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Research Article

PRODUCTION OF RHAMNOLIPIDIC BIOSURFACTANT BY *PSEUDOMONAS LIBANENSIS* STRAIN IHB 17501 AND ITS ANTIBACTERIAL AND CYTOLYTIC EFFECT ON *STREPTOCOCCUS PNEUMONIAE* ATCC 49619

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ABSTRACT

The study is primarily aimed to investigate the potential antibacterial and cytolytic activity of rhamnolipidic biosurfactant produced by *Pseudomonas libanensis* strain IHB 17501 on *Streptococcus pneumoniae* ATCC 49619. Rhamnolipid production were carried out at these optimized shake flask culture conditions – incubation at 30°C in an orbital shaker set at 200 rpm for 7 days in mineral salts medium. Upon characterization using Thin Layer Chromatography (TLC) and Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR – FTIR), results confirmed that the biosurfactant produced is of rhamnolipid type. TLC chromatogram was observed to have one predominant spot (R_f value of 0.8) that migrated at a similar mobility of methyl ester forms of rhamnolipid (R_f value of 0.84). FTIR analysis of the biosurfactant revealed the most important adsorption bands located at 2928.43cm-1 (CH band: CH_2-CH_3 hydrocarbon chains), dominant aromatic C=C at 1707.28 cm-1, C-O stretching bands at 1011.79 cm-1 that confirm the presence of bonds formed between carbon atoms and hydroxyl groups in the chemical configuration of rhamnose rings. Antibacterial activity of the produced rhamnolipid extract was evaluated through MIC and MBC determination. Rhamnolipid extract produced by *Pseudomonas libanensis* strain IHB 17501 was able to demonstrate antibacterial activity against *Streptococcus pneumoniae* with MIC at 0.029ug/ml concentration and an MBC of 0.058ug/ml. Morphological changes on the bacterial cell wall were visualized using JEOL JEM 1010 Transmission Electron Microscope. The TEM analysis showed that at MIC, bacterial cell wall sinking and pore formation could be observed appearing as patchy white spots.

Keywords: Biosurfactant, Rhamnolipid, *Pseudomonas libanensis, Streptococcus pneumoniae,* Antibacterial activity, Minimum inhibitory concentration, Minimum bactericidal concentration

INTRODUCTION

In an epoch where ecological technology has become bedrock of the search for sustainable progress, the pressing need for more biosustainable, biocompatible and biodegradable surfactant based products makes the study of biosurfactants an imperative area of research. The use of synthetic surfactants have brought about increasing environmental concerns due to their ability to accumulate aquatic toxicity and ultimate resistance to biodegradation, leading to increased pollution[1,2]. This prompted investigation to microbial derived surface active compounds was essentially due to their low toxicity, high biodegradability and stability at extremes of pH, temperature, and salinity[2, 3]. Surfactants of microbial origin denominated as biological surfactants or biosurfactants, have received considerable attention as a substitute with enormous potential.

Biosurfactants being amphiphilic, have a unique chemical structure that confers them a wide range of properties. Rhamnolipids, principally produced by *Pseudomonas aeruginosa* strains, are the most investigated biosurfactants due to their potential applications in a wide variety of industries[2, 4]. However, other species of *Pseudomonas* are not well studied in terms of bioactive substances[5]. At present, there is inadequate research on biosurfactant compounds produced by bacterial strains isolated from wastewater[6]. The escalating need for innovative antimicrobial agents has drawn much attention to biosurfactants as antibacterial agents[2]. Rhamnolipids were previously reported to have antimicrobial activity against *S. aureus*, *Bacillus sp.* and *Klebsiella pneumoniae*, several yeasts, and fungal strains[7, 8].

A study on the immense potential of rhamnolipid biosurfactant produced by *Pseudomonas species* other than the common terrestrial *Pseudomonas aeruginosa* strains as an antibacterial agent is fitting and pertinent. This study focuses on the production and application of rhamnolipidic biosurfactants produced by *Pseudomonas libanensis* strain IHB 17501 as an antibacterial agent against Streptococcus pneumoniae ATCC 49619, the pathologic agent of pneumococcal infection.

MATERIALS AND METHODS

Procurement of *Pseudomonas* strain

The pure cultures of *Pseudomonas libanensis* strain IHB 17501 isolated from freshwater sediments of Casili, Balamban, Cebu, Philippines were acquired from the Microbiology Laboratory of the University of San Carlos Talamban Campus (USC-TC) Biology Department. The *Pseudomonas* strain was maintained in a *Pseudomonas* isolation agar stored at 4°C prior to screening for rhamnolipid biosurfactant production.

Seed Culture Preparation and Rhamnolipid Production

The potential biosurfactant producer was inoculated to a 5 ml nutrient broth and incubated for 24 hours at 30°C in an orbital rotary shaker set at 200 rpm. The rhamnolipid media utilized in this study contained glucose (10g/L), glycerol (10ml/L), magnesium sulfate (0.5%), potassium dihydrogen orthophosphate (2%), yeast extract (1g/L) and 50% distilled marine water. The inoculated rhamnolipid media were incubated

in a 250 ml shake flask at the same optimized conditions for 7 days. The shake flask culture was filtered by using a 0.2um Whatman membrane filter to obtain a cell free broth which was screened for biosurfactant production.

Rhamnolipid extraction

Rhamnolipid in the cell-free broth was extracted by addition of 50ml of ethyl acetate to the 50 ml cell culture flask maintaining optimized shake flask culture condition for 24 hrs. After 24hrs, the ethyl acetate was separated using separatory funnel. Cell culture flask was washed with ethyl acetate three times. The extracted ethyl acetate was evaporated under reduced pressure using rotary evaporator set at 150 rpm at 35°C leaving behind relatively pure rhamnolipids having brown oil-like appearance.

Screening for Rhamnolipidic Biosurfactant Production

Biosurfactant production was screened through Oil Spreading Assay, Emulsification Assay, Parafilm M Test[8]. Anthrone Assay as described[8] was used to estimate glycolipid content of the extract denoted as dark green color in anthrone solution. Hemolytic activity was screened through addition of the biosurfactant to an isotonic solution of human red blood cells – a positive result indicated by a red to brown color of supernatant fluid after centrifugation of the mixture.

Characterization of the Rhamnolipidic Biosurfactant

Thin Layer Chromatography

Ethyl acetate layers of the strain were spotted at a point of origin near the bottom of the pre-coated plate (silica gel 60, Sigma, USA) using a microcapillary tube. Once dried, the plate was developed in a solvent system of chloroform: methanol: acetic acid in a 65:15:2 v/v ratio. The plate was allowed to air dry above the hot plate with low heat. When fully dried, the plate was dipped in Cerium Ammonium Molybdate (CAM) strain and was heated with low heat until spots were visible. Rf value was then computed in comparison with a standard rhamnolipid solution. Upon visualization, the spot nearer the point of origin corresponds to dirhamnolipids, while the spot further from the point of origin represents monorhamnolipids.

FTIR Spectral Analysis of Biosurfactant

The IR spectra of the partially purified extract were recorded on an ATR- FTIR spectrometer in the 4000–400 cm−1 spectral region at a resolution of 2 cm−1. The analysis was done at the Philippine Nuclear Research Institute Chemistry Research Section Atomic Research Division. The characteristic peaks were matched from spectra reported in literature.

Antibacterial Activity

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC):

Antimicrobial activity of rhamnolipid was tested against the test cultures by the macro-broth dilution method using 13 sterile test tubes. One (1) ml of Mueller Hinton Broth was added to each tube. One (1) ml of the extract was added to tubes 1 and 2. Tubes 2 to 10 were serially diluted by removing 1ml from tube 2 then subsequently transferring 1 ml to the succeeding tubes until tube 10. One ml from tube 10 was discarded. One (1) ml bacterial suspension was added to all 13 tubes except for tube 12 which served as the negative control. One (1) ml of 10ug/ml of penicillin standard was added to tube 13. The tubes were incubated for 24 hours at 37°C. The optical density values at 625 nm were determined using a UV/Vis Spectrophotometer. The MIC was determined as the lowest concentration of rhamnolipid that visually inhibits the microbial growth in comparison with the positive control (penicillin) as reflected by a decrease in absorbance reading as compared to the initial absorbance.

MBC was determined by spreading a 0.1 ml sample on nutrient agar plates which were then incubated at 37°C for 24 hours. The samples utilized for this test were obtained from the three tubes with rhamnolipid concentrations higher than the tube showing the MIC concentration. The rhamnolipid concentration from the samples from these tubes is higher than the MIC tube since a serial dilution was performed prior to MIC determination. MBC was determined as the lowest MIC concentration where no viable growth was obtained.

Evaluation of Cytolytic Activity through Transmission Electron Microscopy

Tube showing the MIC was fixed in 2.5% buffered formalin solution. The mixture was then centrifuged at 4000 rpm for 15 minutes and washed with NSS thereafter. Washing was done twice with centrifugation in between washings. Centrifugate was drop – cast onto formvar – coated carbon grids treated with poly-L-lysine and was allowed to stand for 10 minutes. The grids were then washed with 0.45 nm – filtered distilled water and stained with 2% uranyl acetate for 20 seconds. Grids were washed again with 0.45 nm – filtered distilled water and were allowed to air dry. Cells were viewed with JEOL JEM 1010 Transmission Electron Microscope at 80KV accelerating voltage.

Table 1: Screening Results for Biosurfactant Production by *Pseudomonas libanensis* strain IHB 17501

Strain	Oil Spreading Assay	Parafilm M test	Emulsification Assay	Anthrone Assay
Pseudomonas libanensis strain IHB 17501	+	+	+	+

Table 2: Absorbance Readings of Anthrone Assay

	INITIAL ABSORBANCE	CORRECTED ABSORBANCE
Sample	1.588	1.453
Standard	2.099	1.964
Reagent Blank	0.135	-

Table 3: MIC Determination Absorbance for Each Tube at 625 nm

Tube # (C=ug/ml)	Initial	Final	Tube #	Initial	Final
1 (0.932ug/ml)	1.965	1.650	8 (0.007ug/ml)	0.097	0.257
2 (0.466ug/ml)	1.908	1.539	9 (0.004ug/ml)	0.055	0.141
3 (0.233ug/ml)	1.820	1.450	10 (0.002ug/ml)	0.052	0.125
4 (0.117ug/ml)	0.944	0.912	11 **NEG. CTRL**	0.048	0.121
5 (0.058ug/ml)	**0.362**	**0.311**	12 **QC TUBE**	0.043	0.043
6 (0.029ug/ml)	**0.155**	**0.129**	13 **POS. CTRL**	0.042	0.031
7 (0.015ug/ml)	0.089	0.546			

Table 4: Computation of Rhamnolipid Extract Concentration in a Two-fold Serial Dilution

Tube #	RL Concentration (ug/ml)	Tube #	RL Concentration (ug/ml)
1	0.932	8	0.007
2	0.466	9	0.004
3	0.233	10	0.002
4	0.117	11	NEGATIVE CONTROL
5	0.058	12	QUALITY CONTROL TUBE
6	0.029	13	POSITIVE CONTROL
7	0.015		

NOTE: *Final concentration = initial concentration / dilution factor
*Tube 1 represents the original rhamnolipid concentration since it was undiluted
*Tube 11 contains Mueller Hinton Broth added with the test organism
*Tube 12 contains only Mueller Hinton Broth
*Tube 13 contains Mueller Hinton Broth added with the test organism and 10ug/ml of penicillin

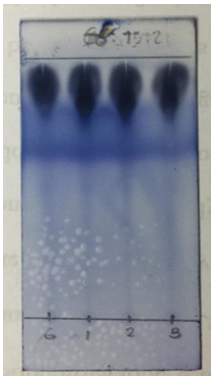

Figure 1: TLC plate

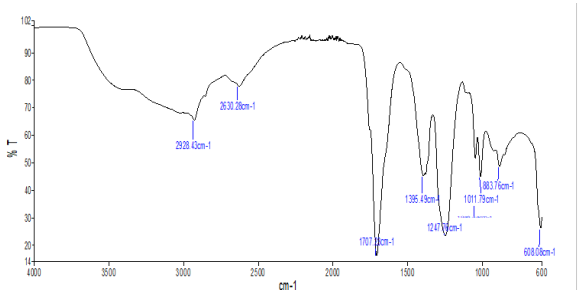

Figure 2: FTIR spectral analysis of biosurfactant produced by *Pseudomonas libanensis* strain IHB 17501

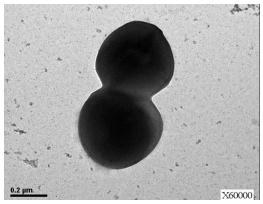

Figure 3: *Streptococcus pneumoniae* under TEM

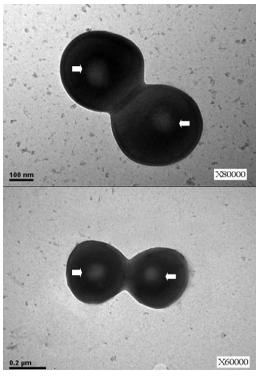

Figure 4: *Streptococcus pneumoniae* at MIC concentration
***Cell wall pore formations pointed by arrows.**

RESULT AND DISCUSSION

Screening for Rhamnolipidic Biosurfactant Production

The potential of *Pseudomonas libanensis* strain IHB 17501 to produce biosurfactant were screened through Oil Spreading Assay, Emulsification Assay, Parafilm M Test, and Anthrone Assay. The biosurfactant screening showed positive results for all screening tests (Table 1) therefore elucidating the impression that *Pseudomonas libanensis* strain IHB 17501 was able to produce a biosurfactant at these optimized conditions: incubation at 30°C in an orbital shaker set at 200 rpm for 7 days. In addition to this, the use of glycerol (10ml/L) and glucose (10g/L) as carbon sources were to shown to produce single type of glycolipid biosurfactant as seen during characterization of the biosurfactant.

Employing anthrone assay, measurement of the amount of rhamnose in the glycolipid was done using UV/Vis spectrophotometer at 625 nm. The absorbances summarized in Table 2 are directly proportional to the rhamnolipid concentration. The initial absorbance was corrected by subtracting from the original absorbance, the reagent blank absorbance, owing to the fact that colored reagents greatly contribute to the absorbance of the solution.

The rhamnose concentration of the sample was computed using Beer – Lambert Law as follows:

$$\text{Rhamnose concentration} = \frac{\text{Absorbance of sample x Concentration of STD}}{\text{Absorbance of STD}}$$

$$= \frac{1.453 \; x \; 0.371}{1.964}$$

= 0.274 ug/ml

There is a need to multiply the rhamnose concentration by a correction factor since the rhamnose moiety embodies only a fraction of the rhamnolipid molecule. Rhamnolipid values were determined by multiplying rhamnose values by a factor of 3.4 obtained from the correlation [y= (0.0139 x -0.0058) x 0.68] of pure rhamnolipids/rhamnose[7, 9]. Based on the computations shown below, the extract obtained from the seed culture had a rhamnolipid concentration of 0.932ug/ml.

Rhamnolipid concentration = rhamnose concentration x factor
= 0.274 ug/ml x 3.4
= 0.932 ug/ml

Characterization of the Biosurfactant

Presence of the rhamnolipid biosurfactant was further evaluated using TLC and FTIR. TLC analysis of the extract suggested that the isolated microbial surface – active compound from *Pseudomonas libanensis* strain IHB 17501 was composed of rhamnolipid (Figure 1). The extract was separated on TLC plates alongside a commercially available rhamnolipid sample. The extract was observed to have one predominant spot (R_f value of 0.8) that migrated at a similar mobility of methyl ester forms of rhamnolipid (R_f value of 0.84). FTIR analysis of the biosurfactant revealed the most important adsorption bands located at 2928.43cm-1 (CH band: CH_2-CH_3 hydrocarbon chains), dominant aromatic C=C at 1707.28 cm-1, C-O stretching bands at 1011.79 cm-1 that confirm the presence of bonds formed between carbon atoms and hydroxyl groups in the chemical configuration of rhamnose rings (Figure 2). These TLC and ATR – FTIR findings strongly suggested that the biosurfactant produced is of rhamnolipid type.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) against *Streptococcus pneumoniae* ATCC 49619

Using ultraviolet-visible spectrophotometric method to determine end point measurements, bacterial growth was denoted as an increase in absorbance compared to the baseline, whereas, bacterial growth inhibition was reflected by a decrease in absorbance in any of the tubes from 1 to 13 in the standard macrodilution procedure for MIC determination (Table 3). The Mueller Hinton broth had an initial absorbance reading of 0.043, whereas the bacterial suspension used had an initial absorbance reading of 0.093. The computed rhamnolipid concentration was 0.932ug/ml. During MIC determination, the rhamnolipid extract was diluted into a two-fold serial dilution from tube 1 until tube 10. Tubes 11 to 13 were considered as control tubes and do not contain rhamnolipid extract. The final concentration of rhamnolipid extract in each tube was computed using the formula: Final concentration = initial concentration/dilution factor where, dilution factor is the reciprocal of the dilution used for each tube. The dilution factor is 2 (two-fold serial dilution). Computation of the final concentration of the diluted rhamnolipid extract in each tube is summarized in Table 4 below.

Final absorbance from tubes 1 to 6 was noted to decrease considerably as compared to its initial absorbance, whereas from tubes 7 to tube 10, an increase from the initial absorbance was remarkable. These noteworthy findings point toward bacterial growth inhibition from tubes 1 to 6 and bacterial growth from tubes 7 to 10. The last tube showing a decrease in absorbance is tube 6 which signifies that the least concentration of rhamnolipid which caused inhibition of bacterial growth was contained in this tube and any concentration below this can no longer inhibit bacterial growth, as supported by increasing absorbance from tubes 7 to 10. By adherence to MIC definition, the tube considered to be showing the minimum inhibitory concentration is tube 6 having a rhamnolipid concentration of 0.029ug/ml. Since MIC was determined to be at this rhamnolipid concentration, any concentration below its MIC cannot inhibit bacterial growth which explains why there was remarkable increase in absorbance from the initial reading from tubes 7 to 10. Being a negative control tube, visible growth should be observed at tube 11 as reflected by an increase in absorbance from the initial reading, since it was not added with any antibacterial agent. If the broth used in the test is viable and not contaminated, it is expected that there should be no change in absorbance at tube 12 since it contains only Mueller Hinton Broth. A decrease in absorbance should be observed at tube 13 added with penicillin (positive control drug) that indicates bacterial growth inhibition, since *Streptococcus pneumoniae* ATCC 49619 was noted to be penicillin sensitive.

For Minimum Bactericidal Concentration (MBC) determination, a portion (0.1 ml) of the contents of two to three tubes above the MIC tube (i.e. tubes 3, 4, and 5) were plated into a melted nutrient agar. After incubation for 24 hours at 37°C, plates were examined for colonial growth on the media. No bacterial growths were noted from all the plates inoculated with the samples. Since the rhamnolipid concentration decreases from tubes 1 to 10 being serially diluted two-fold, tube 1 represents highest rhamnolipid concentration since it is undiluted and tube 10 the lowest. Having no bacterial growths at tube 3 ($RL_{conc.}$ = 0.233ug/ml), tube 4 ($RL_{conc.}$ = 0.117ug/ml) and tube 5 ($RL_{conc.}$ = 0.058ug/ml), these results strongly suggested that at tube 5 with rhamnolipid concentration of 0.058mg/ml, is the least possible rhamnolipid extract concentration required to kill the test organism.

Cytolytic Activity on *Streptococcus pneumoniae* ATCC 49619

The cytolytic activity of rhamnolipid on *Streptococcus pneumoniae* ATCC 49619 is seen as morphological changes (deformations, lysis, cellwall sinking into the bacterial body, patchy white spots, and invaginations) in the bacterial cell membrane[10]. The TEM analysis showed that at MIC, morphological alterations in the bacteria could be observed. Cell wall sinking into the bacterial body is reflected as patchy white spots as shown in Figure 4. With an MIC of 0.029ug/ml, the membrane bilayers begin to disintegrate and coexist with the rhamnolipid – phospholipid mixed micelles which results to trans-bilayer pore formation, softening of the bilayer and enhancement of bilayer fluctuations perceived as pale staining white spots. At MBC of 0.058ug/ml, only rhamnolipid-phospholipid mixed micelles exist with more holes developing causing the vesicles to rupture into few pieces of bilayers and eventually disintegrate which explains why at this concentration no cell was visible upon TEM analysis. In contrast, the untreated cells (Figure 3) appeared intact with no cell body sinking and the cell wall was not deformed.

CONCLUSION

Based on the findings it can be concluded that *Pseudomonas libanensis* strain IHB 17501 is capable of rhamnolipid biosurfactant production. Furthermore, rhamnolipid had antibacterial and cytolytic activity against *Streptococcus pneumoniae* ATCC 49619.

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