acid precipitation (b) White colour precipitate obtained on organic solvent extraction and (c) Light brown colour precipitate obtained by zinc sulphate precipitation.

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