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TO INVESTIGATE THE SUPPRESSION OF FUNGAL AND BACTERIAL BIOFILMS BY A BIOSURFACTANT PRODUCED BY THE BACTERIUM PSEUDOMONAS AERUGINOSA.

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ABSTRACT:

Pseudomonas aeruginosa is the primary producer of rhamnolipids, which are extracellular secondary metabolites with surface-active properties. The main goal of this research was to look at Pseudomonas' potential to produce biosurfactants. When compared to commercially available surfactants, their low toxicity, biodegradability, efficiency, and specificity drew more attention, making them suitable candidates for use in new generations of microbial dispersal agents and as an adjuvant to control surface-growing communities of microorganisms. Biosurfactant dispersal properties have been shown to rival those of conventional inhibitory agents against bacterial and yeast biofilms. There is mounting evidence that this biosurfactant plays a role in various stages of this bacterium's biofilm development.Furthermore, rhamnolipids have significant anti-adhesive and disrupting potential against established biofilms formed by a variety of bacterial and fungal species.The purified biosurfactant's inhibitory activity against Candida and bacterial biofilms, which are groupings of microorganisms that may stick to inanimate surfaces, resulting in the spread of various illnesses, was the second focus of the research.

Keywords: Rhamnolipids, Anti-adhesion, biosurfactant, Biofilm producers.

INTRODUCTION

Biosurfactants are extracellular or cell membrane surfactants produced by bacteria, yeasts, and fungi(Karanth, Deo, and Adi 1999; Mulligan 2005; Tabatabaee et al. 2005).They are a structurally varied collection of surface-active compounds produced by microbes. Where the hydrophobic moiety can be a carbohydrate, an amino acid, a cyclic peptide, a phosphate, a carboxylic acid, or alcohol, among others, and the hydrophilic moiety can be a carbohydrate, an amino acid, a phosphate, a carboxylic acid, or alcohol, among others (Satpute et al. 2010). These compounds diminish surface and interfacial tension in aqueous solutions as well as hydrocarbon mixtures, making them promising candidates for improving oil recovery and demulsification processes (Desai and Banat 1997; Muthusamy et al. 2008; Youssef et al. 2004).They are amphipathic molecules that can form specialized structures that are essential to their action. (Christofi and Ivshina 2002).They increase the area at the aqueous hydrocarbon interface, which improves hydrocarbon bioavailability to microbial cells. This accelerates hydrocarbon dissolution and utilization by microorganisms. (Tuleva, Ivanov, and Christova 2002).

Bacterial attachment to surfaces and biofilm formation have serious consequences in the food, environmental, and biomedical fields. Biofilm is a bacterial community that adheres to biotic and abiotic surfaces and is embedded in a polymeric matrix that is primarily composed of polysaccharides, proteins, and nucleic acids. (Flemming and Wingender 2010). Bacteria found in natural, clinical, industrial and food-processing environments are referred to as biofilms. Biofilms are now thought to be the source of many pathogen outbreaks. (Aarnisalo et al. 2007; Lapidot, Romling, and Yaron 2006)and bacterial biofilms are responsible for more than 80% of microbial infections in the body and bacterial biofilms account for more than 80% of microbial infections in the body (Moreau-Marquis, Stanton, and O'Toole 2008; Wu et al. 2016). In biofilms, bacteria are typically well protected from the effects of antibiotics, disinfectants, and the host immune system. Biofilms are very challenging to eradicate because biofilm bacteria are up to 1000 times more resistant to antibiotics and host immune responses than planktonic bacteria. (Burmølle et al. 2010; Kamali et al. 2021). Therefore, searching for novel compounds or strategies to inhibit biofilm formation or disperse preformed biofilm is needed. The presence of biofilm in food processing environments can result in spoiling and disease transmission, posing a health concern to consumers. (Zezzi do Valle Gomes and Nitschke 2012). There is sufficient data to suggest that the biofilm mode of life leads to greater resistance to antimicrobial agents. (Satpathy et al. 2016).

C. Albicans is a fungus that commonly occurs as a commensal in the gastrointestinal and vaginal flora of many healthy individuals. It is also an opportunistic pathogen: this microorganism can cause severe infections in case of a deteriorated immune

system of the patient (Tournu and Van Dijck 2012). For instance, cancer patients, HIV patients, elderly people, and patients who take immunosuppressive drugs or antibiotics, have a higher risk of uncontrolled Candida proliferation (Ramage et al. 2001).

Pseudomonas rhamnolipid, a glycolipid biosurfactant, has numerous applications in these fields. The application of biosurfactants to a surface changes its hydrophobicity, interfering with microbial adhesion and desorption processes; in this respect, biosurfactant production by Pseudomonas bacteria in vivo can be viewed as a defense against other colonizers. (Dafforn, Lewis, and Johnston 2011; van Hoogmoed et al. 2000; Joseph et al. 2001).

The current study sought to assess the antibacterial and anti-adhesive effects of this biosurfactant against a variety of pathogenic microorganisms. Biosurfactants have been demonstrated to prevent pathogenic organism adhesion to solid surfaces or infection sites; consequently, prior attachment of biosurfactants to solid surfaces may constitute a new and effective method of countering pathogenic microorganism colonization. (Harshada 2014; Singh and Cameotra 2004).The amount of biofilm generated by Salmonella typhimurium, Salmonella enterica, E. coli, and Proteus mirabilis was reduced by pre-coating vinyl urethral catheters with a surfactin solution before inoculation with the medium. (Mireles, Toguchi, and Harshey 2001).

2. MATERIALS AND METHODS

2.1. MICROORGANISM COLLECTION

The biosurfactant-producing Pseudomonas aeruginosa NDBS strain was identified from oil-contaminated soil used for production in the Latur district. The isolated organism is morphologically, biochemically, and genetically identified using 16s rRNA sequencing. Similarly, hemolytic activity, oil displacement, and drop collapse tests are used to screen biosurfactant producers.

2.2. PREPARATION OF INOCULUMS FOR BIOSURFACTANT SYNTHESIS

Isolates were grown in 100 ml of sterile minimum media (MM) containing 2% vegetable oil as a carbon source. The suspension was incubated for 48 hours at 37 °C. The culture flask inoculum was subcultured in nutrient agar. (Sharma and Saharan 2016). The isolates were kept in nutrient broth. 2% (v/v) glycerol stock before use in the current study. For the formation of biosurfactant, a 5% inoculum of a 24-hour-old culture was employed. Before use, the optical densities of pseudomonas were read at a wavelength of 600nm, and the growth of pseudomonas was recorded at 24-hour intervals.

2.3. PRODUCTION MEDIA AND CULTIVATION CONDITIONS

The defined medium's composition was (per liter) minimal agar medium with dextrose (1gm/lit), dipotassium phosphate (7.0gm/lit), mono potassium phosphate (2.0gm/lit), sodium citrate (0.5gm/lit), magnesium sulphate (0.1gm/lit), ammonium sulphate (1.0 gm/lit) with 2 percent (v/v) cheap carbon source such as vegetable oil with PH 7.0 ±0.2 Inoculated production media with 5% (v/v) of selected isolate pure culture and incubated for 72 hours at 37 °C at 150 rpm min^{-1}.

2.4. BIOSURFACTANT PURIFICATION AND EXTRACTION

2.4.1. ACID PRECIPITATION

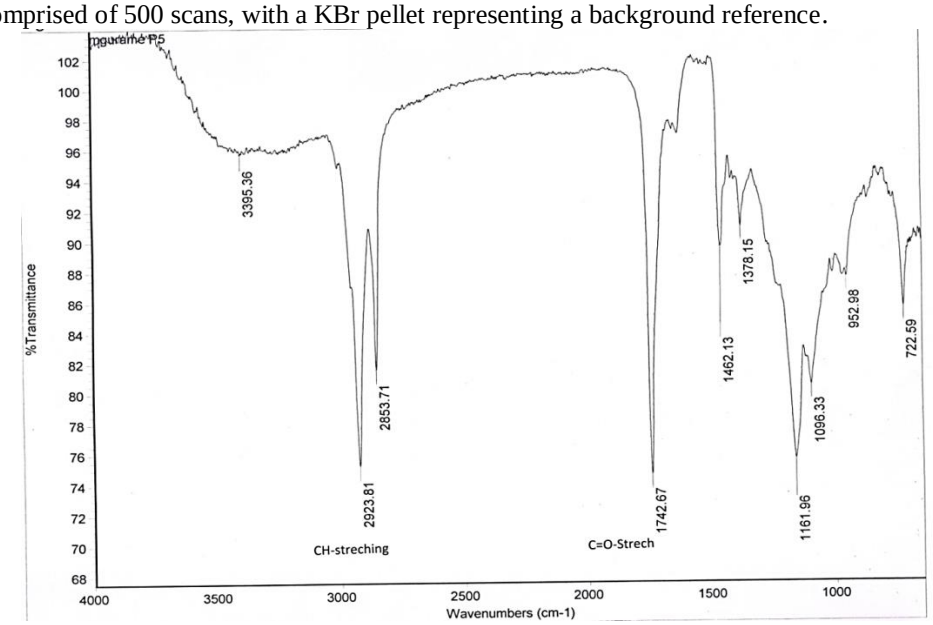
To remove cells, the culture was centrifuged at 7000g for 15 minutes. The supernatant was then acidified with hydrochloric acid to a pH of 2.0. After 30 minutes of centrifugation at 7000g, the precipitate was extracted with a chloroform-ethanol solvent (2:1). Extraction was used to collect and weigh the precipitate. The partially purified biosurfactant was dissolved in 10 mL chloroform and purified further using column chromatography. The column was packed with silica G-500, and different fractions were collected with $CHCl_3$:CH_3OH in 50:3, 50:5, and 50:20 ratios. The resulting product was used to characterize and investigate biofilm inhibition.

2.5. BIOSURFACTANT CHARACTERIZATION

The presence of aliphatic hydrocarbon chain groups was revealed by FTIR analysis of the biosurfactant's wave numbers for C-H bonds. The presence of alkanes was confirmed by C-H bonds of the CH3, CH2, and CH groups observed at wavenumbers 3,355 and 2923 cm1, 2853, 1462, and 1378 cm1. The presence of C-O bonds was indicated by the wavenumber 1,762,1142 cm1. The above data from the respective wave numbers confirmed the biosurfactant's glycolipid nature. The biosurfactant's mass spectrometric analysis supports the biochemical and FTIR results, with peaks observed at m/z = 597, 521, 332, 323, & 242 indicating the presence of carbohydrate and lipid moieties.

2.5.1. FOURIER TRANSFORM INFRARED SPECTROSCOPY

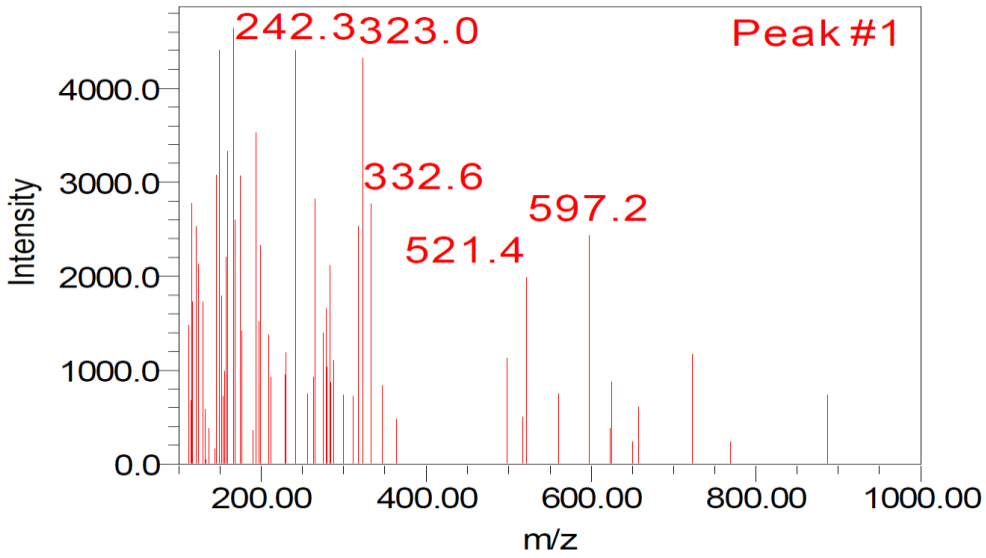
Fourier transform infrared spectroscopy (FTIR) is most useful for identifying distinct types of chemical bonds (functional groups), and can thus be used to identify some components of an unknown mixture. To obtain translucent pellets, freeze-dried crude biosurfactant (10 mg) was ground with 100 mg of KBr and pressed with 7,500 kg for 30 seconds. Infrared absorption spectra were recorded using a Thermo Nicolet AVATAR 330 FTIR system with a spectral resolution of 4 cm1 and a wavenumber accuracy of 0.01 cm^{-1}. All measurements were comprised of 500 scans, with a KBr pellet representing a background reference.



FTIR spectrum of *Pseudomonas aeruginosa* biosurfactant.

2.5.2. MASS SPECTROMETRIC ANALYSIS OF BIOSURFACTANT

The biosurfactant was dissolved in methanol and thoroughly mixed. The biosurfactant was mass spectrometrically analyzed using UV-MS in an LCMS Quadrupole ion-trap mass spectrometer. At a flow rate of 10 µl min^{-1}, standard solutions and samples under investigation were infused into the mass spectrometer. The nitrogen and auxiliary gas flow in the UV-MS were kept at 50 and 5 ml/min, respectively, and are arbitrary values set by the software. The scanning was done in negative ion mode and at 2,000 *m/z*.



Pseudomonas aeruginosa biosurfactant LC-MS spectrum.

2.6. FORMATION OF BIOFILM

Biofilm growth quantified using crystal violet measurement of biofilm formation is one of the most often used techniques for evaluating the efficacy of biosurfactants and biofilm inhibitory agents (O'Toole 2011).In a 96-well microtiter plate, a microbial biofilm is stained with 1% crystal violet. Instead of only staining the biofilm's component cells, the crystal violet also discolors the biomass of the entire biofilm, including EPS and extracellular proteins.(Banat, De Rienzo, and Quinn 2014).

2.6.1. GROWING A BIOFILM

1. Grow a pathogenic strain of *E. coli*, *S. aureus*, *P. Vulgaris*, *S. Typhi*, and *Candida albicans* in a rich medium (i.e. Muller Hinton /PDB) overnight.
2. For biofilm assays, dilute the overnight culture at 1:100 in a fresh medium. A standard biofilm assay medium that promotes planktonic growth and the formation of a more robust biofilm.
3. The dilution in each well of a 96-well dish Incubate the microtiter plate at 37°C for 4-24 hours.

2.6.2. STAINING THE BIOFILM

1. After incubation, turn the plate over and shake out the liquid to remove the cells.
2. Place the plate in a small tub of water and gently submerge it. Shake the water out. Repeat this procedure a second time. This step assists in the removal of unattached cells and media components that can be stained in the following step and significantly reduces background staining.
3. Fill each well of the microtiter plate with 125 L of a 0.1% crystal violet solution in water. Incubate the microtiter plate for 10-15 minutes at room temperature.
4. Rinse the plate 3-4 times with water by immersing it in a tub of water as described above, shaking it out, and blotting vigorously on a stack of paper towels to remove all excess cells and dye.

2.7. RESULTS

Biosurfactant acts as an anti-adhesive agent, preventing the growth of biofilms produced by *Proteus Vulgaris*, *Staphylococcus aureus*, and *Candidaalbicans* wherever planktonic cell proliferation occurs.

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