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EXTRACTION OF BIOSURFACTANT FROM *PSEUDOMONAS AERUGINOSA* AND ITS EFFECTS ON SOME PATHOGENIC BACTERIA

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Abstract

Pseudomonas aeruginosa was isolated from clinical sources and tested for biosurfactant production when growing in the mineral salt medium (production medium) that containing glycerol as carbon and energy source. Rhamnolipid with emulsification properties were extracted from *P. aeruginosa* by organic solvent methods (methanol/chloroform/acetone), purified bysilica gel G60 column chromatographic and then characterized by TLC where it turn out that the biosurfactant is composed of both mono- and di-rhamnolipid. The partial Purified rhamnolipids exhibited emulsion activity of 88.18% towards Kerosene oil as oil-in-water, while the surface activity was 55mm. Biosurfactant that extracted from *P. aeruginosa* showed antibacterial activity by reducing growth of several pathogenic bacteria (*Staphylococcus aureus, Klebsiella pneumonia and Escherichia coli*). The inhibitory activity of biosurfactant against pathogenic bacteria studied by using Kirby-Bauer disk diffusion method, where the biosurfactant had higher inhibitor activity against *S. aureus*, where highest diameter of inhibition zone was (30mm).

Key words: Antimicrobial activity, Biosurfactant, *Pseudomonas.*

Introduction

Several pathogenic bacteria, such as *Staphylococcus aureus, Klebsiella pneumonia and Escherichia coli*have been recognized as the most important cause of nosocomial infections (Tian *et al*., 2018). The virulence factors of these bacteria involve;toxins, enzymes, adhesion proteins, and cell-surface proteins, factors that help the bacteria to defense the innate immune defense and antibiotic resistance mediate survival of the bacteria and tissue invasion at the site of infection (Ribet & Cossart, 2015).

The high demand for new antimicrobial agents following increased resistance shown by pathogenic microorganisms to existing antimicrobial drugs has drawn attention to biosurfactants as antibacterial agents (Gudiña *et al*., 2013). Some of these biosurfactants have been described for their potential as biological active compounds and for their applicability in the medical field. Therefore, they are a suitable alternative to synthetic medicines and antimicrobial agents and can be used as safe and effective therapeutic agents. Recently, there has been an increasing interest in the effects of biosurfactants on human and animal cells and cell lines (Rodrigues and Teixeira, 2010).

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Microorganisms can produce a broad range of molecules. They include amphipathic molecules called biosurfactants (Willem, 2012). Biosurfactants are compounds produced on microbial cell surfaces or extracellularly excreted, which contain both hydrophilic and hydrophobic moieties (Sambanthamoorthy *et al*., 2014).

There are several significant advantages to the use of biosurfacatnts rather than synthetic ones, for example biosurfactants are biodegradable, cost-effective, low toxicity, selectivity, specific activity at extreme temperatures, pH and salinity, the possibility of their production by fermentation, their potential applications in environmental protection and management, the recovery of crude oil, as antimicrobial agents in the health and food processing industries (Johny, 2013; Silva *et al*., 2014).

Biosurfactants can be classified by their chemical structure and by their microbial origin. Rosenberg and Ron (1999) classify them into two categories; high molecular weight, and low molecular weight molecules. Low-mass surfactants include glycolipids, lipopeptides and phospholipids, where polymeric and particulate surfactants are the high-mass surfactants (Singh, 2012).

Up to now, the most commonly isolated and best studied groups of biosurfactants are those of glycolipid compounds. Rhamnolipids are mainly produced by *Pseudomonas aeruginosa*, They are a group of biosurfactants of glycolipid nature, composed of a hydrophilic head formed by one or two rhamnose molecules, known as monorhamnolipids and dirhamnolipids, and a hydrophobic tail containing fatty acids (Bendaha *et al.*, 2016).

Several Biosurfactants exhibit anti-bacterial, anti-fungal and anti-viral activity, making them suitable candidates for infections control (Sambanthamoorthy *et al.*, 2014). Rhamnlipid has shown antimicrobial activity against several microorganisms such as the gram-positive bacteria, *Staphylococcus aureus*, *Clostridium perfringens*, *Bacillus subtilis* and Gram-negative bacteria like *Salmonella typhimurium*, *Klebsiella pneumonia*, *Escherichia coli*, *Enterobacter aerogenes* (Magalhaesand Nitschke, 2013).

The present study was carried out to produce microbial surfactants from *Pseudomonas aeruginosa* isolated from clinical source and study the inhibitory effect of microbial surfactants against some pathogenicbacteria. The microbial derived Biosurfactants have some of the potential applications of in pollution and environmental control, hydrocarbon degradation heavy metal removal, hexa-chloro cyclohexane degradation and antimicrobial activity (Sachdev & Cameotra, 2013).

Materials and methods

Isolation and identification of *Pseudomonas aeruginosa*

Forty two clinical isolates were collected from hospitalsanddiagnosed depending on the cultural characteristics, microscopic characteristics, biochemical tests, and then confirmed by Vitek system.

Cultivation of *P. aeruginosa* and biosurfactant Production

The mineral salt medium utilised for biosurfactant production was composed of the following: 0.1% K_2HPO_4,0.1% KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.002% $CaCl_2 \cdot 2H_2O$, 0.005% $FeCl_3 \cdot 6H_2O$, 0.2% $NaNO_3$ and 3% glycerol as the main carbon and energy source, with the pH of the medium adjusted to 6.8 and the medium was autoclaved for 15 minat 121°C (Silva *et al*., 2010). Five ml of the mineral salt medium was inoculated with a fresh culture of *P. aeruginosa* and incubated at 30°C, 200 rpm with an incubation period of 18–24 h, and further used as the seed culture for the biosurfactant production (Dhivya *et al*., 2014).

Production of biosurfactant was performed out according to Shah *et al*., (2016). One liter of mineral salt medium, with divided into two erlenmyer flasks. 2 % of seed culture was added to each container after cooling to 50°C. The containers were incubated for 120 hrs and shaken (200) rpm at (30)°C.

Screening for rhamnolipidbiosurfactant production

Ability of *P.aeruginosa* isolates to produce biosurfactant have been checked for both supernatant and precipitate. Isolates were cultured in mineral salt media and then centrifuged for 30 min at (10000) rpm,4°C. The Surface activity was tasted by theoil spreading assay and emulsification index (E24).

A. Oil spreading assay

To apply the oil-spreading test, the oil was layered over water in a petri plate and a drop of supernatant and pellet was added to the surface of oil. The diameter of the clear zone on the oil surface was measured in 5 replications for each isolate. A water drop was used as a negative control (Luepongpattana *et al*., 2014).

B. Emulsification index (E24)

The emulsification index test was done to method described by Sharma *et al*., (2014) used to detect activity of rhamnolipidas biosurfactant, as follows: Emulsifying capacity of isolates was evaluated by an emulsification index (E24) for kerosene oil. To do so, 1.5 mL of Kerosene was added to 1.5 ml of supernatant and pellet in a test tube, which was vortexed at high speed for 2 min and allowed to stand for 24 h. The percentage of the emulsification index was calculated using the following equatio:

E24 = (height of the emulsion layer / total height of the mixture) *100

Extraction and partial purifcation of the biosurfactants

After cooling centrifuge the supernatant containing biosurfactant was treated with the mixture of extraction solvent (methanol/ chloroform/ acetone, 1:1:1 by volume). The mixture was continuously shaken at 200 rpm, 30°C for 5 hrs inside incubator shaker. Two layers of precipitate were obtained. The lower layer was discarded (Shah *et al*., 2016).

The extracted viscous honey-colored rhamnolipid product was collected for purification using silica gel chromatographic. A sample of rhamnolipid was dissolved in methanol. The column (1.5cm × 35cm) was packed with silica gel and not allowed to dry. The sample was applied on the surface of the column. After that, it was eluted with methanol gradually (Darvishi *et al*., 2011).

Each fraction was evaporated on a rotary evaporator and its oil displacing area was measured. The fractions that demonstrated the oil displacement test were further separated by thinlayer chromatography (TLC) using aluminum silica gel 60 F254 plates and a chloroform : methanol : 20% aqueous acetic acid (65 : 15 : 2) solvent system. Rhamnolipids can be visualized using the Molisch test, with reagents specific for sugars (Moussa *et al*., 2014).

The effect of biosurfactant against some pathogenic bacteria in vitro

Mueller Hinton agar was prepared and poured in a sterile petriplate. The plates were allowed to solidify. Sterile filter paper disc (6) mm were Saturated with partial purified biosurfactant. 24 hrs. broth culture of pathogenic bacteria (*Staphylococcus aureus, Klebsiella pneumonia and Escherichia coli*) was swapped with sterile cotton swabs on Muller Hinton, then distribute the discs into the plates. The plates were incubated at 37°C for 24 hours. After incubation period, the zone of inhibition was observed around the disc and it was measured (Cao *et al*., 2009).

Results and Discussion

Initial studies

The bacterial isolate was examined based on its morphological and biochemical characteristics. All isolates grew on Cetrimide agar. This media is selective for isolation and identification of *P. aeruginosa* (Banerjee *et al*., 2017). Pale yellow colonies on MacConkey agar because this pathogen is not fermenting lactose (Anderson & Cindy, 2013). The bacterial strain was a non-spore forming, gram negative, rod-shaped, motile bacterium. The oxidase and catalase tests were positive. Further identification was performed based on the Vitek2 system (MacFaddin, 2000).

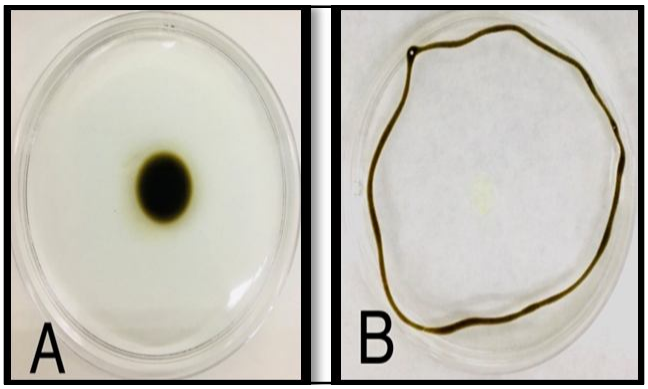
Screening for biosurfactant production

In this study, *P. aeruginosa* isolates were screened for biosurfactant production by two methods; Surface activity by Oil spreading test and Emulsification index (E24).

Surface activity by oil spreading test

Oil spreading assay is a reliable method to detect biosurfactant production by diverse microorganisms based on the ability of the biosurfactants present in the supernatant of isolate solutions capable of spreading the oil and producing a clear zone. This clearing zone on the oil surface correlates to surfactant activity, also called oil displacement activity (Walter *et al*., 2010). Oil spreading assay results were in corroboration with emulsification assay results.

Most isolates of *P. aeruginosa* were positive for the oil spreading assay and *P.aeruginosa* isolate (P_{11}) isolate showed the highest oil spreading activity. Results are given in Fig. 1.



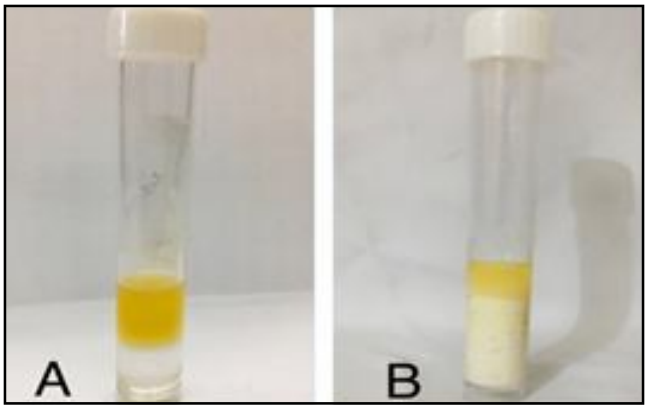
A- Oil without biosurfactant (control) B- Oil with biosurfactant.

Fig. 1: Oil spreading assay.

The oil spreading method is rapid and easy to carry out, requires no specialized equipment, and only requires a small volume of sample (Shoeb *et al*., 2015).

Emulsification index (E24)

Emulsification assay is an indirect method used to screen biosurfactant production obtained surface tension reductions between 2 mm and 13.6 mm (Shoep *et al*., 2015) Fig. 2.



A-Oil wthbiosurfactant B- Oil with PBS as control

Fig. 2: Detection of biosurfactant activity by E24 assay.

Extraction and partial purifcation of the biosurfactants

Biosurfactant produced by *P.aeruginosa* was extracted with organic solvents byusing methanol/ chloroform/acetone (1 : 1 : 1) (Kazim *et al*., 2017). Johny *et al*., (2013) and Sharma *et al*., (2015) have also concluded that organic solvent using methanol/ chloroform/acetone was the best technique in extraction of biosurfactant. This is due to the presence of the

hydrophobic end of the biosurfactant, making them soluble in organic solvent (Shah *et al*., 2016).

The recovery of biosurfactant from cell free culture was done bysilica gel column chromatography to separate rhamnolipid with a suitable elution protocol (Jie *et al*., 2019). The yield of biosurfactant was (20.04 g/l) from *P. aeruginosa* in the presence of glycerol as sole source of carbon. The partial Purified rhamnolipids biosurfactant had surface activity 55 mmwith emulsification index 88.18%.

The results of thinlayer chromatography (TLC) suggested that the mono-/di-rhamnolipids produced by *P. aeruginosa* (P_{11}) were completely separated. In the TLC chromatogram, the lower spots are di-rhamnolipid structures and the higher spots contain mono-rhamnolipid molecules Fig. 3.

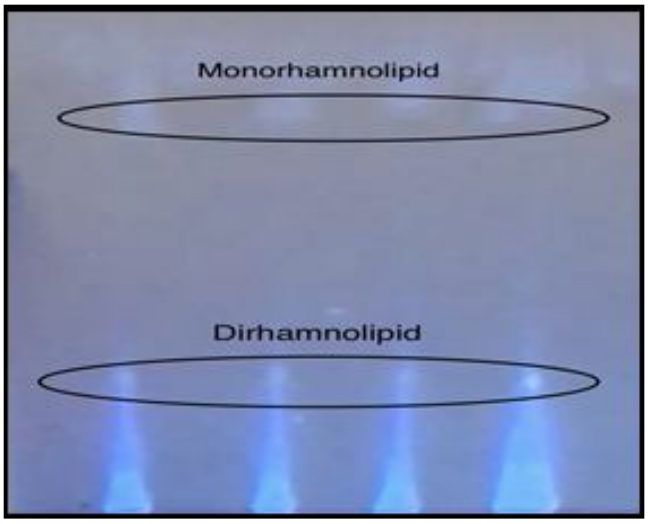


Fig. 3: TLC analysis of mono- and di-rhamnolipids separated by silica gel column chromatography.

Antibacterial activity of biosurfactant

The antimicrobial activity of biosurfactant was tested against some pathogenic bacteria included: *S. aureus*, *K. pneumonia* and *E. coli*. It was observed that the bio surfactant reduced the growth of these bacteriaas in table 1, the highest effect was observed on the growth of *Staphylococcus aureus*. Fig. 4.

Mechanism of antibacterial activity of biosurfactant is act on the integrity of cell membranes, which leads to cell lysis. There are different ways in which the biosurfactant affect on the membrane integrity. The rhamnolipid, a glycolipid which are thought to act on the lipid part of cell membranes or outer proteins, causing structural fluctuations in the membranes (Lopes *et al*., 2014).

Table 1: Antimicrobial activity of partial Purified rhamnolipidsbiosurfactant produced by *P.aeruginosa*.

Bacterial isolates	1g/ml	0.5g/ml
E. coli	21	17
S. aureus	30	25
K. pneumonia	20	14

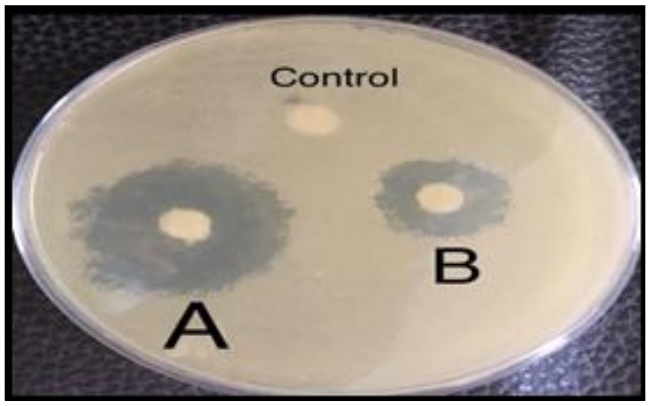


Fig. 4: Shows the effect of biosurfactant on *S. aureus*.
A- Represent 1g/ml concentration of biosurfactant.
B- Represent 0.5g/ml concentration of biosurfactant.

The result suggests that the rhamnolipid molecule having both hydrophobic and hydrophilic groups could insert its fatty acid components into a cell membrane that caused considerable alteration in the ultrastructure of the cell such as ability of the cell to interiorize the plasma membrane **(**Bharali *et al*., 2013).

Conclusions

The rhamnolipidbiosurfactant produced by *P. aeruginosa*had antimicrobial activity against different species of microorganisms. Thus the produced antimicrobial compound can be further characterized and can be applied as a biocontrol agent.

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