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ORIGINAL ARTICLE

Characterization of glycolipid biosurfactant from *Pseudomonas aeruginosa* CPCL isolated from petroleum-contaminated soil

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Keywords
critical micelle concentration, drop collapse test, haemolysis assay, oil spreading test, orcinol/sulfuric acid method, rhamnolipid.

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Abstract

Aims: To isolate and characterize the biosurfactant-producing micro-organism from petroleum-contaminated soil as well as to determine the biochemical properties of the biosurfactant.

Methods and Results: A novel rhamnolipid-producing *Pseudomonas aeruginosa* (GenBank accession number GQ241355) strain was isolated from a petroleum-contaminated soil. Surface active compound was separated by solvent extraction of the acidified culture supernatant. The extract was able to reduce the surface tension of water from 72 to 44 mN m^{-1} at a critical micelle concentration of 11·27 ± 1·85 mg l^{-1}. It showed better activity (based on microdilution method) against Gram-positive (≤ 31 mg ml^{-1}) bacteria and filamentous fungi (≤ 50 mg ml^{-1}) than Gram-negative bacteria (≥ 125 mg ml^{-1}) with mild toxicity (HC_{50} – 38 ± 8·22 μg ml^{-1}) to red blood cells. Fourier transform infrared spectroscopy revealed the presence of aliphatic chain, hydroxyl groups, ester and glycosidic bonds. Presence of nineteen rhamnolipid homologues with variation in chain length and saturation was revealed from liquid chromatography coupled to mass spectrometry with electrospray ionization.

Conclusion: The results indicate that the isolated biosurfactant has a novel combination of rhamnolipid congeners with unique properties.

Significance and Impact of the Study: This study provides a biosurfactant, which can be used as a biocontrol agent against phytopathogens (*Fusarium proliferatum NCIM* 1105 and *Aspergillus niger NCIM* 596) and exploited for biomedical applications.

Introduction

Microbial surfactants are produced by a variety of micro-organisms such as bacteria, fungi and yeast from renewable resources (Lin 1996). These molecules have received considerable interest in recent years because of their superior properties including higher surface activity, lower toxicity, higher biodegradability and environmental compatibility when compared to the synthetic surfactants (Arutchelvi *et al.* 2008). Glycolipids are reported to have much higher yield when compared to other classes of biosurfactants (Kitamoto *et al.* 2002). Rhamnolipid belongs to this class, and its production is very often reported by *Pseudomonas* sp., (Sharma *et al.* 2007). This micro-organism produces a mixture of rhamnolipids that contains mono- and dirhamnolipids (Deziel *et al.* 1999). Rhamnolipids find applications in bioremediation of hydrocarbons, removal of heavy-metals and cleaning of oil spills (Mulligan 2004). They are highly valued for their antimicrobial activity with lesser toxicity (Lang and Wullbrandt 1999; Haba *et al.* 2003) when compared to their chemical counterparts. They can also be applied successfully against zoosporic plant pathogens (Sharma *et al.* 2007) and are used as biocontrol agents against damping-off disease in chilli and tomato plants in field trials (Sharma *et al.* 2007). The composition of the rhamnolipid mixture is

determined by the bacterial strain, type of carbon source used and the process strategy adapted for its purification (Lang and Wullbrandt 1999). Rhamnolipid homologues vary in the number of rhamnose units, number, chain length and saturation of fatty acids which in turn determine their physico-chemcial and biological properties (Abalos *et al.* 2001). Hence, in search of identifying a potential biosurfactant with improved properties, we anticipated rhamnolipid as a right choice.

Therefore, an attempt was made to isolate a novel rhamnolipid-producing bacterial strain from a petroleum waste site using hexadecane as one of the carbon source. Hexadecane was chosen because it is one of the contaminants released from petrochemical industries (Zhu *et al.* 2001; Callaghan *et al.* 2009), and it enriches the biosurfactant-producing micro-organisms from the soil population. In addition, this study deals with the isolation, production, chemical characterization, anti-infective properties and ocular toxicity of the isolated rhamnolipid.

Materials and methods

All the chemicals used for the experiments were procured from Sigma Aldrich (Bangalore, India), Himedia Laboratories Pvt. Ltd., and SRL (Mumbai, India). All the plastic wares were obtained from Tarson, Kolkata, India. Soil samples were collected from a petroleum waste site located in Chennai (India).

Isolation of biosurfactant-producing micro-organisms

Soil samples were collected in a sterile bag from a petroleum-contaminated sites located near Manali (Latitude 130°10′7″N, Longitude 80°15′54″E), Chennai, Tamil Nadu, India. One gram of the soil was added to 10 ml of sterile water. One millilitre of this soil suspension was added into two different 250-ml Erlenmeyer flasks containing 100 ml of minimal medium (MM) and mineral salt medium (MSM) (see Data S1–S4) (Arutchelvi *et al.* 2009), inside the laminar hood under aseptic conditions. These flasks were then incubated in a temperature-controlled shaker at the temperature of 37°C with the shaking speed of 180 rev min^{-1} for 10 days (Arutchelvi *et al.* 2009). One millilitre of filter sterilized hexadecane [1% (v/v)] was also added to MM and MSM to enrich the biosurfactant-producing micro-organisms. After incubation, samples (0·1 ml) of the enrichment culture were serially diluted and spread onto nutrient agar plates. There appeared several colonies, and pure cultures of each morphologically distinct colony were obtained by repetitive streaking onto solid nutrient agar. Isolation was repeated four times. Ample precautions were taken to maintain aseptic conditions.

Screening for biosurfactant-producing micro-organism

Isolated single colonies were inoculated into a tube containing 3 ml of MM/MSM and was incubated for 24 h in a shaker (37°C and 180 rev min^{-1}). The cell pellet was removed (centrifugation at 3214 ***g*** for 10 min), and the culture supernatant was tested for the presence of surface active agents using drop collapse and oil spreading tests (Youssef *et al.* 2004). Water and sodium dodecyl sulfate were used as a negative and positive controls, respectively. Uninoculated MM/MSM was also tested to eliminate the false positive results arising from media components other than surface active molecule produced by the micro-organisms.

In drop collapse test, a drop of culture supernatant placed on parafilm (hydrophobic surface) spreads/collapses, if the culture supernatant contains biosurfactant. This is considered as a positive result. Negative result does not show spreading of liquid/culture supernatant on parafilm because of the absence of biosurfactant/surface active compound. Similarly, in oil spreading test, positive result shows the zone of clearance of oil which placed on water layer, whereas in negative result no clearance zone appears. Water and sodium dodecyl sulfate give negative and positive results, respectively, with the above-mentioned tests. Hence, they are considered as negative and positive controls, respectively. The colonies that showed the positive result with these tests were maintained on nutrient agar plates at 4°C and subcultured once every month.

Identification of the biosurfactant-producing micro-organism

The shortlisted micro-organisms were further characterized with Gram staining; catalase and oxidase tests (Cappuccino and Sherman 2004). Plating of this micro-organism on *Pseudomonas* agar supplemented with cetyltrimethyl ammonium bromide (CTAB) was carried out to confirm the genus (Lowbury and Collins 1955). 16S rDNA sequencing was performed through an external agency (Chromous Biotech Pvt. Ltd., Bangalore, India), and the nearest homologues to this 16S rDNA gene (1448 bp) sequence was obtained from Seqmatch in Ribosomal database project [RDP (http://rdp.cme.msu.edu/)]. Phylogenetic tree was constructed using Tree Builder in RDP, and a distance matrix was generated using the Jukes-Cantor corrected distance model (Bruno *et al.* 2000).

Biosurfactant production, isolation and determination of surface activity

For biosurfactant production by the chosen micro-organism, 100 ml of MSM with trace elements (see Data S1–S4;

Benincasa *et al.* 2002) was inoculated with one millilitre of the seed culture. This was incubated for 96 h in a temperature-controlled shaker at a temperature of 37°C with the shaking speed at 180 rev min^{-1}. The incubation conditions were decided for this experiment based upon the earlier reports (Wei *et al.* 2005). Two millilitres of the culture was aliquated at every 6 h for 4 days. Half of this culture sample was used to quantify the concentration of biomass and rhamnolipid. The cells were separated from the culture (centrifuged at 3214 ***g*** for 10 min), and dried (at 105°C) biomass was weighed. The pH of the supernatant was adjusted to 2·0 with 6 mol l^{-1} H_2SO_4, followed by extraction with a mixture of chloroform and methanol (2 : 1) (Heyd *et al.* 2008). This extract was concentrated by rotoevaporation, and the amount of rhamnolipid here was quantified using orcinol/sulfuric acid method (Heyd *et al.* 2008) followed by multiplying rhamnose values by 3 (Heyd *et al.* 2008). The remaining half of the culture sample was used to estimate the growth of the micro-organism by measuring the optical density at 600 nm and the number of colony forming units (CFU ml^{-1}) on nutrient agar plates (unpublished results).

Critical micelle concentration (CMC) of the extract was estimated by measuring the surface tension at 25°C of a 0·05 mol l^{-1} sodium bicarbonate (pH 8·6) solution with varying concentrations (0–100 mg l^{-1}) of the extract using a surface tensiometer (NIMA technology model DST9005; Nima Technology Ltd, Coventry, UK) which operates based on the principle of Wilhelmy plate method (Abalos *et al.* 2001). All the experiments were repeated twice.

Determination of functional groups using FTIR

The functional groups and the type of bonds present were determined with a Fourier transform infrared spectrometer (FTIR; Jasco N4200, Tokyo, Japan), after dissolving the extract in chloroform and measuring the spectra using a sodium chloride disc in the 4000–400 cm^{-1} spectral region, at a resolution of 4 cm^{-1}. The system was calibrated with polystyrene standards.

Identification of rhamnolipid homologues using LC/ESI-MS

The extract was separated using reverse phase HPLC (Agilent 1100 series; Agilent Technologies India, Bangalore, India) with C18 column (5 μm pore size; Purospher® STAR RP-18e column; 250 × 4·6 mm). Acetonitrile–water gradient system was applied at a flow rate of 0·8 ml min^{-1}, starting with 50% acetonitrile for 10 min, raised to 70% in 6 min, which was kept constant for 13 min and reduced to 50% in 6 min. The molecular masses of the fractions were determined in a triple quadruple mass spectrometer (API 3000; Applied Biosystems, CA) in positive ion mode, and the masses were recorded in full scan mode from *m/z* of 50–750. The ion spray voltage and ion source temperature were 5000 V and 450°C, respectively.

Determination of the haemolytic activity

Biosurfactant has application in topical pharmaceutical and cosmetic preparation; hence knowing its skin and ocular toxicity is imperative. Haemolytic assay quantifies the adverse effects of surfactants on the cytoplasmatic membrane and is a specific *in vitro* tool for evaluating the potential ocular irritation induced by surfactants (Martínez *et al.* 2007). This is an alternative method to the Draize *in vivo* test (Balls *et al.* 1995).

Peripheral blood was collected from human volunteer by venipuncture, and 0·3% of EDTA was added to it. Red blood cells (RBC) were separated (centrifugation at 800 ***g*** for 10 min) and washed thrice in phosphate-buffered saline (PBS) (pH 7·4). RBC suspension (cell density of 5×10^8 cells ml^{-1}) (Nishida *et al.* 2007; Hemaiswarya and Doble 2009) was kept in an ice bath throughout the experiments. A stock solution of the extract (1 mg ml^{-1}) was prepared by dissolving it in 200 mmol l^{-1} of sodium bicarbonate. Various concentrations of this solution (ranging from 2·5 to 100 μg ml^{-1}) were incubated with erythrocyte suspension [to arrive at a final erythrocyte concentration of 1% (v/v)] for 1 h at 37°C. The per cent haemolysis was determined by measuring the optical density of the supernatant at 540 nm (Nishida *et al.* 2007; Hemaiswarya and Doble 2009) of the supernatant. The half-maximal haemolysis value (HC_{50}) was calculated by fitting the data to a four-parameter sigmoidal curve by using SIGMAPLOT 11 (Systat Software Inc., Bangalore, India) (Nishida *et al.* 2007).

The results were compared with completely haemolysed blood with distilled water (100%), 100 μl of 200 mmol l^{-1} sodium bicarbonate and 900 μl of PBS without the glycolipid. Each experiment was repeated twice.

Antibacterial activity

Thirty-five milligram of the isolated glycolipid extract was dissolved in 1 ml of 100% DMSO (stock solution). *In vitro* minimum inhibitory concentrations (MIC) were determined against seven bacteria namely, *Bacillus subtilis* (NCIM 2718), *Escherichia coli* (NCIM 2931), *Enterobacter aerogenes* (NCIM 5139), *Proteus vulgaris* (NCIM 2813), *Ps. aeruginosa* (NCIM 5029), *Staphylococcus aureus* (NCIM 5021) and *Salmonella typhimurium* (NCIM 2501) by microdilution method in 96-well plates (CLSI standards 1993). Twofold dilutions of glycolipid (range

of concentration of the extract used 2–0·002 mg ml^{-1}) in Mueller Hinton broth were inoculated with 10 μl (which has 5×10^6 CFU ml^{-1}) of the above-mentioned bacterial cultures. Controls in the presence and absence of organic solvents were also included. No inhibitory effects were observed in the presence of DMSO at the highest concentration used (5·5% v/v). Plates were incubated without agitation at 37°C, and MICs were determined at 20 h using resazurin dye as a bacterial respiration indicator. Pink colour indicated growth, whereas blue indicated inhibition of the bacterial growth (Sarker *et al.* 2007). Each experiment was repeated four times.

Antifungal activity

Minimum inhibitory concentration of the glycolipid extract was determined against three fungi namely *Candida albicans* (NCIM 3471), *Aspergillus niger* (NCIM 596) and *Fusarium proliferatum* (NCIM 1105) by microdilution method in 96-well plates (Liu *et al.* 2007). Each well contained 0·1 ml of broth medium (see Data S1–S4; Sivakumar *et al.* 2009). Hundred microlitres of the sample solution (50 mg ml^{-1} of glycolipid in DMSO) was diluted twofold in the wells starting with the working concentration of 3 mg ml^{-1} (range of concentration of the extract used 3–0·003 mg ml^{-1}) and 50 μg ml^{-1} of amphotericin B was used as the standard antifungal drug. Controls in the presence and absence of organic solvents were also included. No inhibitory effects were observed in the presence of DMSO at the highest concentration used (6% v/v). Ten microlitres of 0·1 mg ml^{-1} of resazurin was added in each well, and the plates were incubated at 37°C for 48 h. MIC was determined based on change in colour of resazurin. The solution was blue in colour when there was no growth, and it turned pink otherwise. Each experiment was repeated four times.

Results

Isolation, screening and identification of biosurfactant-producing micro-organisms

The number of colonies was very less in case of MM because it has only 0·05% glucose and 1% hexadecane, whereas MSM contains 3% glucose with 1% hexadecane. Six morphologically different colonies from the MM and thirteen colonies from MSM were isolated, but only one was found to produce extracellular biosurfactant. The isolated biosurfactant-producing micro-organisms in this study was appeared on nutrient agar plates, where the highest (10^{-7}) dilution of culture was plated. Hence, the isolation of these particular micro-organisms is not by chance. The isolated biosurfactant-producing micro-organism in this study is a Gram-negative rod-shaped bacterium and indicated the presence of catalase and cytochrome oxidase. It was able to grow (bluish green colonies) on *Pseudomonas* agar plate containing CTAB. Based on the 16S rDNA sequence homology and phylogenetic analysis (Supplementary material Fig. S1), the micro-organism was characterized as *Ps. aeruginosa* strain CPCL. Lineage of the isolate is given in supplementary material (Fig. S2). The sequence length of 1448 bp is deposited in GenBank database under the accession number GQ241355.

Growth and Product formation kinetics

The organism exhibited 6 h of lag and 36 h of log phases (Fig. 1). The maximum biomass concentration of 2·43 g l^{-1} was attained in 36 h. Production of glycolipid was initiated after 12 h and increased progressively up to 48 h, where it reached a maximum of 2·7 g l^{-1}. The relationship between dp/dt (rate of product accumulation) and dx/dt (rate of biomass formation) as a function of biomass concentration (x) is shown in Fig. 1 inset. Maximum rhamnolipid formation occurred during the stationary phase.

Determination of functional groups using FTIR

FTIR spectrum of the extract (Supplementary material Fig. S3) was consistent with the rhamnolipid structure already reported in literature (Heyd *et al.* 2008). Vibration at 2700–3000 cm^{-1} region confirms the presence of methyl and methylene groups. The deformation vibrations at 1465 and 1375 cm^{-1} substantiate the presence of alkyl groups. Presence of ester carbonyl groups (1744 cm^{-1}) and glycosidic bond (C–O–C) (1064 cm^{-1}) is also confirmed.

Determination of molecular masses of the rhamnolipid congeners

Nineteen rhamnolipid homologues with the pseudomolecular ions between m/z 305 and 703 were identified using LC/ESI-MS. The main pseudomolecular ions and their relative abundances for all the rhamnolipid congeners are summarized in Table 1. Monorhamnolipid homologues were found to be the major (82·27%) constituent in the mixture with Rha-C_8–C_{10}/Rha-C_{10}–C_8 as a predominant (58·54%) component and its m/z is 475 (Supplementary material Fig. S4a). A dirhamnolipid with m/z of 703 is the major isoform (3·4%) within the dirhamnolipid homologues which correspond to Rha-Rha-C_{12}–$C_{12:1}$/Rha-Rha-C_{10}–$C_{14:1}$ (Supplementary material Fig. S4b).

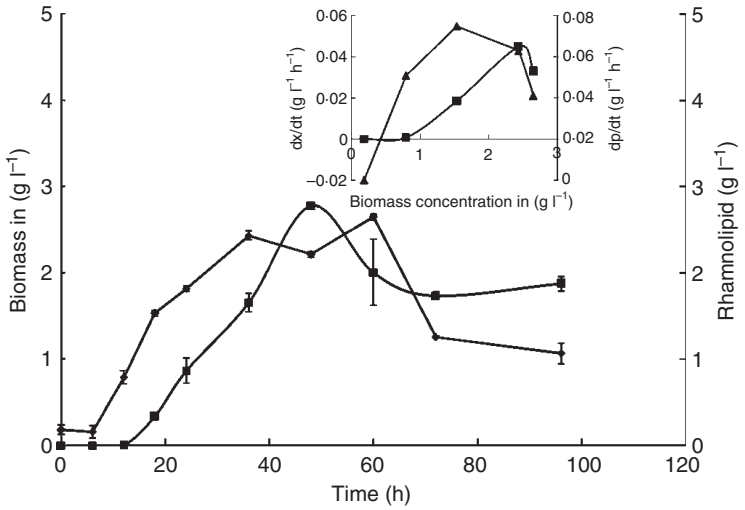

Figure 1 Growth kinetics and glycolipid formation by CPCL: (■) Concentration of rhamnolipid (g l^{-1}) at different time points (◆) Biomass concentration (g l^{-1}) at different time points; Figure Inset: Kono–Asai classification of the kinetic data: (■) Product formation rate (▲) Growth rate–presented in the inset.

Physical and biological properties of the crude extract

The surface tension of water decreased (Supplementary material Fig. S5) from 72·5 to *c.* 44 mN m^{-1}, at a concentration of 11·27 ± 1·85 mg l^{-1} and remained constant thereafter. A concentration-dependent haemolysis of RBC was observed here (Supplementary material Fig. S6). The half-maximal haemolysis (HC_{50}) was calculated as 38 ± 8·2 μg ml^{-1} (R^2–0·97; R^2_{adj}–0·96; *P*-value < 0·0001).

Table 1 Homologues of rhamnolipid produced by *Pseudomonas aeruginosa* CPCL, their average molecular masses (*m/z*) and relative abundances

Rhamnolipid congeners	Molecular masses	Relative abundance (%)
Rha-C_8	305	4·88
Rha-C_{10}	333	6·24
Rha-C_{12}	361	5·46
Rha-C_8–C_8	447	1·23
Rha-C_8–C_{10}/Rha-C_{10}–C_8	475	58·54*
Rha-C_{10}–C_{10}	503	2·93
Rha-C_{10}–$C_{12:1}$/Rha-$C_{12:1}$–C_{10}	529	1·85†
Rha-C_{10}–C_{12}/Rha-C_{12}–C_{10}	531	1·13
Rha-Rha-C_8	451	1·41
Rha-Rha-C_{10}	479	0·57
Rha-Rha-$C_{12:1}$	505	2·24†
Rha-Rha-C_{12}	507	0·98
Rha-Rha-C_8–C_8	593	1·46
Rha-Rha-C_8–C_{10}/Rha-Rha-C_{10}–C_8	621	1·83
Rha-Rha-C_8–$C_{12:1}$/Rha-Rha-$C_{12:1}$–C_8	647	1·21†
Rha-Rha-C_{10}–C_{10}	649	2·15
Rha-Rha-C_{10} : $C_{12:1}$/Rha-Rha-$C_{12:1}$–C_{10}	675	1·27†
Rha-Rha-C_{10}–C_{12}/Rha-Rha-C_{12}–C_{10}	677	1·2
Rha-Rha-C_{12}–$C_{12:1}$/Rha-Rha-C_{10}–$C_{14:1}$	703	3·42†

*Major isoform.

†Rhamnolipid with unsaturated fatty acid chain.

Minimum inhibitory concentrations of the isolated glycolipid against seven bacterial and three fungal cultures are summarized in the Table 2. Antifungal activity of standard amphotericin B against the filamentous fungus and *C. albicans* was 50 and 3·125 μg ml^{-1}, respectively.

Discussion

The isolated organism showed equal similarity with *Ps. aeruginosa* strains isolated from marine as well as from clinical samples. Among the closest micro-organisms, *Ps. aeruginosa* C-3, *Pseudomonas* sp. RJS4 and *Pseudomonas sp.* O-1 are reported to produce biosurfactant.

The rate of accumulation of glycolipid was 0·046 g l^{-1} h^{-1}, and its production during the stationary phase reached a maximum of 2·781 g l^{-1}. This corresponded to a yield factor of 1·37, and the productivity during this phase was 0·094 g l^{-1} h^{-1}. The Kono–Asai classification (Kono and Asai 1969) revealed that the linear increase in the cell growth rate occurred at the exponential phase, whereas the

Table 2 Antibacterial and antifungal activity of the isolated rhamnolipid

Micro-organisms	MIC (mg ml^{-1})
Escherichia coli NCIM 2931	2
Enterobacter aerogenes NCIM 5139	> 2
Pseudomonas aeruginosa NCIM 5029	1
Proteus vulgaris NCIM 2813	0·5
Salmonella thyphimurium NCIM 2501	0·125
Bacillus subtilis NCIM 2718	0·031
Staphylococcus aureus NCIM 5021	0·016
Candida albicans NCIM 3471	3
Fusarium proliferatum NCIM 1105	0·047
Aspergillus niger NCIM 596	0·047

MIC, minimum inhibitory concentration.

product formation rate occurred at the stationary phase. Also, the maximum rates of product and biomass formations occurred at two different biomass concentrations i.e., at 2·5 and 1·5 g l^{-1}, respectively. Hence, it could be concluded that the product formation is a nongrowth-associated process.

Chemical nature of the isolated extract is determined as glycolipid using FTIR. The presence of fragments corresponding to *m/z* of 163 is related to the rhamnose groups and those with *m/z* equal to 103 are related to the cleavage of this sugar (Haba *et al.* 2003). The fragments with *m/z* of 109 indicate the presence of 3-hydroxydecanoic acid ions, (Haba *et al.* 2003) and *m/z* of 141 and 169 are attributed to the presence of fatty acids C_8 and C_{10}, respectively. This is similar to the findings of others (Haba *et al.* 2003; Sharma *et al.* 2007). In addition, the isolated extract has rhamnolipids with unsaturated fatty acid chains (*c.* 10%), as observed by others (Abalos *et al.* 2001; Sharma *et al.* 2007; Pornsunthorntawe *et al.* 2008). Even though the extract contains most of the known homologues of rhamnolipid, there are considerable differences in the composition (82·27% of monorhamnolipids, 17·73% of dirhamnolipids and 10% unsaturation) when compared to the reported ones which makes it unique. This in turn led to the variation in its surface activity from the reported ones. Surface activity is inversely proportional to the CMC, and the observed CMC (11·27 ± 1·85 mg l^{-1}) lies in the lowest range of the values reported in the literature (5–200 mg l^{-1}) for rhamnolipid produced by different strains of *Pseudomonas* sp. (Abalos *et al.* 2001). There are only few reports on monorhamnolipid as a predominant component in the mixture with very low CMC. The CMC of the isolated surfactant is very small when compared to that of classical surfactants such as SDS (CMC = 663 mg l^{-1}), CTAB (CMC = 364·5 mg l^{-1}) and CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate) (CMC = 3·69–6·14 g l^{-1}) (Deleu *et al.* 2003).

HC_{50} of the rhamnolipid isolated in this study is 38 ± 8·2 μg ml^{-1}, which is *c.* 3·4 times greater than its CMC value. HC_{50} value for SDS (41·5 μg ml^{-1}) is much below its CMC (Aparicio *et al.* 2005). Hence, the isolated rhamnolipid can be considered as a milder ocular irritant when compared to chemical surfactants and can be used for biomedical applications. The MIC against Gram-negative bacteria and fungi reported here is above its HC_{50}. Hence, this surfactant can be considered for the treatment against Gram-positive bacteria either as a monotherapy or in combination with other drugs. The extract showed the best activity against *Staph. aureus*, which is a very common infectant in medical implants. Biosurfactant at concentrations greater than CMC provokes changes in the membrane fluidity and causes cellular damage. Among the Gram-negative micro-organisms tested, *Salm. typhimurium* was more susceptible (125 μg ml^{-1}). Rhamnolipid (RL47T2) isolated from *Ps. aeruginosa* 47T2 NCBIM 40044 (Haba *et al.* 2003) demonstrated highest activity against *Ent. aerogenes* CECT 689, whereas in our study, the extract did not show any activity up to 2 mg ml^{-1}. This can be attributed to either the compositional variation of the glycolipid mixture or differences in the strains between the two micro-organisms.

Very few reports are available on the antifungal activity of rhamnolipid. Mild action of the rhamnolipid on yeast cells is reported (Vasileva-Tonkova *et al.* 2008), which is similar to our observations with *C. albicans*. Both the filamentous fungi used in our study are phytopathogens (*F. proliferatum NCIM* 1105 and *A. niger NCIM* 596), and the isolated rhamnolipid showed excellent activity against them. Field studies demonstrated the efficacy of application of rhamnolipid against damping-off diseases in chilli and tomato plants (Sharma *et al.* 2007). *Fusarium proliferatum* is a known wheat pathogen (Kwon and Anderson 2001). In addition, *F. proliferatum* was discovered as an important pathogen of garlic and onion in Europe and there is a potential risk for the accumulation of mycotoxin in these contaminated plants (Stankovic *et al.* 2007). Hence, field studies can be tried out to explore the efficacy of rhamnolipid against black point disease in wheat and other infections in garlic and onion caused by *F. proliferatum*. MIC of the isolated rhamnolipid against *A. niger* (47 μg ml^{-1}) is higher and lower than the activity of rhamnolipid from *Ps. aeruginosa* 47T2 NCBIM 40044 (> 256 μg ml^{-1}) and *Ps. aeruginosa* AT10 (16 μg ml^{-1}), respectively (Abalos *et al.* 2001; Haba *et al.* 2003).

Our study showed that the isolated biosurfactant is different in composition of the rhamnolipid mixture from the previously reported mixtures. It has efficient surface activity with lesser ocular toxicity when compared to the synthetic surfactants. This extract can also be exploited for applications such as anti-adhesive agent against Gram-positive bacteria especially *Staph. arureus* (infections in medical implants) and as biocontrol agent against *F. proliferatum* (phytopathogen).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 16S rDNA sequence analysis of CPCL.

Figure S2 Lineage of the isolated micro-organism.

Figure S3 FTIR of the extract from *Pseudomonas aeruginosa* CPCL.

Figure S4 (a) Mass spectrum of the peak eluting at 1·81 min which corresponds to major monorhamnolipid with the *m/z* of 475 (Rha-C_8–C_{10}/Rha-C_{10}–C_8) (b) Mass spectrum of the peak eluting at 1·71 min which corresponds to major dirhamnolipid with the *m/z* of 703 (Rha-Rha-C_{12}–$C_{12:1}$/Rha-Rha-C_{10}–$C_{14:1}$).

Figure S5 Surface activity of the isolated crude extract using Wilhelmy plate method.

Figure S6 Haemolytic activity of isolated crude extract.

Data S1 Composition of Minimal medium (MM).

Data S2 Composition of Mineral salt medium (MSM).

Data S3 Composition of MSM with trace elements.

Data S4 Composition of Broth medium (used for determining antifungal activities).

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