Production, characterization and antimicrobial activity of biosurfactants from indigenously isolated *Bacillus wiedmannii* MWS5

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Abstract

Biosurfactants are fascinating bioproducts because they are amphiphilic molecules with a wide range of functions, environmental acceptability and have several industrial applications. Due to non-toxicity, biodegradability and costeffectiveness, biosurfactants are considered as excellent substitute to the chemically synthesized surfactants. The aim of present study is isolation and characterization of novel potent biosurfactant producer bacterial strains of water habitat. At sea port Karachi, ten water samples were collected from two different oil spill water points. After isolation and enrichment of the bacterial communities, they were screened for the biosurfactants production by employing blood hemolysis, drop collapse assay, oil spreading technique, and emulsification activity. All bacterial strains from oil-contaminated environment showed highest biosurfactants production potential. *Bacillus wiedimanii* (MWS5) was identified as the top biosurfactant producer by morphological, biochemical, and 16S rRNA techniques. Lipopeptide type biosurfactant was confirmed by Fourier transform infrared spectroscopy (FTIR) and thin-layer chromatography (TLC). The extracted biosurfactant showed antibacterial potential against different pathogenic bacterial strains.

Introduction

Biosurfactants (BS) are surface active substances capable of lowering surface tension in both hydrocarbon and aqueous media compared to their chemically synthesized equivalents. They could be produced by using renewable resources including plants, animals, and oils, as well

as petrochemical feedstock (Adebajo *et al.*, 2020). The main criteria used to categorize biosurfactants are their chemical structure and microbiological source. The hydrophobic component is made up of chains of hydrocarbons or unsaturated or saturated fatty acids, whereas the hydrophilic component is produced by the combination of an acid, cations or anions, and mono, di, or polysaccharides (Diez *et al.*, 2022). On the basis of molecular weight, microbial surface active compounds can be divided into two main groups: high molecular weight such as polysaccharides, lipopolysaccharides, proteins, and lipoproteins and low molecular weight such as glycolipids and lipopeptides. High-molecular-weight biosurfactants work more effectively to stabilize oil-water emulsions, while low-molecular-weight biosurfactants are more efficient for surface tension reduction. The biological activities and applications of various biosurfactants are directly related with the molecular structural variations (Eras-Muñoz *et al.*, 2022). Bacillus species able to withstand hard environmental conditions because of their spore forming ability. As a result, they have been shown to be mostly found in soil and water, with a relatively high frequency than in other matrices (Ndibe *et al.*, 2018). Bacillus is a genus of microorganisms that produces a wide range of cyclic lipopeptides/lipoproteins, such as fengycins, surfactins, lichenysins, and bacillomycin. Two distinct parts of lipopeptide BSs are an acyl tail (s) and a brief linear oligopeptide sequence with an amide bond (Ali *et al.*, 2022). Fengycin isoforms derived from marine Bacillus strain efficiently inhibited the growth of a variety of bacteria, including *K. aerogenes, C. fruendii, A. faecalis, M. flavus, E. coli, P. vulgaris and S. marcescens (Asmani et al., 2020)*

The current study aims to isolate and screen bacterial strains that produce biosurfactants from different types of water environment, morphological, biochemical and structural characterization of purified biosurfactants by TLC and FTIR to identify the type of biosurfactant and evalution of antimicrobial potential of extracted biosurfactant.

Material and Methods:

Sample collection and isolation of pure bacterial colonies

Ten oil contaminated water samples were collected from different oil spill points at Karachi sea port (Kolachi Pier Keamari and Oil Pier 1 Jetty Keamari). According to the standard methods for the examination of water and wastewater (Association, 1926), water samples were collected in sterilized 50ml falcon tubes. Serial dilutions of water samples from 10^{-1} to 10^{-6} were prepared.

Spread plate method was used for the inoculation of plate count agar plates with 2 ml of 10^{-4} to 10^{-6} dilutions. All the plates were incubated at 37ºC for one day. Different colonies were isolated, characterized and purified according to the cultural differentiation. A unique code was assigned to each bacterial isolate to be maintained for further research activities (Ewida & Mohamed, 2019).

Morphological and biochemical characterization

Isolated colonies of purified bacterial strains were grown on solidified agar plates. Colour, cell shape, elevation, margins and opacity of each colony was observed (Morikawa *et al.*, 2000). Gram staining was used to examine cells under a microscope for the differentiation of gram positive and gram negative bacteria (Tripathi & Sapra, 2020). Biochemical tests Methyl red, Catalase and Voges- Proskauer were carried out on biosurfactants producing isolates (McDevitt, 2009).

Screening for biosurfactant producers

Blood hemolytic activity, drop collapse assay, oil spreading test and emulsification index were the screening procedures used to determine biosurfactant production potential of each bacterial isolate (Ewida & Mohamed, 2019).

Blood hemolytic activity

Primary screening test to identify biosurfactant producing bacterial isolates was blood hemolytic activity. 5 ml of sheep blood was added to one liter nutrient agar medium to prepare blood agar plates. On blood agar plates, freshly prepared colonies were streaked and incubated for 2-3 days at 37°C. The potential to produce biosurfactants was assessed by clear zone or greenish colour surrounding the colonies (Carrillo *et al.*, 1996).

Oil Spreading Technique

50 ml broth media was inoculated with bacterial isolates and incubated for 72 hours at 37°C. Cell free culture broth (CFB) was collected after the centrifugation of culture media at 6000rpm for 30 mints. They were stored at 4°C and used for the various biosurfactant screening tests. In a large petri dish (10 cm in diameter), 50 mL of distilled water was poured, and then 100 µL of crude oil was added to the surface of the distilled water. After that 20 µL of CFB was also added at the center of crude oil. The displacement diameter was measured in cm (Morikawa *et al.*, 2000).

Drop collapse assay

Hydrocarbon degrader and surface-active agent's producer bacterial strains were screened for the drop-collapse assay. Drop collapse assay was carried out in accordance with Youssef *et al* (2007). In microtiter plate, 2 microliters of crude oil was added and left for 24 hours to equilibrate followed by addition of 5 µL of culture supernant. After 1 minute, size of drop was observed. Round drops were graded as negative, suggesting no biosurfactant production whereas flat drops indicate positive result (Bodour *et al.*, 2003).

Emulsification activity

According to the method described by Bento *et al* (2005), emulsification activity was determined using the emulsification index known as E24%. Emulsification activity was performed by adding 2ml of each of five oils (mobil oil, diesel, kerosene, mustard oil and olive oil) and 2 ml of cell free supernatant of each bacterial isolate. As a control, distilled water was utilized. All mixtures were mixed by vortex for 2 min at high speed and left at room temperature for 24 hours. Emulsification index E24 % with each oil was calculated according to equation.

$$\text{E24 (\%)} = \frac{\text{Height of the emulsion layer}}{\text{Total height of the liquid}} x\ 100$$

Molecular identification

16S rRNA sequencing was used to identify potent biosurfactant producer strain. For this, genomic DNA of bacterial isolate was extracted and used as template for the amplification of 16S rRNA gene by polymerase chain reaction (PCR). Forward Primer: F5'-CCTANGGGNNGCANCAG-3' and Reverse primer: R5'-GGACTACNNGGGTATCTAAT-3' were used for the amplification of 16S rRNA gene. The following thermal parameters were used to perform the PCR reaction: 5 minutes of initial denaturation at 94°C, 1 minute of denaturation at 94°C, 1 minute of annealing at 55°C, 2 minutes of extension at 72°C, and a final 10 minutes of extension at 72°C. The PCR products were analysed by electrophoresis on 1% agarose gel. Sequencing was carried out by BGI (Beijing Genome Institute) China. Using the 16S rRNA nucleotide sequence BLASTN and the nucleotide sequence databases from DDBJ, EMBL, and GenBank, the closest taxonomic groups were determined.

Extraction of biosurfactants:

To remove the bacterial cells, the sample of the selected culture was centrifuged for 15 minutes at 6000 rpm. Supernatant with pH 2 was achieved by adding 6 N HCl. By settling overnight at 4°C, precipitate were formed. Centrifugation for 15 minutes at 10,000 rpm and 45 °C was used

to collect the precipitates. By using 65:15 (v/v) chloroform/ethanol Grey white crude extract was extracted at room temperature after being diluted in deionized water (pH 8.0) to a final pH of 7. Rotary evaporator was used for the evaporation of solvent for the production of pure biosurfactant (El-Gebaly, 2020).

Characterization of the biosurfactant

TLC analysis

After dissolving 0.1 g of the purified biosurfactants in methanol, 20 µL aliquots were placed over silica gel that had already been coated (G60; Merck, Germany) on small glass slides. As the mobile phase, 65:15:2 v/v/v mixture of chloroform, methanol and acetic acid was used. By the exposure with iodine vapours, 1% ninhydrin solution, and p-anisaldehyde for the staining of lipids, free amino groups, and sugars respectively, the major components were identified. Respective reagents were sprayed, then heated to 110 ^{O}C until certain spots appeared (Ibrahim, 2018).

Fourier transform-infrared spectroscopy(FTIR)

One mg of the purified biosurfactant was mashed with 100 mg of KBr, and physical pressure of 7500 kg was applied for 30 seconds to generate translucent pellets. Using a Nexus 670 spectrometer (Nicolet, USA), infrared spectra between 500-4000 cm^{-1} wave number was measured. Using KBr pellet as a background reference, all data was corrected (Zhou *et al.*, 2015).

Antibacterial activity of extracted biosurfactants

Antibacterial activity was determined against *E. coli P. aeruginosa B. subtilis* and *S. enterica* using standard agar well diffusion method. Diameter of zones of inhibition were measured in mm, and the results were classified as sensitive, moderate, or resistant. Minimum Inhibitory Concentration (MIC) was defined as the lowest biosurfactant concentration at which the growth was inhibited (Basit *et al.*, 2018). Rifampicin was used as positive control while methanol as negative control.

Results and Discussion

Isolation, purification and identification of biosurfactant producing bacterial strains

By using spread plate and dilution procedures, ten morphologically distinct bacterial isolates were screened out of the oil-contaminated water samples. By using the hemolytic test, drop collapse assay, oil spreading activity, and emulsification index, they were further evaluated for

biosurfactant potential (Table 1). For the primary screening of biosurfactant producer bacterial strains, multiple screening method should be used (Sharma *et al.*, 2015). On blood agar plates, eight of the ten bacterial isolates showed high levels of hemolytic activity (β), two displayed low levels of hemolytic activity (α), and none displayed negative hemolytic activity (ɣ). The ability of isolates to completely lyse red blood cells, or hemolytic potential, is one of the attribute that makes them outstanding biosurfactant producers. (Rashedi *et al.*, 2005). All the isolate showed positive drop collapse test. Stability of liquid droplets depends on biosurfactant concentration (Adebajo *et al.*, 2020). In the oil spreading test, highest zone of clearance was observed by MWS5 (5cm) while lowest was 2 cm by MWS9. Another sign of biosurfactant activity in the oil spreading assay is the cell free supernatant's capacity to displace the oil and formation of higher clean zone. The emulsification test was also used to confirm the biosurfactant production potential. This test was selected because, in contrast to surface tension measurement, which takes a long time and is laborious, making it difficult to screen a large number of isolates, it is accurate, fast, reliable, and simple to perform (KATEMAI, 2012). Out of five oils used for the emulsification activity, MWS5 produced highest emulsion layer with mobil oil. It is interesting that each isolate showed different and distinct emulsifying response when exposed to each of the used oil.

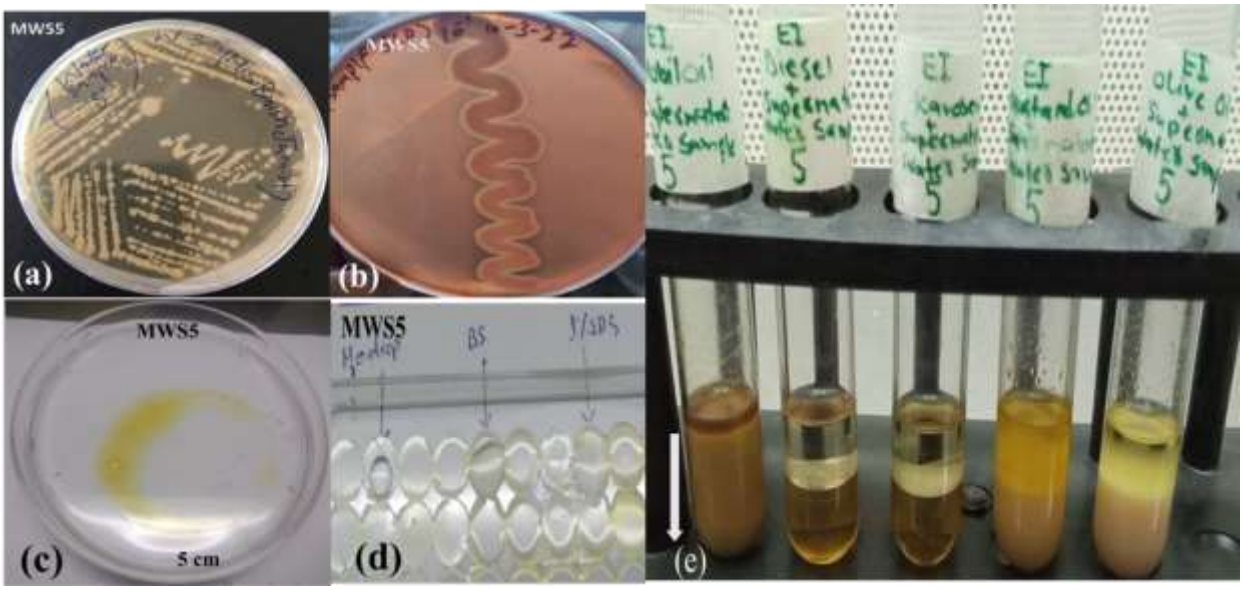

Fig 1. Screening tests of potent biosurfactant producer (MWS5) (a) Colony morphology **(b)** Hemolytic activity **(c**) Oil spreading activity **(d)** Drop collapse test **(e)** Emulsification index

Table 1. Screening of Bacterial Isolates from Oil Contaminated Sea Water

No. of Isolates	Bacterial isolates	Oil displacement Activity (cm)	Drop collapse assay	Hemolytic Activity
1	MWS1	4	+	β-haemolysis
2	MWS2	2.5	+	α –haemolysis
3	MWS3	3	+	β-haemolysis
4	MWS4	2.5	+	β-haemolysis
5	MWS5	5	++	β-haemolysis
6	MWS6	2.5	+	β-haemolysis
7	MWS7	3	+	β-haemolysis
8	MWS8	3.5	+	β-haemolysis
9	MWS9	2	+	α –haemolysis
10	MWS10	3.5	+	β-haemolysis

Table 2. Emulsification Index of Bacterial Isolates from Oil Contaminated Sea Water

Bacterial Isolate	E24 (%)				
	Mobil oil	Diesel	Kerosene	Mustard oil	Olive oil
MWS1	40	60	44.34	54.48	50
MWS2	28	55	27.14	40	35.71
MWS3	34.44	43.57	33.57	45.38	49.25
MWS4	37.69	55	33.44	46.15	33.44
MWS5	82.58	45	55.80	65.51	64.28
MWS6	35	37	44	48	49
MWS7	43	42	55	37	38
MWS8	52	39	39	28	42

MWS9	34	43	53	30	40
MWS10	55	33	38	44	35

Morphological, biochemical and molecular identification

Based on all screening results, the most efficient biosurfactants producer strain was determined to be MWS5. The morphological and biochemical characterization of the selected strain (Table 3) revealed a high degree of similarity (99%) to Bacillus. Top matches to the *Bacillus wiedmannii* strain were found when partial 16S rRNA gene sequence was aligned with the NCBI GenBank. The unknown strain was identified as *Bacillus wiedmannii* MWS5 because of the consistent sequencing similarities with *Bacillus wiedmannii*.

Table 3. Morphological and biochemical characteristics of selected strain + = positive result; – = negative result.

Bacterial Isolate	**MWS5**
Colony colour	Creamy
Cell shape	Rod shape
Elevation	Flat
Margins	Entire
Opacity	Opaque
Gram staining	+
Catalase Test	+
Methyl Red Test	-
Voges-Proskauer Test	+

Extraction and structural characterization of biosurfactant

Through solvent extraction method, MWS5 produced 1.5 g/L biosurfactant. The extracted biosurfactant was submitted to TLC and FT-IR analysis. Staining with iodine vapours and ninhydrin results in development of yellow and purples spots indicating the presence of lipid and free amino acid in the extracted biosurfactant. The strong absorption bands in the region of 3250–3500 cm^{-1}were due to the stretching vibrations of –NH and –OH groups indicating the

peptide (Sharma *et al.*, 2018). Peptide component molecules exhibit characteristic bands at 1650–1700 cm^{-1} (CO–N bond stretching mode) and at 1520–1550 cm^{-1}, which can be attributed to the combination of the N–H bond stretch mode and the C–N stretch mode. Peak at 1409 cm^{-1} is associated with lactam in its cis form (Lyu *et al.*, 2018). However, it was also noted that an aliphatic chain with C–H modes at 2840–3000 cm^{-1} was present. A carbonyl group was responsible for the band seen at 1735–1750 cm^{-1} (Diez *et al.*, 2022). As a result, it was anticipated that the biosurfactant generated by the MWS5 strain would have peptide and fatty acid moieties, indicating that it was a lipopeptide. In several Bacillus species, lipopeptides are the most extensively researched and characterized biosurfactants (Sharma *et al.*, 2018).

Table 4. Results of TLC characterization of biosurfactant

+ = positive result; − = negative result.

Isolate	Protein detection			Carbohydrate Detection	Lipid Detection	Interpretation
	Ninhydrin		Biuret			
TLC				TLC	TLC (Iodine vapors)	
MWS5	+	+	+	_	+	Lipopeptide

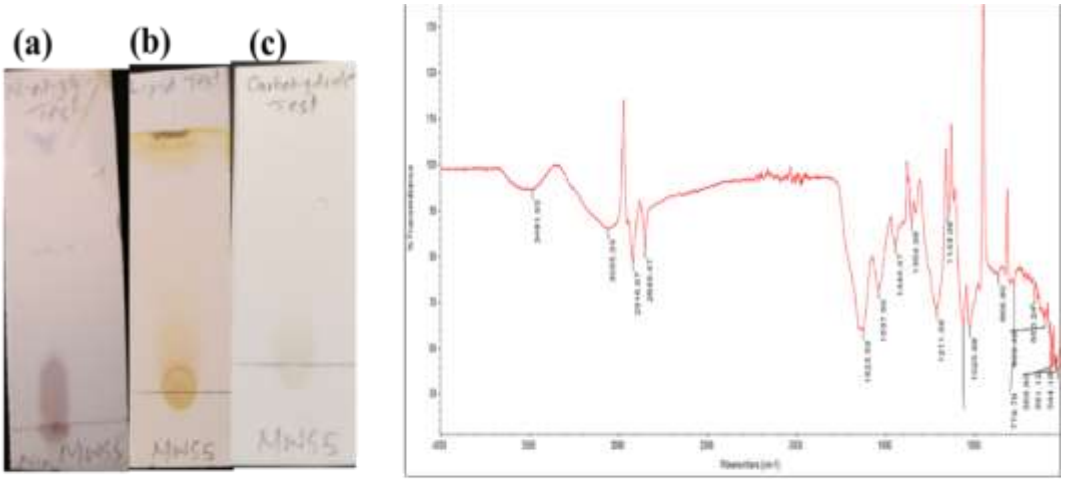

Fig 2. (A) TLC characterization of biosurfactants by MWS5 (a) stained with ninhydrin (b) stained with iodine (c) stained with p-anisaldehyde **(B)** FTIR spectra of MWS5

Antimicrobial assessment of extracted biosurfactant

Using the agar well diffusion method, the purified biosurfactants were tested for their antibacterial efficacy against both gram positive and gram negative bacteria. Table 5 represents the inhibition zones of bacterial growth of the tested bacteria upon addition of tested biosurfactan. MWS5 represented the highest antibacterial activity for *S.enterica (*26.6±1.1) mm at 20mg/ml as compared to other three strains (Table 5 & Fig 3). The antimicrobial function of lipopeptide biosurfactant is one of its possible applications as a bioproduct. The lytic membrane characteristics of lipopeptide biosurfactants have demonstrated their antibacterial action (Basit *et al.*, 2018).

Table 5. Antimicrobial activity of MWS5

Diameter of zone of inhibition (mm) (Mean± Standard deviation)				
Bacterial isolate	***E. coli***	***P. aeruginosa***	***B. subtilis***	***S. enterica***
MWS5	21.3±0.5	23.6±0.5	22.3±0.5	26.6±1.1

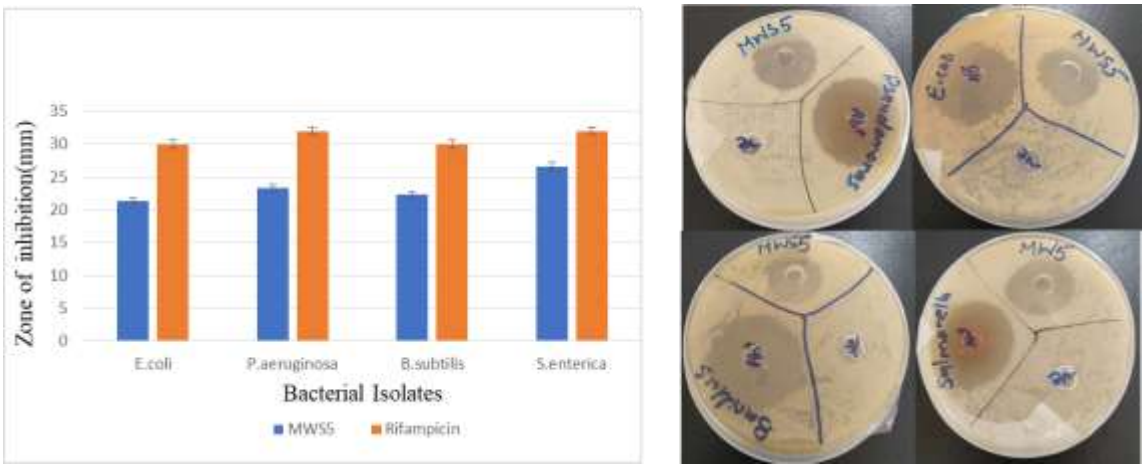

Fig 3. Antimicrobial activity of biosurfactants produced by MWS5

Conclusions

This study revealed that indeginous strain *Bacillus* wedimannii isolated from oil contaminated water environment have been confirmed biosurfactant producer with ability to produce assayable biosurfactants. The extracted biosurfactant belongs to the lipopeptide class and is a valuable tool for a variety of industrial and environmental activities, particularly oil spill bioremediation.

Biosurfactant also possessed very good antibacterial activity. The bacterial strain found in this study may prove to be a useful source of innovative, eco-friendly biosurfactants that could eventually displace synthetic surfactants.

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