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In vitro Cytotoxicity and Antimicrobial Activity of Biosurfactant Produced by *Pseudomonas aeruginosa* Strain PB3A

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ABSTRACT

In the present study *Pseudomonas aeruginosa* PB3A strain isolated from oil contaminated soil was exploited for the biosurfactant production. Effect of growth parameters for the biosurfactant production were studied which revealed their optimum production occurs at pH 7, with a temperature of 37°C and optimum incubation time of 48 h. Further the characterization studied of partially purified biosurfactants by TLC and FTIR confirms the presence of biosurfactant production. Screening of antibacterial and anticancer studies also reveals that, the extracted biosurfactant effectively controlled the test pathogens and also suppress the proliferation of HeLa cells showing an IC_{50} value of 125 μg mL^{-1}. The present study concludes the biosurfactant produced by *Pseudomonas aeruginosa* PB3A shows significant cytotoxicity activity which is of eco-friendly in nature.

Key words: Biosurfactant, *Pseudomonas aeruginosa*, anti-bacterial activity, anti-proliferative activity, HeLa cell lines

INTRODUCTION

Requirements of surfactants and emulsifiers are vital in the cosmetic, food, petroleum and pharmaceutical industries. Since, most of the surfactants used in industries are of chemically synthesized, surface-active molecules of biological origin that have been described in recent decades (Onbasli and Aslim, 2009).

Due to the advancement in biosciences technology and increasing concerns over environmental issues have led to the discovery of biosurfactants which act as a being a potential alternative to the chemical surfactants (Rahman *et al*., 2002). Chemical surfactants are organic compounds which are mainly used in various sectors of washing powders, detergents, toothpastes, soaps and shampoos which are not degradable in nature and persist in the environment for prolonged time (Siegmund and Wagner, 1991).

Biosurfactants are extracellular macromolecules produced by various microbes like bacteria, yeast and fungi and in particular by natural and recombinant bacteria (Richter *et al*., 1998). Apart from synthetic carbon sources, vegetable oil, hydrocarbons and ethanol have been widely employed for the production of biosurfactant. Biosurfactants are well recognized for their action of enhancing the hydrocarbon emulsification, solubilizing complex hydrocarbon contaminants and increasing their availability in the soil and other areas for effective microbial degradation. They have exceptional properties like lowering the surface tension, interfacial tensions and Critical Micelle Concentration (CMC) both in aqueous solutions and hydrocarbon mixtures similar to chemical synthesized surfactants (Raza *et al*., 2007).

The real bottleneck in producing biosurfactants is its high cost of production. Among the biological surfactants, rhamnolipids have reported to be the most adopted surfactant by the industries as a new class of renewable surfactants (Muller *et al*., 2011).

Recently, there is an increase in the concern about environmental protection leading to the development of cost effective process for biosurfactant production (Morita *et al*., 2011). The microbial biosurfactants are complex molecules of various chemical types including fatty acids, peptides, glycolipids, phospholipids, antibiotics, lipopeptides, etc. Investigation in this area has expanded relatively a lot in the recent years due to its prospective use in different areas. Several biosurfactants are used as antibacterial and antifungal agents which requires very low concentrations, which makes them as highly sought biomolecules for various applications especially in case of biological control agents, pharmaceutical and health care industries. Recent studies have also reported that biosurfactants are not only useful as antibacterial, they also have a potential use in anti tumor activity studies (Kumar *et al*., 2006; Donio *et al*., 2013).

In our present study, *Pseudomonas aeruginosa* strain PB3A was isolated from oil contaminated regions and exploited for the production of biosurfactant and its application in antimicrobial and antitumor property.

MATERIALS AND METHODS

Screening of biosurfactant producing isolates: Different oil contaminated soil samples were collected from nearby Chennai, Tamil Nadu in a sterile container and transported to the laboratory and stored in the refrigerator for further processing. The samples were subjected to pretreatment using enrichment technique using Mineral Salt Medium (MSM) and serially diluted on nutrient agar plates ((g L^{-1}): beef extract 1.0 g, yeast extract 2.0 g, peptone 5.0 g, NaCl 5.0 g) for the isolation of different bacterial strains. Plates were examined and different isolates were maintained on nutrient agar slants for further study.

For the preparation of a mineral salt medium, the following composition (g L^{-1}) was utilized: Na_2HPO_4 (2.2), KH_2PO_4 (1.4), $MgSO_4.7H_2O$ (0.6), $FeSO_4.7H_2O$ (0.01), NaCl (0.05), $CaCl_2$ (0.02), yeast extract (0.02) and 0.1 mL of trace element solution. Composition of trace element solution (g L^{-1}): 2.32 g $ZnSO_4 \cdot 7H_2O$, 1.78 g $MnSO_4 \cdot 4H_2O$, 0.56 g H_3BO_3, 1.0 g $CuSO_4 \cdot 5H_2O$, 0.39 g $Na_2MoO_4 \cdot 2H_2O$, 0.42 g $CoCl_2 \cdot 6H_2O$, 1.0 g EDTA, 0.004 g $NiCl_2 \cdot 6H_2O$ and 0.66 g KI. pH of the medium was adjusted to 7.0.

Screening for biosurfactant production: Pure culture of bacterial isolates were streaked on the freshly prepared blood agar plate and incubated at 37°C for 24-48 h for 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. α-hemolysis, when the colony was surrounded by greenish zone, β-hemolysis, when the colony was surrounded by a clear white zone and γ-hemolysis, when there was no change in the medium surrounding the colony (Carrillo *et al*., 1996).

The isolates which gave positive results in the haemolytic assay were subjected to qualitative drop-collapse test described by Bodour and Miller-Maier (1998). Crude oil was used in this test and 2 μL of oil was applied to the well regions delimited on the covers of 96-well micro plates and these were left to equilibrate for 24 h. Five microlitre of cell free supernatant of each test isolates at end of 48 h incubation in MSM (10 mL) was transferred to the oil-coated well regions and drop size was observed after 1 min with the aid of a magnifying glass. The result was considered positive for biosurfactant production when the drop was flat and those cultures that gave rounded drops were scored as negative, indicative of the lack of biosurfactant production (Youssef *et al*., 2004).

Similarly, 2 mL of each test isolates suspension from mineral salt medium after 48 h of incubation, 2 mL hydrocarbon (oil) was added to each tube and mixture was vortexed at high speed for 1 min, they were allowed to stand for 24 h. The emulsion index (E_{24}) is the height of the emulsion layer (cm) divided by total height (cm), multiplied by 100 Bodour *et al*. (2004):

$$\text{Emulsification index } (E_{24}) = \frac{\text{Height of the emulsion layer}}{\text{Total height}} \times 100$$

In order to determine anionic nature of biosurfactant such as rhamnolipids, methylene blue agar plate technique was performed. Briefly, Mineral salt agar medium supplemented with glucose as carbon source (2%) and cetyltrimethylammonium bromide (CTAB: 0.5 mg mL^{-1}) and methylene blue (MB: 0.2 mg mL^{-1}) were used for the detection of anionic biosurfactant. Thirty microlitre of cell free supernatant of each test isolates grown from MSM (10 mL) was loaded into the each well prepared in methylene blue agar plate using cork borer (4 mm). Similarly, positive (sodium dodecyl sulphate) and negative control (sterile distilled water) were added to the methylene blue agar plate and was then incubated at 37°C for 48-72 h. A dark blue halo zone around the culture was considered positive for anionic biosurfactant production. The test isolates which gave positive results for all the above four screening methods was then inoculated into production medium for further study.

Growth and biosurfactant production: Biosurfactant production by the isolated *Pseudomonas aeruginosa* was carried out in shake flasks containing 500 mL of MSM medium. Each flask was inoculated with 5 mL of a pre-culture prepared using the same culture medium and incubated overnight at 37°C and 120 rpm and incubated for 96 h. After the incubation time, cells were harvested by centrifugation (10,000 rpm, 15 min) and cell dry weight was determined (48 h at 105°C).

Qualitative determination of biosurfactant production: After 48 h of incubation, the cell-free supernatant was collected by centrifuging and the biosurfactant concentration was estimated using orcinol assay method (Wu *et al*., 2008). From the cell free supernatant, 100 µL was mixed with 900 µL of a 0.19% orcinol solution (in 53% H_2SO_4) and heated at 80°C for 30 min. The preparation was then cooled to room temperature and optical density was measured at 421 nm and compared with the standard curve prepared with L-rhamnose and expressed as Rhamnose Equivalents (RE) (mg mL^{-1}).

Purification of biosurfactant: The culture medium was centrifuges at 10000 rpm for 15 min and the cell free supernatant was used for the extraction of biosurfactant. The pH of the supernatant was adjusted to 2.0 with 0.1 N HCl and an equal volume of ethyl acetate was added in a separation funnel. The mixture was then shaken vigorously and allowed to settle until phase separation occurs. The organic phase was collected by repeating the above mentioned steps for 2-3 times and the water was removed using anhydrous sodium sulphate followed by concentrating the extract using a rotary evaporator. The resulting product was considered as the crude biosurfactant. For further purification, the crude biosurfactant was dissolved in 0.05 M sodium bicarbonate. After filtration, the pH of this solution was adjusted to 2.0 using 0.1 N HCl and then the solution was kept at 4-8°C for 24 h. The precipitate was finally collected by centrifugation at 5000 rpm for 15 min, freeze-dried and analyzed for biosurfactant.

Analytical characterization

Thin Layer Chromatography (TLC): Preliminary characterization of the extracted biosurfactant was done by TLC method. A portion of the crude biosurfactant was separated on a silica gel plate using chloroform, methanol and water in the ratio of 70:10:0.5 as developing solvent system with different color developing reagents. Two such reagents like, ninhydrin reagent (0.5 g ninhydrin in 100 mL anhydrous acetone) was used for lipopeptide biosurfactant detection as red spots and anthrone reagent (1 g anthrone in 5 mL sulfuric acid mixed with 95 mL ethanol) to detect glycolipid biosurfactant as yellow spots (Yonebayashi *et al*., 2000).

Infrared spectra (IR): The biosurfactant was extracted from the culture supernatant (2 mL) with chloroform (2 mL), dried with Na_2SO_4 and evaporated using a rotary evaporator. The IR spectra were recorded on the Bruker IFS113v FTIR-spectrometer, in the spectral range of 4000-400 cm^{-1}.

Identification of bacterial strain: The selected isolate PB3A was identified by 16S rRNA gene sequence analysis. Briefly, total genomic DNA was extracted using CTAB (Cetyltrimethylammonium bromide) method, followed by amplification of 16S rRNA gene using universal primers, 16S rDNA primers 27f (5'-GAGTTTGATCCTGGCTCAG-3') and 1492r (5'-TACGGYTACCTTGTTACGACTT-3') followed by purification using QIAquick Gel Extraction kits from Qiagen. Further, sequencing of amplified DNA was performed by DTS cycle sequencing kit and ABI automated DNA sequencer (Kumar *et al*., 2006). The sequences were then aligned and analyzed for similarity comparisons using the BLAST program (Altschul *et al*., 1990).

Applications of biosurfactant

Antimicrobial assay: The crude biosurfactant was tested for its antimicrobial activity against several pathogenic microbial strains by well diffusion method (Joshi *et al*., 2008). The antibacterial assay was carried out using agar well diffusion tests and broth dilution techniques. The antimicrobial activity of the biosurfactants was tested against four standard bacteria species namely *S. aureus* ATCC 29736, *Staphylococcus epidermidis* ATCC 12228*, E. coli* ATCC 8739 and *P. aeruginosa* ATCC 27853 strain. Mueller Hinton agar medium were used for the evaluation of antimicrobial property of biosurfactant against the test pathogens. An overnight nutrient broth culture of test pathogens were prepared and made a lawn culture using sterile swab over the nutrient medium plates. After the lawn preparation, wells were punched using sterile well borer and the samples were loaded in to the respective wells. The plates were incubated for 24-48 h and observed for clear zone formation which indicates the antibacterial activity of the biosurfactants.

Cell culture: The human cervical carcinoma cell line, HeLa (HPV-18) was procured from National Center for Cell Science (NCCS), Pune, Maharashtra, India. The cells were grown in Dulbecco's modified eagle medium supplemented with 10% heat inactivated fetal bovine serum, 2 mM L-glutamine and antibiotics (1% penicillin-streptomycin). The cells were incubated in a 5% humidified CO_2 incubator at 37°C and passaged twice in a week.

Cell toxicity studies on HeLa cells: The cytotoxicity assay of partially purified biosurfactant was tested on HeLa cell lines using MTT assay. The cells were seeded at densities of 1×10^5 cells per well in 96-well tissue culture plates and incubated for 48 h. Then the cells were treated with different known concentrations of partially purified biosurfactant (100-1000 μg mL^{-1}) for 48 h.

After incubation, the wells were washed with Phosphate-Buffered Saline (PBS), pH-7.4 and then 20 μL well^{-1} (5 mg mL^{-1} in PBS) of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl--tetrazolium bromide (MTT) was added in each wells. After 4 h of incubation, 100 μL of dimethyl sulfoxide (DMSO) was added and absorbance was determined at 450 nm using micro plate reader. The readings were used for the calculation of cell viability (%) (Curcic *et al*., 2012). The effect of the partially purified biosurfactant on the proliferation of HeLa was expressed as the % cell viability, using the following equation:

$$\text{Cell viability (\%)} = \frac{\text{A450 of treated cells}}{\text{A450 of control cells}} \times 100$$

Statistical analysis: The Quantitative values obtained in the treatment were converted to percentage inhibition. Regression analysis was applied for inhibition concentration calculation required to produce a 50% reduction in the cell viability (IC_{50}). All the results were expressed as the Mean±SD of values obtained in triplicate from three independent tests. Statistical differences were correlated and evaluated using Student's t-test and the significance was reported with difference of $p<0.05$.

RESULTS AND DISCUSSION

Screening of biosurfactant producing strains: Different bacterial strains were isolated from oil contaminated samples collected from different parts of Chennai, TamilNadu. Among the isolated strains PB3A showed a positive results for all the four screening methods such as, haemolytic assay, drop collapse method, emulsification assay and Methylene blue agar plate assay and used for further study.

Various reports suggested that (Sekhon *et al*., 2012) more than one screening method should be applied for the primary screening of prospective biosurfactant production. Among the bacterial strains isolated, PB3A showed clear hemolytic zone in the blood agar plates confirming the presence of biosurfactant production. Similar reports were also published by various researchers who have found an association between hemolytic activity and surfactant production and they recommended the use of blood agar lysis as a primary method to screen biosurfactant production (Carrillo *et al*., 1996; Mulligan *et al*., 1984; Youssef *et al*., 2004).

The PB3A strain also showed a flat drop appearance in the drop collapse, a sensitive method to detect biosurfactant production as suggested by Jain *et al*. (1991). The drop collapse method works on the principle that a drop of liquid containing a biosurfactant collapses and spreads over the oily surface (Bodour and Miller-Maier, 1998). The strain PB3A shows the maximum emulsification index of 65.5% in the present study, which confirms the production of biosurfactant (Peypoux *et al*., 1999). The CTAB agar plate method is a semi quantitative assay for the detection of anionic surfactants such as rhamnolipidic biosurfactant production (Tsuge *et al*., 1996; Wu *et al*., 2008; Tahzibi *et al*., 2004). The strain PB3A shows a dark blue halo zone around the inculcated well. The test is based on the complex formation between anionic surfactant and cationic methylene blue resulting in the color formation (Fig. 1).

The isolated strain, PB3A was studied for its biosurfactant production using MSM medium for 48 h. The results reveal that the maximum productivity of 0.84 mg mL^{-1} was observed after 48 h of incubation at 37°C. Biosurfactant-producing strain had a turbid and pigmented growth in the MSM broth. The *Pseudomonas* genera are one of the most reported for biosurfactant production

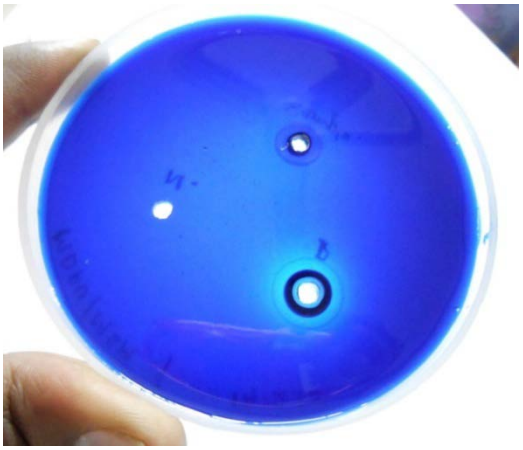

Fig. 1: Methylene blue agar plate test for biosurfactant production

and it has also been reported that the most known biosurfactant that they produce, is a rhamnolipid (Yin *et al*., 2009; Monteiro *et al*., 2009). Similar studies have been reported using mineral salt medium for the production of biosurfactant (Chayabutra *et al*., 2001; Joshi *et al*., 2008).

The partially purified biosurfactant was analyzed using TLC and FTIR spectrum. The thin lay chromatography studies reveals two different spots with Rf value of 0.41 and 0.50 which confirms the presence of glycoplipids. The FTIR spectrum using KBr showed characteristic peaks of peptides at 3451 cm^{-1} assigned to NH stretching mode. The band at 2935 cm^{-1} is due to the presence symmetric stretch ACH of CH_2 in the crude sample. Also, an intense absorption band at 1643 cm^{-1} indicate the presence of ester carbonyl groups (C = O in COOH) in the biosurfactant. The ester carbonyl group was also proved from the band at 1243 cm^{-1} that corresponds to C-O deformation vibrations, although other groups also absorb in this region. Similarly, another strong IR absorption found at 891 cm^{-1} was due to out of plane CAH bending, characteristic of aromatic compounds (Kim and Vipulanandan, 2006; Joshi *et al*., 2008).

The strain PB3A was confirmed at *Pseudomonas aeruginosa* using 16S rRNA sequences. The sequence PB3A showed high similarity of more than 97% with *Pseudomonas aeruginosa* when compared with the existing sequences in the GenBank using BLAST search algorithm. The 16S rRNA sequence was deposited in the GenBank database under the accession number of KF0295931.

Biosurfactant isolated from selected isolate showed a wide activity against the pathogenic strains studied. Zone of inhibition was recorded as a quantitative measurement for the antimicrobial property of the test strain against the pathogens. The biosurfactant shows significant antimicrobial activity against the test pathogens, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *E. coli* and *P. aeruginosa* was detected with a zone of clearance of 10, 11, 10 and 13 mm, respectively. Tsuge *et al*. (1996) explored the antimicrobial activity of the lipopeptide surfactants (biosurfactant) which are due to the potent antibiotics such as surfactin, streptofactin and gramicidin produced by the microorganism (Rahman *et al*., 2002; Satpute *et al*., 2008). Similar results of antimicrobial activity of the biosurfactant from the *C. antartica* have been successfully demonstrated against gram-positive bacteria (Kiran *et al*., 2010).

Different concentrations of partially purified biosurfactants were treated against cervical cancer cell line (HeLa) was given in the Fig. 2a-b. The present study reveals that IC_{50} value of 125 µg

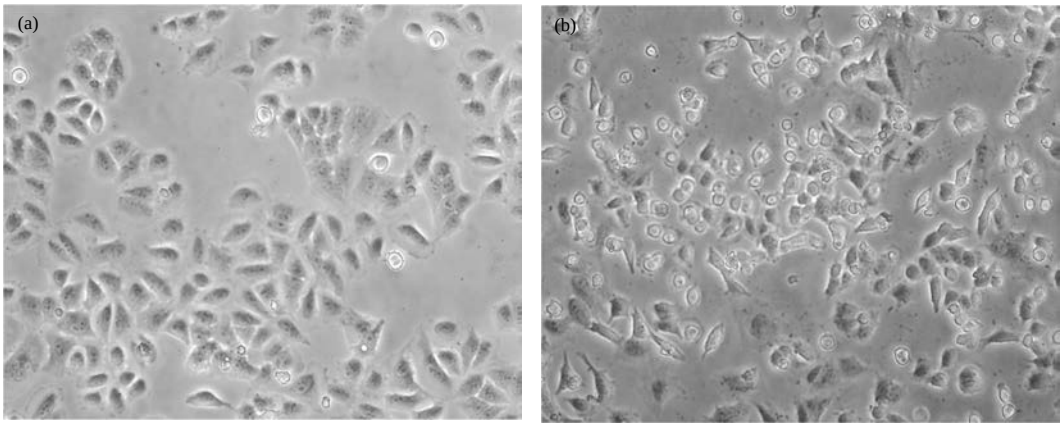

Fig. 2(a-b): *In vitro* anticancer activity against HeLa cells

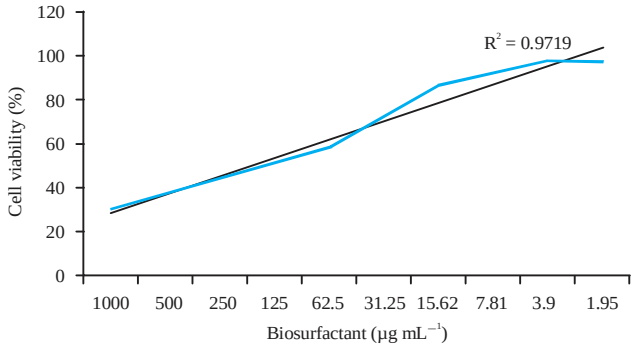

Fig. 3: IC_{50} analysis of biosurfactant against HeLa cells

against the HeLa cells and a maximum suppression was observed up to of 30.49% at a concentration of 1000 µg mL^{-1} (Fig. 3). The above results reveal that the partially purified biosurfactants extracted from *Pseudomonas aeruginosa* strain PB3A shows significant anti proliferation activity against HeLa cell lines, which gives way to explore the potential of these components in the healthcare and pharmaceutical industries (Donio *et al*., 2013).

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