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ISOLATION AND IDENTIFICATION OF BIOSURFACTANT- PRODUCING BACTERIA FROM MECHANIC WORKSHOP CONTAMINATED SOIL AND ITS ANTI-ADHESIVE ACTIVITY

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ABSTRACT A biosurfactant-producing bacterial strain was identified and screened from contaminated mechanic workshop soil. Hemolytic assay, drop collapse test, emulsification index, and oil displacement tests was used to screen biosurfactant generating Bacillus sp. Biosurfactant producing bacteria was discovered that positive oil spreading technique in Bacillus sp. It has high emulsifying activity and it was 38.8%, whereas it was around 26.31% in Staphylococcus aureus. The isolated biosurfactant effectively reduced biofilm formation. These findings suggest that the Bacillus sp biosurfactant has the potential to be a beneficial antibacterial and antibiofilm substance as an alternative to antibiotics or other chemically manufactured harmful agents.

KEYWORDS : Biosurfactant, Anti-adhesion; Bacillus sp; Biofilm

INTRODUCTION

Surfactants are a group of amphiphilic chemical compounds (i.e., having both hydrophobic and hydrophilic domains) that form an indispensable component in almost every sector of modern industry. A major drive-in recent decade has been toward the discovery of surfactants from biological/natural sources—namely bio-surfactants—as most surfactants that are used today for industrial applications are synthetically(Christina Nikolova and Tony Gutierrez ,2021),

In comparison with synthetic surfactants, biosurfactants have better surface activity, lower toxicity, they can bind heavy metals, have higher biodegradability, selectivity and biological activity (Vatsa *et al*., 2010). Synthetic surfactants have applications in the biodegradation processes of organic compounds, but they are environmentally.

A number of microorganisms, such as filamentous fungi, yeasts, and bacteria are producing biosurfactants (Sobrinho *et al*., 2013). Bacteria belonging to genera *Pseudomonas, Bacillus, Corynebacterium*, Klebsiella, Acinetobacter, A*rchromobacte*r, and *Proteobacteria* have been reported to produce efficient biosurfactants (Ahmad *et al*., 2016)

In recent years, biosurfactants are considered as potential drug candidates and looked up for their explicit biomedical applications such as antibacterial, antifungal, antiviral and anti-adhesive agents against several pathogens (Gudina *et al*., 2010). Recently biosurfactants were found disrupting biofilm formation (Diaz De Rienzo et al., 2016). Biofilms are conglomerations of bacterial cells protected by self-synthesized extracellular polysaccharide matrices (EPS). Biofilm infections are extremely challenging to treat because antimicrobials are less effective than planktonic cells (Anderl et al., 2000), thus making clearance more challenging.

Biosurfactant have been shown to modify the surface properties of bacterial cells and reduce their adhesive properties (Ahimou et al., 2000). In this study, our goal was to characterize the biosurfactant-producing Bacillus spp. isolated from mechanic workshop contaminated soil. To analyse their antimicrobial, anti-adhesive and anti-biofilm abilities of biosurfactant produced by Bacillus sp.

METHODS

Sample collection

Soil samples were collected according to the method described by Tambekar and Gadakhl (2013) with little modifications. Soil samples were randomly collected from machine shops.

Screening and isolation of biosurfactant producing bacteria from soil

5 g of each soil sample was inoculated into 100 ml mineral salts medium (MSM, 1 ml petroleum was added to the flasks, and all flasks were incubated at 37°C for 72 hours. After incubation, samples were serially diluted 10-fold with sterile distilled water in test tubes. From the dilutions (10-6, 10-7 and 10-8) the streak plating technique was performed on nutrient agar plates. The inoculated medium was incubated at 37°C for 24 hours. Obtained pure isolates were stored on nutrient agar slants at 4 °C.

Screening of Biosurfactant

The isolated colonies were obtained in pure cultures and tested for their biosurfactant production by the following methods.

Oil spreading technique

Oil spreading experiment was performed using the method described by Morikawa et al, 2000

Blood hemolysis test

On blood agar plates, the newly formed single colonies from the isolated cultures were streaked. These plates were incubated for 48 to 72 hours at 37° C. When the plates were examined, the clear zone around the colonies revealed the existence of organisms that produce biosurfactant (Anandaraj and Thivakaran, 2010).

Drop-collapse test

The bacterial strains were inoculated in mineral salts medium with 0.1% crude oil (petrol) and incubated for 48 hours. Drop collapse test was performed to screen the biosurfactant production (Jain *et al.,* 1991).

Emulsification assay

Utilizing petroleum, an emulsification test was performed by Cooper and Goldenberg, 1987. The emulsification activity was measured and computed using the following formula:

E24 = (Total height of the emulsion layer/height of the aqueous layer) ×100

Extraction of Biosurfactant

Cold Acetone precipitation

Cold acetone precipitation method (Pruthi and Cameotra ,1997) was used for the recovery of biosurfactants from culture supernatant. Three volumes of chilled acetone were added to the supernatant and allowed to stand for 10h at 4°C. Centrifugation was used to separate the precipitate, which was then evaporated to eliminate any remaining acetone before being redissolved in sterile water.

Detection of biofilm producing bacteria

Direct Method

SEM

SEM was used to analyse the generated biofilm.

Indirect method

Congo red agar

As a selective medium, Congo red agar was employed to isolate the organisms that produce biofilm. Brain heart infusion agar was used to create the medium, along with sucrose and Congo red. A loopful of bacterial culture was inoculated in such a way to get isolated colonies. Inoculated plates were incubated at 37°C for 24-48 hours. After incubation black colonies with a dry crystalline consistency indicated biofilm production.

Antibacterial activity against selected biofilm forming bacteria

Muller Hinton Agar media was prepared. Plates were swabbed with E. coli, S. aureus and Pseudomonas aeruginosa cultures. Two wells were made in the agar pates using a sterile well maker and then 100% of the

biosurfactant was added in one separate well and one well for control. The plates were kept in incubation at 37° C for 24°h. The clear zone diameter was noted (Saravanakumari and Mani 2010).

Biosurfactant activity against biofilm forming E. coli

Antibiofilm activity of biosurfactant on hydrophilic surface (test tube method). This technique was developed by modification of the standard method of Christensen et al., (1985) to test biofilm production.

Biosurfactant inhibited the adhesion of E. coli

Biofilm of E. coli was grown on glass slides for the light microscopic observation (Packiavathy et al 2014). In a 5 ml test tube, sterile square glass sides measuring (1×1 mm) were put. The test tube was then filled with 500 µl of various biosurfactant concentrations (0.1 mg, 0.2 mg, and 0.3 mg) in TSB with 1% glucose and 500 µl of logarithmic phase E. coli inoculum. The test tube was then incubated at 370 C for 24 hours. The glass slides were then stained with 0.4% crystal violet and rinsed three times with PBS. The slides were air dried after being rinsed once more with distilled water to remove the excess discoloration after 5 minutes. Use of a light microscope was used to examine the dyed biofilms.

Result

Colony Morphology

Ten different organisms found from the soil of mechanic workshop, which showed good growth results on the nutrient agar plates. These were purified and characterized on the basis of biochemical tests.

Screening of Biosurfactant

Biosurfactant oil spreading assay

Oil spreading assay results showed IS-1 (Staphylococcus sp) and IS- 4 (Bacillus sp) gave positive result with a clear zone of 3.6 cm and 6.7 cm respectively, whereas E. coli gave negative test for oil spreading assay. The emulsification assay is an indirect method used for the screening production of biosurfactant.Maximum emulsification was observed in Bacillus sp (38.8%) and minimum emulsification was observed in Staphylococcus sp (26.31%) and control (Nil)

Drop collapse assay

Bacillus sp and Staphylococcus sp showed positive results with a flat droplet. While control gave round shaped droplet indicative of negative result for drop collapse assay

Detection of biofilm producing bacteria by SEM

Figure-1 showed the 3D structure of the exopolysaccharide was absorbed.

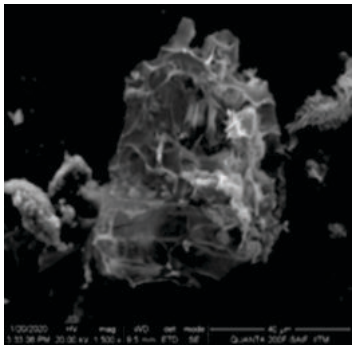

Fig:1, 3D Structure of the exopolysaccharide

Indirect method

Congo red agar

The Congo red agar method applied on Congo red agar medium. The presence of black crystalline colonies of biofilm producer and pinkish red colonies shows the presence of non -biofilm producer. Antimicrobial activity of biosurfactant against selected biofilm forming bacteria

The antimicrobial activity of extracted biosurfactant was tested against selected biofilm forming bacteria such as Staphylococcus aureus, Pseudomonas aeruginosa, and Escherichia coli (Table:1). The extracted biosurfactant efficiently inhibited the growth of the bacteria. The maximum zone was found to be Escherichia coli (28 mm). The minimum zone was found to be Pseudomonas aeruginosa (11 mm) followed by Staphylococcus aureus (17mm).

Table1 Antibacterial activity of biosurfactant against selected biofilm forming bacteria

Bacteria	Diameter of zone of inhibition (mm)
Escherichia coli	28
Staphylococcus aureus	17
Pseudomonas aeruginosa	11

Biosurfactant Activity Against Biofilm Forming E. Coli On Hydrophilic Surface

Biosurfactant displayed strong biofilm inhibition, and the variation in biofilm formation between control and biosurfactant treated tubes. The control test tube indicates the biofilm production. Biosurfactant treated test tube indicates the lacking of biofilm formation due to the addition of biosurfactant. Bacillus sp formed biosurfactant was gradually reducing the biofilm formation.

Biosurfactant inhibited the adhesion of E. coli

To visually disclose the impact of extracted biosurfactant on E. coli biofilm, the changes in morphology and architecture of biofilm were observed by light microscope (Fig-2). The light microscope images captured after crystal violet staining displayed the overall morphology of E. coli biofilm, which revealed that extracted biosurfactant caused a huge collapse on the biofilm and a remarkable decrease in the number of adherent E. coli cells.

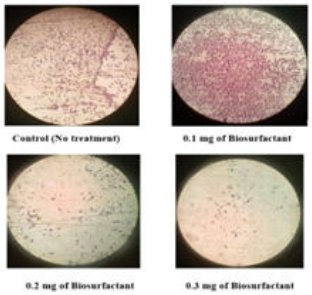

Fig:2 ,Biosurfactant inhibited the adhesion of E. coli (Light Microscopic view)

E. coli exposed to biosurfactant produced scant biofilms organised by small bacterial clumps or even single cells. Additionally, part of E. coli cells was damaged and some debris was loosely attached to the glass surface. The suppressed production and loosed architecture of E. coli biofilm caused by biosurfactant would ultimately attenuate the resistance of bacteria.

Discussion

In our study among 10 isolates, 2 isolates were showing a good growth on utilization of crude oil i.e., petrol. These were further screened for the production of biosurfactant. The isolates were screened for ability to grow on petroleum fractions like petrol, diesel, kerosene, lubricant oil, paraffin oil, crude oil as sole source of carbon table (Neekita Charan and Shivangi Patel, 2017).

The drop collapsing test, oil activity assay test and emulsification activity measurement were used to screen the biosurfactant producer (Dhail 2012; Amiriyan et al., 2004). The drop collapse method depends on the principle that a drop of liquid containing a biosurfactant collapses and spreads over the oily surface. Based on these two criteria, we concluded that Bacillus sp was as an efficient biosurfactant-producer.

The high level of β-haemolytic observed in Bacillus species. It could be attributed to the growth of the isolates on hydrocarbon contaminated soil (Paola Sandra Elenga-Wilson et al., 2021). Similar result was obtained in our study, Bacillus sp which was isolated from the mechanic workshop showed the β-haemolytic colonies on the blood agar plate.

The Bacillus isolates showed oil displacement zone formation. Bacillus species showed the highest oil spreading or displacement ability of 6.60 mm (John et al., 2020).

The ability of the isolates obtained in the present study to produce biosurfactant which emulsifies petrol suggests that they can be used industrially as emulsifying agents. Bacillus species to produce biosurfactant was further corroborated by reduction in surface tension.

Decrease in surface tension indicates the ability of the isolates to produce biosurfactant with surface activity (John et al.,2020).

The antimicrobial activity of the extracted biosurfactants against E. coli, Pseudomonas aeruginosa (gram negative bacteria) and Staphylococcus aureus (gram positive bacteria) were investigated by Muller-plate technique. A similar previous study by (Kaustuvmani et al., 2017) reported that B. subtilis, S. aureus, K. pneumonia, and E. coli were susceptible to the purified biosurfactant where it showed antibacterial properties that inhibited both Gram +ve and Gram –ve strains. In our study demonstrated that the antimicrobial activity of extracted biosurfactant was tested against selected biofilm forming bacteria such as Staphylococcus aureus, Pseudomonas aeruginosa, and Escherichia coli. of the

CONCLUSION

Biosurfactants may have very interesting potentials to reduce the hazardous effects of biofilms; in fact, Bacillus sp biosurfactant has been shown to increase the biofilm eradication efficacy. These findings indicate that the Bacillus sp biosurfactant can potentially be useful or can become a potent antibacterial and antibiofilm compound, as an alternative to antibiotics or other chemically synthesized toxic agents. Hence, we recommend more investigations to be conducted to have a better understanding about the biosurfactants based antiadhesive potential application in various industries such as petroleum, pharmaceutical and cosmetics.

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