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Research Article

PRODUCTION AND EXPLORATION OF ANTIBACTERIAL POTENTIAL OF BIOSURFACTANT FROM ENVIRONMENTAL BACTERIAL ISOLATE

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ABSTRACT

In the present study a number of bacterial cultures were isolated from environmental soil samples. For this, motor repairs shops are one of the possible site of interest were chosen. Isolated bacterial cultures were further screened for biosurfactant production by using oil displacement method and surface tension measurement. Minimum surface tension reduction value was found 40 dynes/ cm as compared to control (Nutrient broth) 56 dynes/cm. Selected strain also showed β-hemolysis and oil displacement while showed negative result in Cetyltrimethylammonium bromide (CTAB) assay which confirm the nature of biosurfactant. It was found that biosurfactant was not of anionic nature. Identifcation of selected environmental isolate was done using 16s rRNA and was found as *Bacillus cereus*. Crude biosurfactant extracted with chloroform was found to show antibacterial against methicillin resistant *Staphylococcus aureus* (MRSA) 43300, *Pseudomonas aeruginosa* (*P. aeruginosa*) PAO1, *Klebsiella pneumoniae* (*K. pneumoniae*) B5O55 and *Salmonella typhi* (*S. typhi*). Thin layer chromatography and autobiography assay to find antimicrobial compound was done.

Keywords: Biosurfactant, Cetyltrimethylammonium bromide, Surface tension, Autobiography.

1. INTRODUCTION

Organic pollutants are commonly considered as pervasive environmental pollutants to survive for microorganism but some kind of microbial community is able to survive in such environment. These microorganisms showed an important role in cleaning these harsh evironmental organic pollutants [1, 2]. The microorganisms such as *Bacillus*, *Pseudomonas, Stenotrophomonas, Acinetobacter* and *Streptomyces* are some of the major genera which produce biosurfactants [3, 4]. The biosurfactants (BS) are the evolving molecule in present time, as they are better than chemical surfactants. But high production cost of biosurfactants limit their applications [5]. Synthetic surfactant contains a number of substances which are toxic and non-biodegradable. Biosurfactants are broadly classified into six major classes such as lipopeptides, glycolipid, neutral lipid, phospholipid, fatty acid and lipopolysaccharides [6]. These are known for their increased applications in industries and medical field [7]. These molecules have a lot of antimicrobial and therapeutic applications like antibiofilm, antifungal, wound healing, antibacterial, antitumour and antiviral [8-13]. These applications of biosurfactants in medical field make them potential candidate to combat many microbial diseases. With passes of time, need for effective and new antimicrobial agent always be in demand. Pathogenic microorganisms are evolving so fast for the present antimicrobial agents so here an ample need for the new antimicrobial agents arises. The genus *Bacillus* is known to produce a broad category of lipopeptides like surfactin, kurstakins, fengicyn, iturin, bacillomycins and mycosubtilin. Among these, surfactin showed broad range of antibacterial activities [14]. In the present study, we have used soil sample from motor repair shops to isolate the biosurfactant producing bacterial strain and its antibacterial activity was also evaluated against methicillin resistant *Staphylococcus aureus* ATCC 43300 (MRSA).

2. MATERIAL AND METHODS

2.1. Sample collection and isolation of bacterial culture

Twenty Soil samples were collected from motor repair

shops of Chandigarh (UT) and Una (Himachal Pradesh), India. Soil samples were further processed for isolation of different types of bacteria. Enrichment of soils samples were done in Bushnell Hass Broth (BHB) (Himedia) containing supplemented with nutrient broth (NB) components (pH 7.0) at 37°C under shaking conditions at 150 rpm for 24 hrs. Enriched samples were spread on Nutrient agar (NA) plates. Different types of bacterial isolates were selected on the basis of colony characteristics like shape, size, color and texture. The colonies were further streaked on NA plates for further use.

2.2. Screening of biosurfactant producing microorganism and selection of isolate

Each isolated bacterial culture was inoculated in Supplemented BHB medium and incubated for five days on shaking condition of 150 rpm at 37°C. After 5 days of incubation, the culture was centrifuged at 10,000 rpm at 4°C for 10 minutes [15]. Cell free supernatant (CFS) was used for screening of bacterial isolate for biosurfactant production. Pure Culture of positive isolates were stored in 50% glycerol stokes at -20°C for future use.

2.2.1. Oil displacement methods

Oil displacement experiment was performed as described by Morikawa et al., [16]. Briefly, 20 ml of distilled water was added to a petri dish followed by 500 µl mustard oil on the surface of water. After that, 100 µl of CFS was added in center of oil fill without disturbing the water oil interface. Oil displaced from the center for those samples which found positive for biosurfactant production.

2.2.2. Surface tension measurement

Du Nouy ring method [17, 18] was used to measure the surface tension of CFS. Production media and distilled water were used as control. In this method, we slowly lifted the ring of platinum wire. The force (Dynes/ cm) was measured which was required to raise the loop from the surface of liquid. We selected the bacterial culture on the basis of oil displacement method and surface tension reduction. Further, the isolate was characterized on the basis of screening methods to screen for biosurfactant production.

2.2.3. Hemolytic activity

Blood agar was prepared by using 5% v/v sheep blood in autoclaved media containing (g/L) yeast extract 5g, tryptone 10g and agar 15g [19]. Selected isolate was streaked on blood agar and incubated at 37°C. After 24 hrs of incubation, the zone of hemolysis was observed around the colonies.

2.2.4. CTAB agar method

CTAB agar method is a semi-quantitative method for the detection of anionic surfactants or extracellular glycolipids. It is specific for anionic biosurfactant and method was developed by Siegmund and Wagner [20]. Anionic biosurfactants, if secreted by microbes, form a dark blue insoluble ion pair with CTAB methylene blue and sodium dodecyl sulphate (SDS); 20 mM, was used as positive control. It was performed by using supernatant as sources of biosurfactant and supernatant was added to well punctured on CTAB agar.

2.3. Identification of selected isolate

Matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) was used for characterization of isolate, further MALDI-TOF results were confirmed by 16s rRNA sequencing. For MALDI Bruker Daltonik MALDI Biotyper, facility at PGIMER, Chandigarh, was used. Briefly, for 16s rRNA sequencing, isolate was grown in Tryptic soy broth (TSB) for overnight. Cells of this overnight incubated culture were centrifuged and further processed for DNA isolation using ZR Fungal/Bacterial DNA miniprep™ Kit (Zymo Research). Isolated DNA was ensured by running gel electrophoresis and further followed by polymerase chain reactions to amplify 16s rRNA gene using 27F 357F 786F primers. Ampliefied product was recovered using GENETIX PCR clean-up kit and purity was again checked using agrose gel electrophoresis. Sequencing of purified DNA was done at the central facility for nucleotide sequencing at Institute of Microbial Technology (IMTECH), Chandigarh.

2.4. Biosurfactant production and polarity determination of product

Selected isolate was first inoculated in NB medium and placed at 37°C for 24 hrs for inoculum preparation. 1% of inoculum was used to inoculate the 100 ml of BHB supplemented production media in 500 ml of Erlenmeyer flask. Production media was incubated at 37°C for 5 days at 150 rpm. After 5 days of incubation, centrifugation was done at 4°C at 10,000 rpm for 10 min. Before CFS was extracted with chloroform in 1:1, pH of supernatant was adjusted to 2 [21]. The solvent

was separated using separating funnel and completely evaporated under reduce pressure at 32°C using Rotavapor (Buchi R-210). Remaining material was dissolved in methanol and used as crude biosurfactant for the further use [22]. Extraction of supernatant was done with different solvents like ethyl acetate, chloroform:methanol (2:1) and chloroform to know the polarity of product. Resins (HP 20 Diaions and XAD-16) were also used for maximum recovery of product.

2.5. Antibacterial activity of crude bio-surfactant

Our aim of this study was to evaluate the antibacterial potential of biosurfactants. Antibacterial assay was performed against some of the most important pathogenic bacteria such as MRSA ATCC 43300, *P. aeruginosa* PAO1, *K. pneumoniae* B5O55 and *S. typhi* standard. Seeded Muller Hinton agar (MHA) (0.8% agar) plates were used. 1% of (10^5 CFU/ml) of each tested organism was used to inoculate the MHA and stored at 4°C for future use. Holes of 6 mm diameter were punched with sterile cork borer and 100 µl of crude biosurfactant was introduced in each well [23].

2.6. Thin layer chromatography and autobiography analysis

Methanol dissolve sample was chromatographically analysed. 10 µl of aliquots was applied to pre coated silica gel (10 x 2 cm) (G60; merck, Germany). Different ratios of chloroform and methanol solvents (1.85:1, 1:1, 1:1.857, 1:3, and 1:9) were used to develop the TLC. Main components of sample was detected by exposure of iodine vapors for lipids, Molish's reagent for sugar, 1% H_2SO_4 followed by heating of silica gel plate at 110°C for 20 min for glycolipids and 0.2-1% ninhydrin solution in ethanol for free amino groups and lipopeptide with red pinkish spot [22, 24]. TLC bioautography (agar diffusion or contact bioautography and agar overlay method) assay was done with best developed TLC plate to locate the active component on TLC plate [25, 26].

3. RESULTS AND DISCUSSION

3.1. Isolation and qualitative analysis for biosurfactant production

From soil samples of motor repair shops, around 50 types bacterial culture were found on the basis of colony characteristics which were further screened primarily by using oil displacement methods and surface tension reduction test. 32 samples were found positive for oil displacement and were taken further for surface reduction test (Fig. 1A). Out of these 32 samples, 12 samples showed surface tension value ≤50 Dynes/cm. Isolate named 3B showed minimum surface tension value i.e. 40 Dynes/cm compared to NB (56 Dynes/cm) and distilled water (70 Dynes/cm) (Table 1). One of this was found best with biosurfactant screening methods. As single method was not sufficient to detect the presence of biosurfactant therefore selected isolate was further screened for β hemolysis (Fig. 1B) and CTAB agar assay (specially done for anionic biosurfactant). Isolate 3B was found positive for β-hemolysis and no precipitation zone was found on CTAB agar as compared to control SDS which showed zone of precipitation (Fig. 1C). Kurniati et al., [27] and Astuti et al., [28] also used hemolytic blood assay and oil-spreading test as preliminary screening method for biosurfactant production and interfacial tension measurement as semiquantitative method for biosurfactant screening. Sidkey et al., [29] and Noha et al., [30] used the hemolytic blood assay and oil-spreading assay which we used in our present study along with this they used drop collapse assay, emulsification index (E_{24}%) as screening methods for biosurfactant producing environmental isolate.

Table 1: Surface Tension value for the isolates showing oil spreading assay (Du-Nouy-Ring Method)

Sample No	Surface tension value (Dynes/cm)	Sample No	Surface tension value (Dynes/cm)
S1	57	S28	49
S2	49	S31	61
S4	**40**	S32	50
S5	59	S33	56
S6	57	S34	63
S7	67	S35	61
S9	48	S36	55
S11	58	S37	49
S14	46	S38	43
S16	71	S39	57
S17	48	S40	52
S18	46	S45	51
S20	50	S46	60
S21	56	S47	46
S22	49	S49	62
S24	55	Nutrient broth	56
S26	51	Distilled water	70

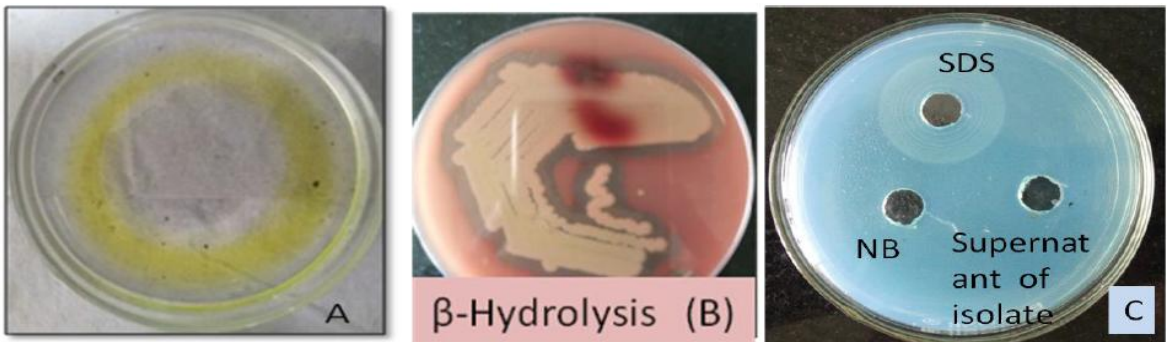


Fig. 1: Screening result of selected isolate (A) oil displacement (B) β-hemolysis (C) CTAB agar assay results

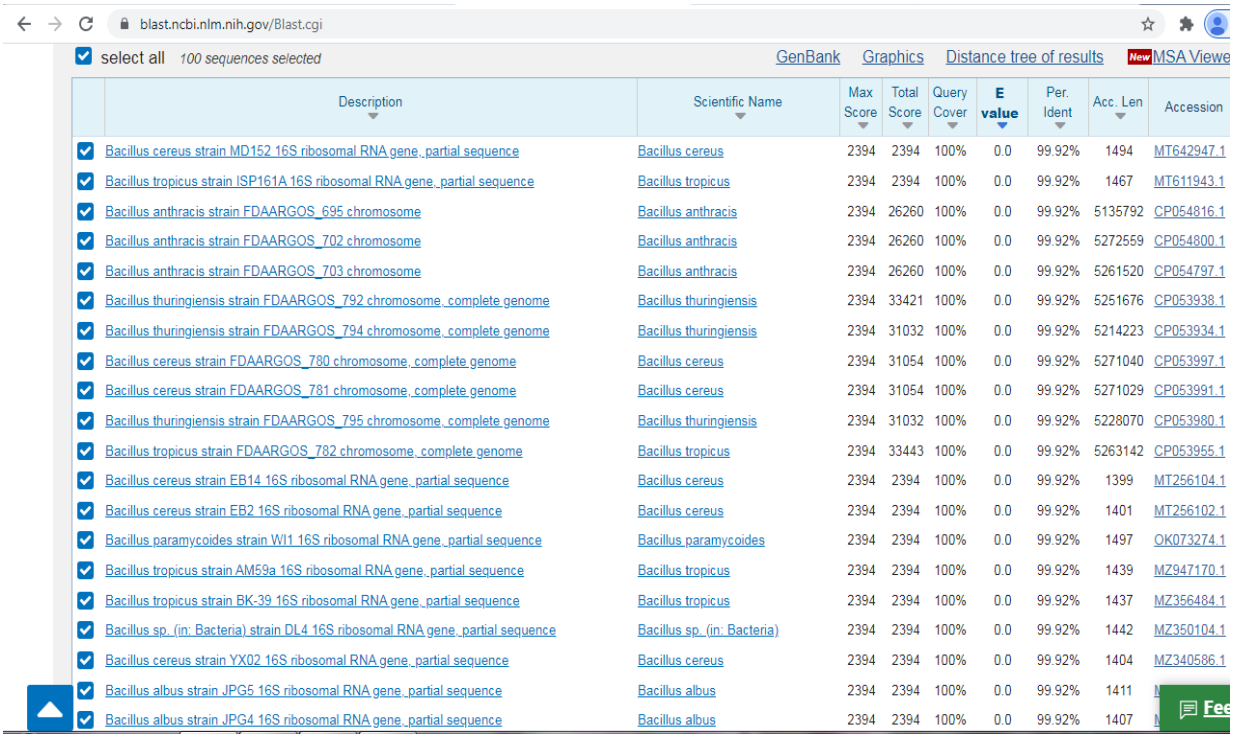
3.2. Identification of selected isolate

The resulting sequences were compared with sequences available in the gene bank data base using Basic Local Alignment Search Tool (BLAST) to obtain the entire stretch of sequence. 16s rRNA sequencing and MALDI-TOF results depicted that selected isolate was *Bacillus cereus*.

TAACACGTGGGTAACCTGCCCATAAGACTGGGA
TAACTCCGGGAAACCGGGGCTAATACCGGATAA
YATTTTGAACCGCATGGTTCGAAATTGAAAGGC
GGCTTCGGCTGTCACTTATGGATGGACCCGCGT
CGCATTAGCTAGTTGGTGAGGTAACGGCTCACC
AAGGCAACGATGCGTAGCCGACCTGAGAGGGT
GATCGGCCACACTGGGACTGAGACACGGCCCA
GACTCCTACGGGAGGCAGCAGTAGGGAATCTTC
CGCAATGGACGAAAGTCTGACGGAGCAACGCCG
CGTGAGTGATGAAGGCTTTCGGGTCGTAAAACT
CTGTTGTTAGGGAAGAACAAGTGCTAGTTGAAT
AAGCTGGCACCTTGACGGTACCTAACCAGAAAG
CCACGGCTAACTACGTGCCAGCAGCCGCGGTAA
TACGTAGGTGGCAAGCGTTATCCGGAATTATTG
GGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTC
TGATGTGAAAGCCCACGGCTCAACCGTGGAGGG
TCATTGGAAACTGGGAGACTTGAGTGCAGAAGA
GGAAAGTGGAATTCCATGTGTAGCGGTGAAATG
CGTAGAGATATGGAGGAACACCAGTGGCGAAGG
CGACTTTCTGGTCTGTAACTGACACTGAGGCGC
GAAAGCGTGGGGAGCAAACAGGATTAGATACCC
TGGTAGTCCACGCCGTAAACGATGAGTGCTAAG
TGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGT
TAACGCATTAAGCACTCCGCCTGGGGAGTACGG
CCGCAAGGCTGAAACTCAAAGGAATTGACGGGG
GCCCGCACAAGCGGTGGAGCATGTGGTTTAATT
CGAAGCAACGCGAAGAACCTTACCAGGTCTTGA
CATCCTCTGACAACCCTAGAGATAGGGCTTCTC
CTTCGGGAGCAGAGTGACAGGTGGTGCATGGTT
GTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA
GTCCCGCAACGAGCGCAACCCTTGATCTTAGTT
GCCATCATTAAGTTGGGCACTCTAAGGTGACTG
CCGGTGACAAACCGGAGGAAGGTGGGGATGAC
GTCAAATCATCATGCCCCTTATGACCTGGGCTA
CACACGTGCTACAATGGACGGTACAAAGAGCTG
CAAGACCGCGAGGTGGAGCTAATCTCATAAAAC
CGTTCTCAGTTCGGATTGTAGGCTGCAACTCGC
CTACATGAAGCTGGAATCGCTAGTAATCGCGGA
TCAGCATGCCGCGGTGAATACGTTCCCGGGCCT
TGTACACACCGCCC.

3.3. Antibacterial activity of crude bio-surfactant qualitative analysis by agar well diffusion method

Crude biosurfactant (1mg/ml) showed good zone of inhibition (ZI) against all four tested pathogenic microorganism methicillin resistant *Staphylococcus aureus* 43300 (13mm), *Pseudomonas aeruginosa* PAO1 (6mm), *Klebsiella Pneumoniae* B5O55 (12mm) and *Salmonella typhi* (10mm). It was found much effective against gram positive MRSA (fig. 3) while the effect was little less for gram negative strains. Biosurfactants from probiotic showed antimicrobial activity potential against many potential pathogenic microorganisms [31, 32]. Crude biosurfactant from *Lactobacillus jensenii* and *Lactobacillus rhamnosus* showed antimicrobial and antibiofilm activity against *S. aureus* [33]. Biosurfactant from *Bacillus subtilis*, surfactin showed broad range of antimicrobial activity as well as considered as substitute for antibiotic and probiotic biosurfactant, has been explored well for their antimicrobial potential [34, 35]. Surfactin and rhamnolipid from *Bacillus amyloliquefaciens* and *Pseudomonas aeruginosa,* respectively, showed pronounced antimicrobial effect against *Staphylococcus aureus*, *Escherichia coli* and the pathogenic yeast *Candida albicans* [36].



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Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Bacillus cereus strain MD152 16S ribosomal RNA gene, partial sequence	Bacillus cereus	2394	2394	100%	0.0	99.92%	1494	MT642947.1
Bacillus tropicus strain ISP161A 16S ribosomal RNA gene, partial sequence	Bacillus tropicus	2394	2394	100%	0.0	99.92%	1467	MT611943.1
Bacillus anthracis strain FDAARGOS_695 chromosome	Bacillus anthracis	2394	26260	100%	0.0	99.92%	5135792	CP054816.1
Bacillus anthracis strain FDAARGOS_702 chromosome	Bacillus anthracis	2394	26260	100%	0.0	99.92%	5272559	CP054800.1
Bacillus anthracis strain FDAARGOS_703 chromosome	Bacillus anthracis	2394	26260	100%	0.0	99.92%	5261520	CP054797.1
Bacillus thuringiensis strain FDAARGOS_792 chromosome, complete genome	Bacillus thuringiensis	2394	33421	100%	0.0	99.92%	5251676	CP053938.1
Bacillus thuringiensis strain FDAARGOS_794 chromosome, complete genome	Bacillus thuringiensis	2394	31032	100%	0.0	99.92%	5214223	CP053934.1
Bacillus cereus strain FDAARGOS_780 chromosome, complete genome	Bacillus cereus	2394	31054	100%	0.0	99.92%	5271040	CP053997.1
Bacillus cereus strain FDAARGOS_781 chromosome, complete genome	Bacillus cereus	2394	31054	100%	0.0	99.92%	5271029	CP053991.1
Bacillus thuringiensis strain FDAARGOS_795 chromosome, complete genome	Bacillus thuringiensis	2394	31032	100%	0.0	99.92%	5228070	CP053980.1
Bacillus tropicus strain FDAARGOS_782 chromosome, complete genome	Bacillus tropicus	2394	33443	100%	0.0	99.92%	5263142	CP053955.1
Bacillus cereus strain EB14 16S ribosomal RNA gene, partial sequence	Bacillus cereus	2394	2394	100%	0.0	99.92%	1399	MT256104.1
Bacillus cereus strain EB2 16S ribosomal RNA gene, partial sequence	Bacillus cereus	2394	2394	100%	0.0	99.92%	1401	MT256102.1
Bacillus paramycoides strain WI1 16S ribosomal RNA gene, partial sequence	Bacillus paramycoides	2394	2394	100%	0.0	99.92%	1497	OK073274.1
Bacillus tropicus strain AM59a 16S ribosomal RNA gene, partial sequence	Bacillus tropicus	2394	2394	100%	0.0	99.92%	1439	MZ947170.1
Bacillus tropicus strain BK-39 16S ribosomal RNA gene, partial sequence	Bacillus tropicus	2394	2394	100%	0.0	99.92%	1437	MZ356484.1
Bacillus sp. (in: Bacteria) strain DL4 16S ribosomal RNA gene, partial sequence	Bacillus sp. (in: Bacteria)	2394	2394	100%	0.0	99.92%	1442	MZ350104.1
Bacillus cereus strain YX02 16S ribosomal RNA gene, partial sequence	Bacillus cereus	2394	2394	100%	0.0	99.92%	1404	MZ340586.1
Bacillus albus strain JPG5 16S ribosomal RNA gene, partial sequence	Bacillus albus	2394	2394	100%	0.0	99.92%	1411	
Bacillus albus strain JPG4 16S ribosomal RNA gene, partial sequence	Bacillus albus	2394	2394	100%	0.0	99.92%	1407	

Fig. 2: BLAST results of 16s rRNA sequences of identified isolate

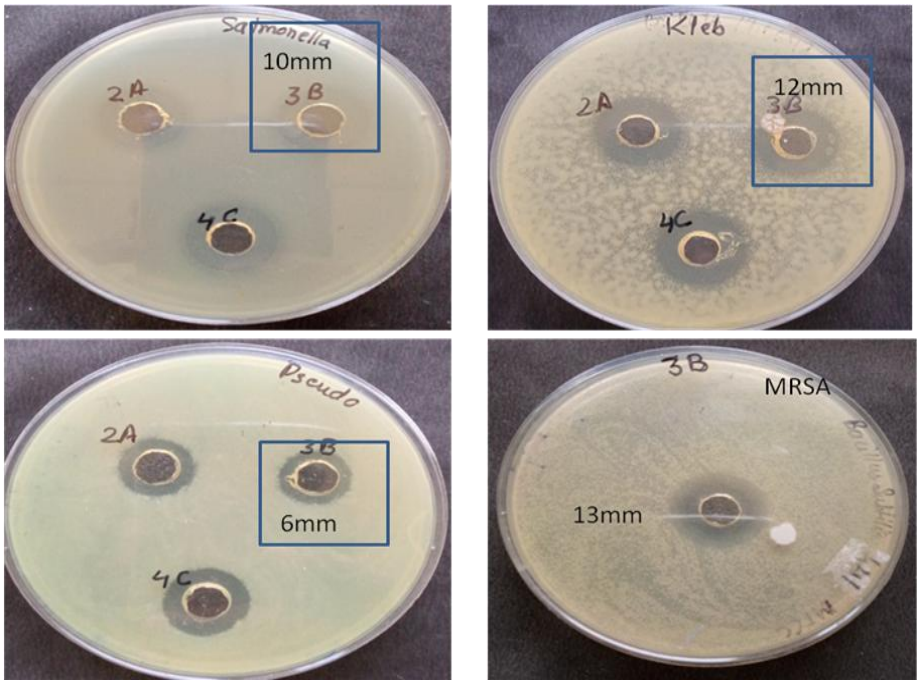

Fig. 3: Antibacterial activity of biosurfactant from selected isolate against methicillin resistant *Staphylococcus aureus* 43300, *Pseudomonas aeruginosa* PAO1, *Klebsiella Pneumoniae* B5O55 and *Salmonella typhi*

3.4. Thin layer chromatography and autobiography analysis

TLC was run with different solvent systems and best results were observed with chloroform:methanol (1:9) which indicate that compound showed more mobility with polar solvent. Spray with ninhydrin (0.2%) solution resulted in development of violet-pinkish spot which inferred that possible compound is a kind of

lipopeptide (fig. 4). Besides this, autobiography assay was done to check the antibacterial effect of semi-purified compound on TLC plate. 1mg/ml of MTT was sprayed on overnight incubated TLC plate and clear zone showed the antibacterial effect of spotted compound on TLC, rest of background was blue area due to formazone formation by living cells (fig. 5). Some researchers have used autobiography assay to detect the antimicrobial compound [37, 26]. This method was very useful in detecting antibacterial compound on TLC as the compound diffused to soft agar which ultimately showed antibacterial effect in soft agar seed media. Alajlani and co-worker [38] also used the bioassay method with sensitive microorganism to detect the bioactive fraction on TLC and active fraction was scraped from TLC which was further purified by reverse phase HPLC. In present study, we have used TLC method to find active fraction against MRAS by contact autobiography assay.

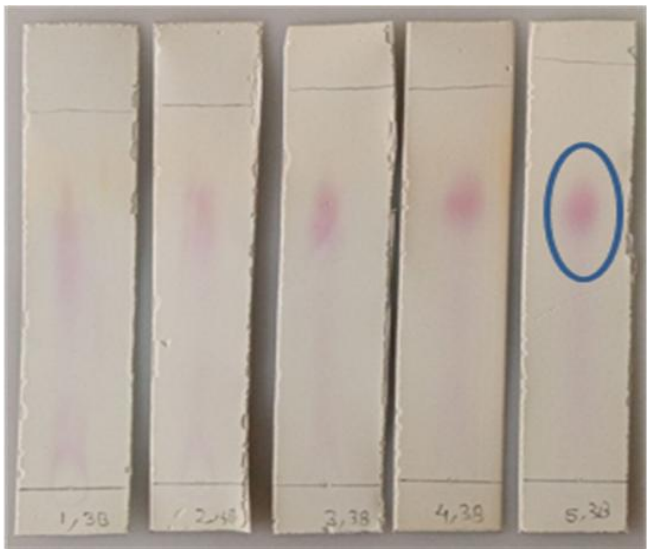

Fig. 4: Chloroform: Methanol (1.85:1), (1:1) (1:1.857) (1:3) (1:9) Rf 0.76 (lipopeptide) TLC plate was developed by 0.2% ninhydrin spray

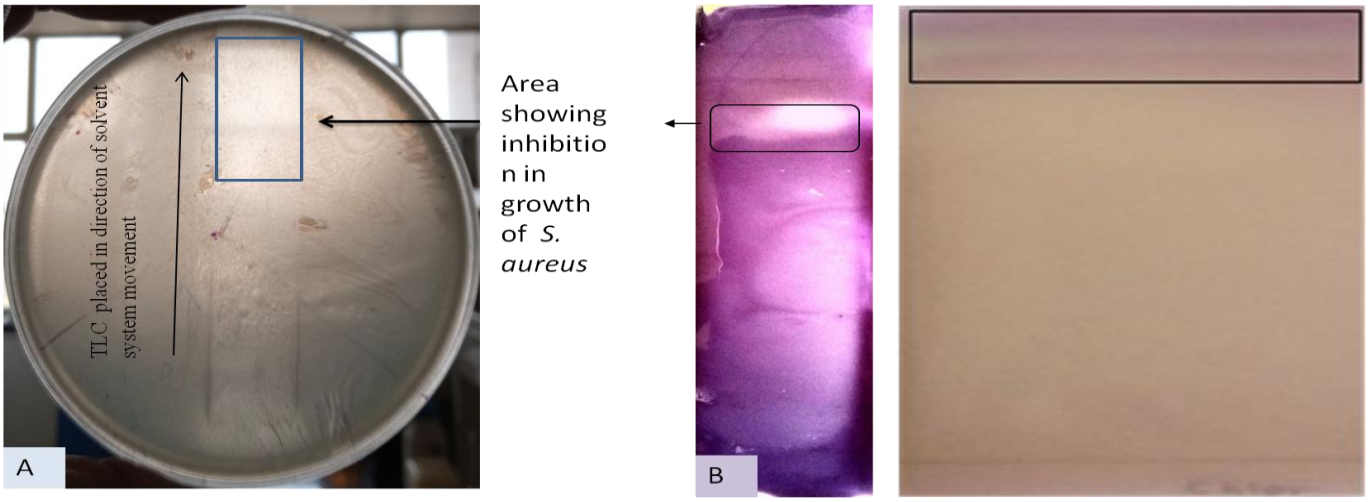

Fig. 5: (A) Agar Diffusion or Contact Bioautography (B) Agar overlay, a bioautography assay, done TLC plate showing clear zone due to the diffusion of active component from TLC plate to seeded agar media, for visualization 1mg/ ml MTT dye was used (C) Preparatory TLC was run showed band of inhibition of methicillin resistant *Staphylococcus aureus* (MRSA) 43300, for visualization MTT was used

4. CONCLUSION

From last few year, lipopeptides which is a most common type of biosurfactant produced by *Bacillus* spp. and rhamnolipids produced by *Pseudomonas* spp., have been explored for their various medical applications. Most common dwelling site for bacterial community produces biosurfactant is mostly oil spillage site. By keeping this in mind, our present study was aimed to explore the soil samples from motor repairs shops for biosurfactant producing bacterial isolated. Isolates were further screened by oil displacement method in which supernatant of culture reduce the surface tension at inter face and oil displace from there. Antibacterial activity was also checked by agar well diffusion method against MRSA ATCC 43300 and other pathogenic microorganisms and further TLC method was used to purify the compounds of interest and autobiography assay was done to check the presence of bioactive fraction over there which showed zone of inhibition. As biosurfactant have been explored for various medical

and industrial applications though some of areas are still there to explore.

5. ACKNOWLEDGEMENT

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Conflict of interest

The authors do not have conflict of interest regarding this paper.

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