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Research Article – Microbiology

Comparative study of antimicrobial efficiency of biosurfactant producing *Pseudomonas* spp. from different soil samples

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Abstract

Amphiphilic biosurfactants are surface-active biological molecules secreted by hydrocarbanoclastic microorganisms. Biosurfactants are eco-friendly, less toxic, biodegradable, and low-cost material, so it has more advantages over chemical surfactants. In this research, *Pseudomonas spp*., biosurfactant producing microorganisms isolated from different sources of soil samples. IS1, IS2, IS3, IS4 isolates obtained from Garden soil sample; Metal contaminated soil sample; Petroleum contaminated soil sample; Oil contaminated soil sample; respectively. Each isolate identified as *Pseudomonas spp.* Furthermore, screened for biosurfactant producers. Each isolate showed a positive outcome for the hemolysis test, drop collapse test, oil displacement test, and emulsification test. All isolate incubated in mineral salt medium for biosurfactant production. Biosurfactant extracted from IS1, IS2, IS3, IS4 showed 35%, 65%, 20%, 52% emulsification index respectively. Antimicrobial activity of extracted biosurfactants against pathogenic microorganisms checked by agar cup method. IS2 isolate shows the highest antimicrobial activity among all. All isolate showed a higher zone of inhibition against gram-positive microorganisms than gram-negative microbes. The purpose of this study involves the assessment of the antimicrobial activity of biosurfactant producers from the soil environment.

Keywords: Biosurfactant, contaminated soil sample, *Pseudomonas spp*., antimicrobial activity.

Introduction

When microbes grow in an environment rich in hydrocarbon, they undergo many adaptations, such as biosurfactant production. These biomolecules are naturally occurring amphiphilic surface-active compounds obtain from microbes, which produced either on cell surfaces or secreted extracellularly (Santhini and Parthasarathi, 2014) known as biosurfactant. It has both hydrophilic and hydrophobic molecules that help to solubilise hydrophobic substrates into the cell. Biosurfactants have low molecular weight compounds mainly comprising of glycolipids and some short-chain lipopeptides, while bioemulsifier have high molecular weight polymeric and lipopeptides surface-active agents (Satpute *et al.,* 2010). Biosurfactants and bioemulsifier help in the reduction of surface tension (SFT) and interfacial tension (IFT) (Satpute *et al.,* 2010). This property specifies application in agriculture, food production, chemistry, cosmetics, and pharmaceuticals (Pacwa-Plociniczak *et al.,* 2011). It also demonstrates antimicrobial activity against various pathogenic microorganisms by interaction with biological membrane systems, especially phosphatidylethanolamine moiety, that disrupts microbial membranes (Das *et al.,* 2014).

Material and methods

Sample used

Various soil samples have collected from different natural sources such as Garden soil, Metal contaminated soil, Petroleum contaminated soil, oil contaminated soil from Navi Mumbai region for research.

Enrichment and isolation

Each soil sample inoculated in sterile Nutrient broth (Himedia) and incubated at 37^0 C for 24-48 hours. Depending on the development of growth, loopful of growth transferred to sterile Cetrimide broth (Himedia), which is a selective medium for *Pseudomonas spp.* Furthermore, all flasks incubated at 37^0 C for 24-48 hours. On next day, it was streaked on a sterile cetrimide agar plate and incubated at 37^0 C for 24-48 hours. Each strain from the different samples subjected to enrichment in sterile cetrimide broth. Characterization of isolates obtained by performing a biochemical test based on Bergey's manual (Devnath *et al.,* 2017; Mahulkar Ankita 2020).

Screening of isolates for detection of biosurfactant producer

Oil displacement test

15 µL of diesel placed on the surface of 40 ml of distilled water placed in a petri dish, on which supernatant of 10 µL of each culture added on the surface of the oil film. The diameter of the clear halo zone measured after 30 seconds (Bendaha *et al.,* 2012).

Drop collapse test

On the surface of a glass slide, A drop of the supernatant of each isolate broth placed on which a thin layer of oil added previously. The teardrop shape on the surface of the oil observed after 1 min (Bendaha *et al.,* 2012).

Blood agar plate method

A loopful of each isolate, spot inoculated on a blood agar plate and incubated at 37^0C for 24 hours (Santhini and Parthasarathi, 2014).

Hydrocarbons emulsification

The supernatant of each culture broth tested on Diesel for emulsification activity (Bendaha *et al.*, 2012).

Biosurfactant Extraction and Purification

The Luria Bertani broth inoculated with isolates with/without olive oil was centrifuged at 10,000 rpm at 4ºC for 15 min to get supernatant. The supernatant liquid was filtered through a Whatman filter to remove cells and residual oil (if used) and other visible contaminants. Supernatant pH adjusted to 2.0 by using 6 N HCl and kept it overnight at 4^0 C, and the precipitate was collected. The collected precipitate extracted with a mixture of the extraction solvents with the following ratio methanol/chloroform /1-butanol (1:1:1V/V) then continuously shaken at 200 rpm and 30ºC for 5 hours. After 5 hours, the lower layer extracted and the solvent present in the mixture evaporated by exposing at room temperature (Santhini and Parthasarathi, 2014).

Identification of the composition of extracted biosurfactant

Extracted biosurfactants were subjected to the following test to check its composition, as described by Mahalingam and Sampath (2014).

Ninhydrin test

A few drops of 2% ninhydrin solution were added in 1 ml biosurfactant solution and kept in a water bath for 5 minutes. The development of a violet color signifies the presence of amino acid.

Orcinol assay

A few drops of orcinol reagent were added in 1 ml biosurfactant solution and kept in a water bath for 30 minutes. The development of a green color signifies the presence of carbohydrates.

Lipid solubilization test

A few drops of biosurfactant solution were added in 3 ml of methanol and water in separate tubes and shaken thoroughly, allowed to stand.

Emulsification test

Emulsification activity carried out using mineral oil. 3 ml of mineral oil added to 3 ml of supernatant, i.e., A ratio of 1:1, the mixture was vortexed vigorously for 2 min. After 24 h, the emulsification index (E_{24}) estimated as follows (Bendaha *et al.*, 2012):

E_{24}=HEL/H*S* x100

Where E_{24}**:** Emulsification activity after 24 hours,

HEL: Height of emulsion layer, HS: Height of the total liquid column.

Antimicrobial activity of extracted Biosurfactant

1 ml culture suspension of pathogenic organisms such as *Candida albicans, Aspergillus niger, Salmonella typhi, Klebsiella pneumoniae, Staphylococcus aureus, Bacillus subtilis* inoculated in sterile nutrient agar (Himedia) for bacteria (Govindammal and Parthasarathi, 2013) and potato dextrose agar (Himedia) for yeast and fungi (Gumaa, 2012), and poured in the sterile Petri plate and wells made by using sterile cork borer and filled with extracted crude biosurfactant of I1, I2, I3, I4 isolate as described in Onbasli and Aslim (2008) and (Mahulkar, Ankita, 2020). Then plates incubated at respective temperatures for 24-48 hours and checked for the zone of inhibition.

Result and discussion

Sample and isolation of microorganisms

The *Pseudomonas spp.* obtained from each contaminated soil sample. Identification and characterization of the isolate were carried out by biochemical tests, as mentioned in Bergey's manual of systematic bacteriology.

Table 1. Identification of bacterial isolate

	GS	CA	C	M	O	GH	SH	TSI	I	HL		DN
										O	F	
IS1	Gram negative rod	+	+	+	+	+	+	Slant: Alk Butt: Alk Gas/H_2S-No	+	+	-	+
IS2	Gram negative rod	+	+	+	+	+	+	Slant: Alk Butt: Alk Gas/H_2S-No	+	+	+	+
IS3	Gram negative rod	+	+	+	+	+	+	Slant: Alk Butt: Alk Gas/H_2S-No	+	+	-	+
IS3	Gram negative rod	+	+	+	+	+	+	Slant: Alk Butt: Alk Gas/H_2S-NO	+	+	+	+

Note: GS – Gram's Staining, M – Motility,CA – Catalase, C-Citrate, O-Oxidase, GH- Gelatine Hydrolysis, SH –Starch Hydrolysis, TSI-Tripple ion sugar test(Alk-alkaline), HL- Hugh &Leiffson medium(O-oxidative and F-Fermentative), DN – denitrification.

Screening test for biosurfactant producer

According to a result, all strains showed a positive outcome for screening tests (Fig. 1). In the drop collapse test, biosurfactants make the latter falls and spreads over the blade when biosurfactants placed on a glass slide on which a layer of oil added previously. All tested strain shows positive results for drop collapse test indicate the occurrence of biosurfactant. The oil displacement test was closely related to the biosurfactant presence in the supernatant of the tested bacteria. The existence of a surface-active compound in extracted biosurfactant causes the oil to repel, forming a clear zone. The diameter of the formed zone correlates with the activity of the tensioactive compounds in the supernatant.

All tested strains showed a positive result for oil displacement with different diameters. The formation of a clear zone around the colony showed the presence of biosurfactants. All tested strains showed β-haemolysis on a

Table 2. Screening test for determination of biosurfactant producer

Sample	Drop collapse test	Blood agar test	Blood agar test	Oil displacement test
IS1	+	+	+	+
IS2	+	+	+	+
IS3	+	+	+	+
IS4	+	+	+	+

Table 3. Identification of the Biosurfactant compound

Sr.no	Test	Observations	IS1	IS2	IS3	IS4
1.	Amino acid Ninhydrin test	No colour change	- ve	- ve	- ve	- ve
2.	Carbohydrate Orcinol assay	Formation of green color	+ ve	+ ve	+ ve	+ ve
3.	Lipid Solubility test Saponification	Insoluble in water but soluble in organic solvents (methanol). Formation of soap bubbles	+ ve	+ ve	+ ve	+ ve

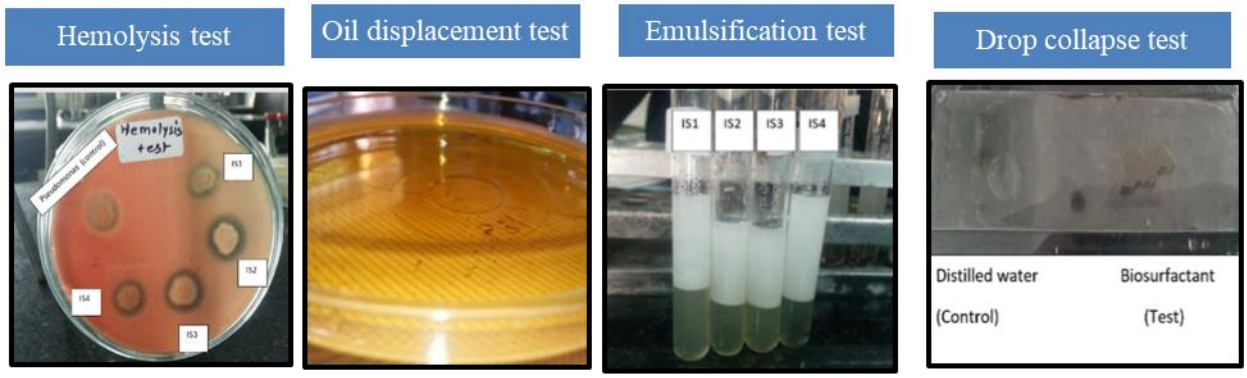


Fig. 1. Screening test for determination of biosurfactant producer

blood agar plate. Our observations (Table 2) are in agreement with those of Bendaha *et al.*, (2012) and Santhini and Parthasarathi, (2014).

Production of biosurfactants

After mass cultivation, the broth culture was extracted and purified using chloroform and methanol method. Purified biosurfactant showed the presence of carbohydrate and lipid in its composition, indicates glycolipid nature (Table 3).

Emulsifying index of extracted biosurfactants

Biosurfactant extracted from IS1, IS2, IS3, IS4 showed 35%, 65%, 20%, 52% emulsification index respectively (Graph 1). Glycolipid biosurfactants are generally composed of carbohydrate heads and lipid tail, which has the ability to reduce the surface tension between water and oil that form microemulsions (Satpute *et al.*, 2010). Microemulsions are thermodynamically stable dispersion of two immiscible liquids, i.e., oil and water stabilized by surfactant films (Satpute *et al.*, 2010). The primary role of biosurfactants is to solubilize hydrophobic molecules by trapping them in a pseudohydrophobic phase formed by micelles, which increase their solubility (Bendaha *et al.*, 2012). The various isolated organisms solubilize the oil and form a pseudohydrophobic phase, thus increases its solubility. IS2 shows higher emulsifying activity than others do; IS1 isolate showed low emulsifying activity (Fig. 2). Emulsion indexes (E_{24}) varies from one strain to another, according to the rate of biosurfactants production.

Determination of antibacterial activity

The antimicrobial activity of the extracted biosurfactant from *Pseudomonas spp.* was checked by the agar cup method. Biosurfactant derived from IS2 isolate showed a higher zone of inhibition amongst all. The result indicates that the antimicrobial activity of extracted biosurfactant against Gram-positive bacteria was more and less in Gram-negative bacteria (Fig. 3). Mechanism of antimicrobial action of biosurfactant involves disturbing cell membrane integrity through interaction with phospholipids as well as membrane proteins (Samadi *et al.*, 2012). The presence of lipopolysaccharide of Gram-negative bacteria membranes limits the entry of hydrophobic and amphipathic molecules of biosurfactant (Samadi *et al.*, 2012). Less zone of inhibition observed in fungi, but yeast was not inhibited by biosurfactant due to the insufficient concentration of biosurfactant fails to disturb the cell membrane leads to acquiring tolerance against biosurfactant. The variation observed in results is due to the differences in the composition of the biosurfactant, which affect emulsification property as well as the lipid membrane composition of the cells. The majority of the pathogenic organism shows better antibacterial activity than commonly used antibiotics. Since these pathogenic organisms, developed resistance against widely used antibiotic one can make use of this biosurfactant after clinical trials conducted to achieve restoration of antibiotic sensitivity.

Conclusion

Our finding has demonstrated that the isolated *Pseudomonas* strains of the contaminated soil sample from Navi Mumbai region was found to be the potent producer of biosurfactant. All isolate produced a surfactant that was composed of glycolipid. IS2 isolate of metal contaminated soil shows a

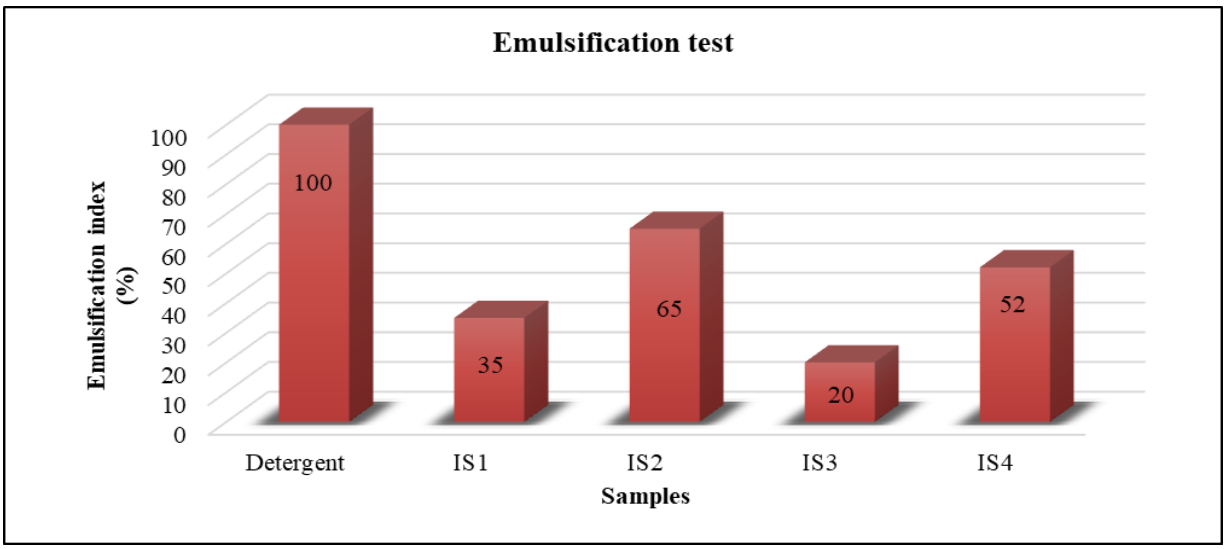
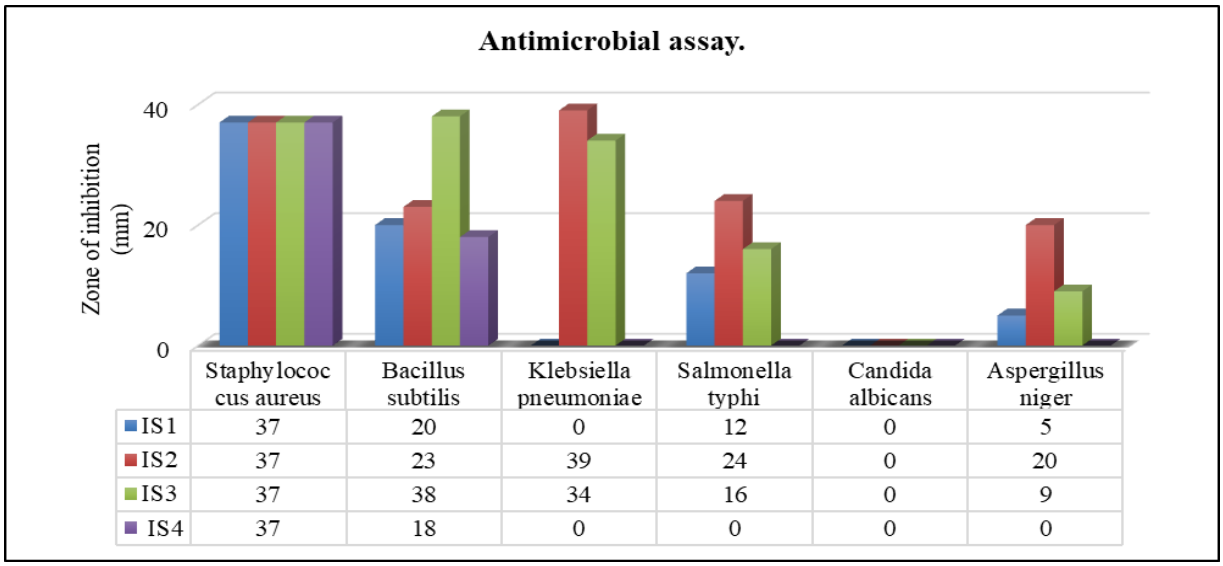


Fig. 2. Emulsification index of extracted biosurfactants



	Staphylococcus aureus	Bacillus subtilis	Klebsiella pneumoniae	Salmonella typhi	Candida albicans	Aspergillus niger
IS1	37	20	0	12	0	5
IS2	37	23	39	24	0	20
IS3	37	38	34	16	0	9
IS4	37	18	0	0	0	0

Fig. 3. Comparison of antimicrobial activity of biosurfactant produced by different strains of *Pseudomonas spp.*

higher emulsification index as well as the highest antimicrobial activity against pathogenic strain; thus, it may use as an antimicrobial agent than antibiotics after clinical trials.

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