RESEARCH ARTICLE

Isolation and Characterization of Biosurfactant-producing *Cytobacillus oceanisediminis* MS-05A

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Abstract

Biosurfactants are amphiphilic molecules that can reduce the surface tension of liquid. They are non-toxic, biodegradable and eco-friendly. Chemically synthesized surfactants are toxic to human health and causes skin diseases. So, there is an extensive need of non toxic and Biodegradable biosurfactant. Attempt has been made for screening and isolation of biosurfactant producing bacteria from Ratnagiri sea side soil. The isolates from soil samples were screened by using hemolytic activity, drop collapse test, tilted glass test and oil displacement activity. The potent isolate MS-05A was characterised at molecular level. It showed 99.64% similarity with *Cytobacillus oceanisediminis* H2 (GQ292772). The growth of isolate MS-05A was optimized in Zobell marine broth for different environmental and nutritional parameters like salt, pH, temperature, agitation and sugar concentration. Optimal conditions were used for growth, emulsification activity and extraction of biosurfactant. The crude biosurfactant of MS-05A was characterized with thin layer chromatography (TLC) and fourier transform infrared spectroscopy (FTIR). Washing performance test and antimicrobial activity of crude biosurfactant were tested. The isolated MS-05A showed optimal growth in Zobell marine broth containing 3% sucrose, 5% salt, at 35°C,120 RPM agitation, pH 7 for 4 days incubation. The cell free supernatant of isolate showed emulsification activity 55% for sunflower oil. The crude biosurfactant of MS-05A showed good washing performance and antimicrobial activity. It may be used as a biocontrol agents in agriculture.

Highlights:

- Isolation and screening of biosurfactant-producing bacteria.
- Optimization of growth of *Cytobacillus oceanisediminus* MS-05A.
- Application of crude biosurfactant as an antimicrobial agent and for washing activity.

Keywords: Emulsification activity, Washing activity**,** Antimicrobial activity.

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Introduction

Surface tension is the force per unit length present at the point of contact between two immiscible phases. Here, the intramolecular force is higher than the intermolecular force. This intramolecular force is reduced as the addition of surfactant (Adamson and Gast, 1997). A Known chemical anionic surfactant like alkyl benzene sulphonates (ABS). These are toxic to aquatic life, such as fish, by damaging the gills and directly affecting osmoregulation. It may be toxic to humans health and cause skin irritation. In higher doses, it could cause dermal cancer (Rebello *et al*., 2014). Commercial detergents may contain builders, softners and other necessary products but may cause harmful effects. Phosphate used as water softener in chemical detergents. It may causes eutrophication and affects aquatic ecosystems (Helmy *et al*., 2020). Whereas biologically produced surfactants are biodegradable, eco-friendly and has very low toxicity. Biosurfactants are amphiphilic molecules that contain both hydrophilic and hydrophobic parts. These biosurfactants may stand in extreme conditions like high temperature, salinity and pH. In Europe, people are aware of the toxicity of chemical detergents, so the use of biosurfactants has increased. The market value of biosurfactants is more than 4.41 billion USD, which may increase by 3.5% annually (Dhanarajan & Sen, 2014).

The marine ecosystem mainly specializes due to its physicochemical, structural and functional diversity. The microbes isolated from marine habitats are highly salt, pH, temperature, pressure tolerant and grow at low nutrient availability. A hydrocarbon degrading microbe has ability to secrete biosurfactants such as *Thiobacillus thiooxidans* and *Corynebacterium* sp. or yeast. Bacterial genera like *Bacillus, Pseudomonas* and *Halomonas* are predominantly found in marine habitats (Floris *et al*., 2018; Wu *et al*., 2019).

During autumn fungal infection results in black coloration on the surface of the plant leaf. It forms a black layer on the leaf surface, reducing the photosynthesis rate (Wood *et al*., 1988). The overall crop loss due to fungal disease in the US is more than $23.5 billion annually (Rossman, 2009). Chemicals antifungal is not safe for plant health, human beings and soil environment; there is a need an eco-friendly approach to biosurfactants (Mnif *et al*., 2015). A well-known opportunistic pathogens and foodborne disease-causing laboratory bacterial isolate such as

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Escherichia coli, Salmonella sp., *Pseudomonas arogenionsa* and *Bacillus cereus* were used for the study. Although *E. coli* is part of the normal intestinal flora, it can cause diarrhea, dysentery, urinary tract infections (UTIs), respiratory infections, and sepsis (Pokharel *et al*., 2023). *Salmonella* species cause foodborne diseases worldwide due to the consumption of contaminated chicken or eggs (Gast & Porter, 2019). Another opportunistic pathogen, *Pseudomonas aeruginosa*, causes infections in patients with cystic fibrosis and is frequently associated with wound infections characterized by blue-green pus (Brindhadevi *et al*., 2020). Additionally, *Bacillus subtilis* and other *Bacillus* species may cause foodborne illnesses characterized by heat-stable toxins and diarrheal syndromes (Apetroaie-Constantin *et al*., 2009; Jovanovic *et al*., 2021).

Detergents are necessary for maintaining hygiene in daily life. A chemically synthesized detergent contains additives such as fillers, builders, foaming agents, wetting agents and more than 20 different types of chemical agents. These chemicals are toxic to the environment globally and are not easily degraded (Mukherjee, 2007). A lipopeptide produced from *Bacillus subtilis* SPB1 is used as a biosurfactant for laundry detergent (Bouassida *et al*., 2018). *Serratai rubidaea* KAP produces a biosurfactant having effective washing performance (Pendse & Aruna, 2020). After literature survey, showed that little work was done on biosurfactant as laundry detergents and antifungal activity against phylloplane fungi. So, the present study focused on isolation, optimization of biosurfactant producer, chemical characterization of biosurfactant and their use as washing performance and antimicrobial activity.

Material and Methods

Collection of samples

A marine soil was meticulously collected from the Ratnagiri sea side (latitude18º9' 2.3868''N, longitude 73°18'0.0108''E). The upper layer of about 5 to 10cm was removed the lower part of the soil samples was collected in sterile polyethylene bags and kept in an ice-cold box (Mallisetty *et al*., 2023).

Isolation of biosurfactant producing bacteria

With utmost precision, the collected samples were removed from the ice box 30 min before use. Aseptically, 10gm of soil was inoculated in 100ml of sterile saline and serial diluted. 0.1ml dilutions were spread on the Zobell marine agar plate (ZMA) (Hi-media)(composition per litre of distilled water: 5 g Peptone, 1g Yeast extract, 0.1g Ferric citrate, 19.45g Sodium chloride, 8.8g Magnesium chloride, 3.24g Sodium sulphate, 1.8g Calcium chloride, 0.55g Potassium chloride, 0.16g Sodium bicarbonate, 0.08g Potassium bromide, 0.034g Strontium chloride, 0.022g Boric acid, 0.004g Sodium silicate, 0.0024g Sodium fluorate, 0.0016g Ammonium nitrate, 0.008g Disodium phosphate, Final pH 7, 30g Agar). These plates were incubated at 35ºC for 2 days. The isolates were screened for biosurfactant production (Haque *et al*., 2020).

Screening for biosurfactant-producing isolates

The isolated colonies on Zobell marine agar were used for screening of biosurfactant production through different test.

Hemolytic activity

Modified blood agar prepared using ZMA with 5% sheep blood. The isolated cultures were spot inoculated on ZMA blood agar, after incubation zone of clearance was observed. (Satpute *et al*., 2010).

Tilted glass test

A single distilled water drop was kept on a clean, grass-free slide. A 24-hour-old culture colony was mixed with distilled water drop using the nichrome wire loop. Water is used as a control (Satpute *et al*., 2010).

Drop collapse test

A thin film of parafilm was attached to the measuring scale. 100µL distilled water was used as the negative control, while 100µL sodium dodecyl sulphate (SDS)1% used as positive control. A 4-day-old culture was used for this test. Drop size after 1 minute was observation (Waghmode *et al*., 2019).

Oil Displacement activity

According to (Waghmode *et al*., 2019) modification, A 90mm petri plate containing 40mL distilled water plate was kept on black background for enhanced visibility. A 20µL volume of crude oil was carefully added to distilled water surface, forming a thin oil layer. Subsequently, 10µL of cell-free supernatant (CFS) of isolate was carefully dispensed at the centre of the oil layer using a micropipette. A presence of a biosurfactant in the CFS indicated by oil displacement activity. For further analysis, only the isolates that showed positive results across all four screening parameters were selected.

Characterization of isolate

The potent isolate was morphologically characterized and used for further study. The isolate was submitted to the National Centre for Microbial Resource (NCMR), Pune, for molecular characterization. The phylogenetically closest relatives were searched on EzBioCloud database by comparing the 16S rRNA gene sequence similarity (Prawan *et al*., 2023). The phylogenetic tree was constructed using the neighbor-joining method and bootstrap 1000 (Chopra *et al*., 2020). MEGA11 software is used for the construction of phylogenetic tree.

Optimization using one-factor-at-a -time growth of potent isolate

Zobell marine broth was used as a basal medium. 24 hours old culture was taken and maintain optical density (O.D.) 0.5 at 600nm (Fooladi *et al*., 2016; Ekpenyong *et al*., 2021). 1mL of this culture was inoculated in 100mL Zobell marine broth for various environmental and nutritional parameters (Dhasayan *et al*., 2015) such as temperature (25, 30, 35 & and 40ºC), salt (1 to 20 %), agitation (90, 120 and 150 RPM) and different sugar (1%) *viz*. glucose, lactose, starch, sucrose were tested (Joshi *et al*.,2008; Joshi-Navare *et al*., 2013). Each parameter was tested in triplicate separately. Growth was measured at 600nm after 4 days (Joshi *et al*.,2008; Chen *et al*., 2018).

Biosurfactant extraction

Four-day-old culture in Zobell marine broths were centrifuged at 7,000 RPM for 30 min. A 6 N HCl were added in supernatant for acid precipitation (final pH 2), kept at 4ºC overnight. The

precipitation was observed at the surface. Ethyl acetate was used for solvent extraction. Repetitive extraction was performed three times. The upper organic layer (ethyl acetate) was collected separately. Sodium sulphate was added to reduce water in it. This extract was concentrated using rotary vacuum evaporate (Heidolph) at 40°C until the honey yellow/ brown color developed. This crude biosurfactant was used for further work (Patowary *et al*., 2017; Haque *et al*., 2020).

Emulsification activity of potent isolate

As per Mukharjee (2007) modification, an equal volume of CFS (3mL) of potent isolate and oil (3mL) is kept within tube and vortexed for 2 minutes. This tube was kept for 24 hr at room temperature. Emulsification activity was calculated by emulsification index (Hussain & Khan, 2018). The emulsification index (E_{24}) was measured as height of emulsification layer to total height of the mixture. Sunflower oil, soybean oil and sesame oil were used. A positive standard was kept 1 gm% sodium dodecyl sulphate and tween 80.

$$E_{24} = \frac{\text{Emulsified layer height (cm)}}{\text{Total height of the mixture(cm)}} \text{X}100$$

Surface tension measurement

The pendant drop technique was used for analysis. This test was carried out in the Department of Chemistry at Savitribai Phule Pune University, Pune. A Contact angle goniometer, KRÜSS DSA 25E, with a KRÜSS ADVANCE 1.11.0.15801 version software. Different concentrations of crude biosurfactant were prepared in sterile distilled water and critical micelle concentrations (CMC) was determined. (Luna *et al*., 2011; Xia *et al*., 2011; Sarubbo *et al*., 2016; Patowary *et al*., 2017 Lopez-Prieto *et al*., 2020). The system's temperature maintains constant at 20°C throughout the experiment. To retain the constant 5µL size of the drop, a 1mm size needle was used with a flow rate of 1 µL/s. The surface tension measurement was taken after ten drops (Chopra *et al*., 2020).

Chemical characterization of crude biosurfactant

TLC analysis

Silica gel-coated aluminum TLC papers were used for analysis. Standard rhamnolipid (AGAE Technologies, LLC, USA), sophorolipid (BioEDGE Aditya Renewtech LLP, India) and crude biosurfactant of MS-05A were spotted on TLC with the help of capillary. Chloroform: methanol: acetic acid (65:15:2) was used as a solvent system. Iodine and p-anisaldehyde were used as developers (Chopra *et al*., 2020).

FTIR analysis

The crude biosurfactant of MS-05A was analysed with Fourier Transform Infrared Spectroscopy (FTIR). The analysis was performed using a Shimadzu- IR Affinity (Diamond ATR) FTIR accessory. The infrared (IR) range scans from 400 to 4000 cm^{-1} with a number of scans 40 (Waghmode *et al*., 2019).

Applications of MS- 05A crude biosurfactant

Washing performance test

According to Pendse and Aruna, (2020), a white cotton fabric measuring 15 X 10 cm was used. Five cotton fabric of this size were stained with 5mL sunflower, 5mL soybean, 5mL tea, 2mL blood and 1mL basic fuchsin (0.5% w/v) respectively. All the stained fabrics were placed in a hot-air oven for one hour. These cotton fabrics were washed with 10mL of different detergents such as 10% (w/v) market detergent, 10% (w/v) sodium dodecyl sulfate, distilled water, ethyl acetate and 4% (w/v) crude biosurfactant. For the soaking purpose, 90ml distilled water was added and kept for 30min. After soaking, cotton fabrics were washed with a small amount of distilled water. The cotton fabric was kept for drying at room temperature and observed the change after 24 hr (Mukherjee, 2007; Pendse & Aruna,2020).

Antibacterial activity

Antibacterial activity of crude biosurfactant was conducted against four different laboratory isolates such as *Escherichia coli* (MCC- 3099), *Salmonella enterica* (MCC- 3910), *Pseudomonas aeruginosa* (MCC- 2265) and *Bacillus subtilis* (MCC- 2110). Well diffusion method was used for it. The broth's cellular concentration was about 10^7 CFU/mL and optical density was recorded at a wavelength of 600 nm. A sterile 20ml Mueller Hinton agar plate was spared with 2ml soft agar of above laboratory isolates. A 6 mm cork borer was used to make a well in it. Tetracycline (Resteclin® -500) and erythromycin (Althrocin® -500) with a final concentration 50 mg/mL were used (positive control). The concentration of crude biosurfactant ranges between 0.4 to 0.000004g/ml were used. The plate was refrigerated for 30 min. The plates were incubated at 37°C for 24 hours, and the zone of inhibition were measured (Yuliani *et al*., 2018). Minimum inhibitory concertation (MIC) was determined using macrodilution broth method (Denney, 2019).

Antifungal Activity

- *Isolation of phylloplane fungi*

The unknown fungus was isolated from infected *Ixora coccinea* plant leaves from the college garden. The molecular analysis of this isolated fungus was done at the National Fungal Culture Collection of India, Agharkar Research Institute, Pune. The sequence was compared with fungal data on NCBI.

- *Antifungal activity*

This isolated fungus was grown in 100mL potato dextrose broth. Well diffusion method was used for it. The fungal spore concentration was prepared (1×10^5 spore/mL). 1-mL spore suspension was spread on PDA. A 6 mm cork borer was used to drill and 30 µL crude biosurfactant was inoculated. The concentration of crude biosurfactant ranges between 0.4 g/mL and 0.0004 g/mL. A standard antifungal tablets Fluconazole (Fluka-150) A standard antifungal tablets Fluconazole (Fluka-150) having 15 mg/mL concentration was used as positive control. was used as positive control. Plates were incubated at 30°C for 48 hours. The zone of inhibition was measured in millimetres using a ruler (Guillen-Navarro *et al*., 2023). The minimum inhibitory concentrations (MIC) were assessed using the macrodilution broth assay, and optical density was recorded at 600 nm after 48 hours of incubation (Kumari *et al*., 2021).

Results and discussion

Screening of biosurfactant-producing bacteria

Five distinct colonies were isolated on Zobell Marine agar plate and were designated as MS-01A to MS-05A.

Table 1.: Screening of biosurfactant-producing bacteria

Code number of isolates	*Hemolytic activity*	*Tilled glass test*	*Drop collapse test*	*Oil displacement activity*
MS-01A	++	--	--	--
MS-02A	++	--	--	--
MS-03A	--	--	--	--
MS-04A	--	--	--	--
MS-05A	++	++	++	++

Abbreviations: ++: Positive test, --: Negative test

Five different isolates were screened for biosurfactant production as shown in Table 1. Out of five isolates three isolates MS-01A, MS-02 and MS-05A were showed zone of hemolysis on modified blood agar (Satpute *et al*., 2010). Out of five isolates only isolate MS-05A showed tilted glass test, drop collapse test positive and shows oil displacement activity. In tilted glass test distilled water flows on the surface of the glass slide due to reduction in surface tension, allowing water drop to move on the glass surface (Hussain & Khan, 2018). In drop collapse test, reduction in the surface tension of broth was observed. It gets spread on the parafilm paper (hydrophobic surface) (Waghmode *et al*., 2020). Four days old culture broth for drop collapse test showed increase in diameter by 2mm. It also changes the shape of the drop from dome to flat as shown in fig. 1. For the oil displacement activity, isolate MS-05A cell free supernatant showed zone of clearance. MS-05A isolate showed all four-screening test positive. It indicates that the isolate has ability to produce biosurfactant.

Characterization of MS-05A isolate

The organism MS-05A isolated from marine soil at Ratnagiri sea side, showed a mucoid colony on Zobell marine agar. Microscopic observations showed Gram-positive, endospore-forming rod-shaped bacteria. The 16SrRNA gene sequence showed 99.64 % sequence similarity with *Cytobacillus oceanisediminis H2 (*Accession number- *GQ292772*) analyzed using EzBioCloud database. The NCBI accession number for 16S rRNA gene sequence of isolate MS-05A clustered with type strain of *Cytobacillus ocenisedminis* H2^{T}is PQ143929 as shown in fig. 2.

Optimization of growth of MS-05A using one- factor-at-a- time method

The growth of MS-05A was found to be optimum at 3% sucrose pH 7, temperature 35°C, and 5% salt on rotary shaker at 120RPM

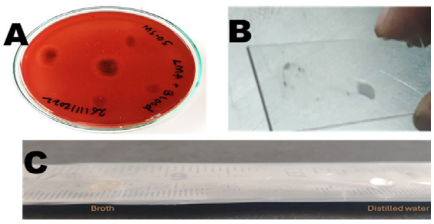

Fig. 1.: Screening of biosurfactant-producing bacteria. A. Hemolytic activity, B. Tilled glass test, and C. Drop collapse test

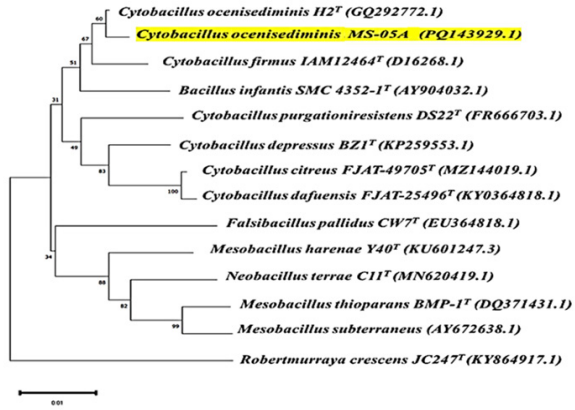

Fig. 2: Neighbor-joining tree based on nearly complete 16S rRNA gene sequences, depicting the relationships between isolate MS-05A and related type strains. Bootstrap values (>50%), derived from 1000 resampled datasets, are shown at the branch points. The scale bar indicates 0.01 substitutions per nucleotide position

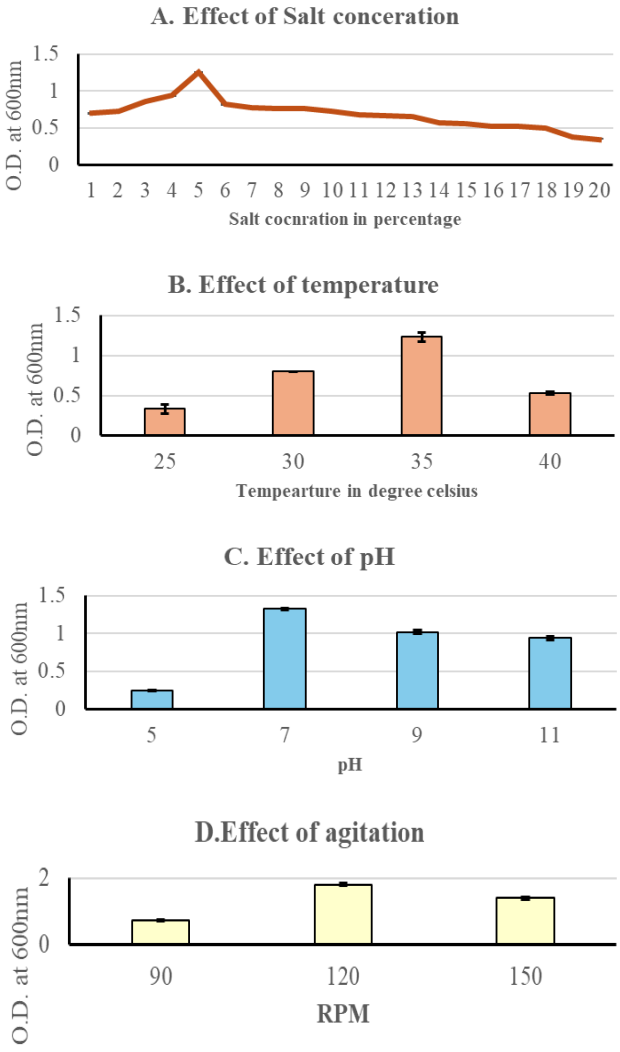

Fig. 3: Effect of environmental parameters on the growth of MS-05A A. Effect of salt, B. Effect of temperature, C. Effect of pH and D. Effect of agitation

agitation in Zobell marine broth (Fig. 3 and Fig. 4). Optimum conditions were used for biosurfactant production. The yield of the crude biosurfactant was found to be 0.67g/L. The known data indicate that *Cytobacillus oceanisediminus* TESV, *Bacillus oceanisediminus*, and *B. oceanisediminis* PM 08 produced 0.64g/L, 1.7g/L, and 1.55g/L of biosurfactant respectively (Javed *et al*., 2022; Chooklin *et al*., 2023; Kumari *et al*., 2024).

Emulsification activity

It was found that sunflower oil has 55% emulsification activity, while 54 and 53% emulsification activity for sesame oil and soybean oil respectively (Fig. 5). The positive control tween 80 showed higher emulsification for sesame oil 72% while soybean oil 53% and low for sunflower oil 50%. The surfactant sodium dodecyl sulphate showed 50% emulsification for all three oil.

The present study showed 53 to 55% emulsification for edible oil *viz*. sunflower oil, sesame oil, and soybean oil. As per reported data on the emulsification activity of edible oil depends on the amount of emulsifier, droplet size, fatty acid composition, solubility parameter, oil extraction method, oil density, pH and temperature (Rahate & Nagarkar, 2007, Iqbal *et al*., 2013, Permyakova *et al*., 2020; Kampa *et al*., 2022). A reported data of emulsification of edible oil is 54 to 56% from different oil-degrading bacteria such as *Pseudomonas arginase, Bacillus subtilis*, MTCC441, *Bacillus cereus* UCP1615 and *Planococcus halototerans* IITR 55 (Chander *et al*., 2012; Gaur *et al*., 2020; Durval *et al*., 2021; Suryawanshi *et al*., 2021).

Surface tension measurement

The crude biosurfactant of isolate MS-05A showed reduction in surface tension of sterile distilled water from 72.10mN/m to 28.90 mN/m at critical micellar concentration (CMC) of 120 mg/L. Several microbial strains have been reported to exhibit significant surface tension-reducing capabilities. For example, the phospholipid- biosurfactant producing actinomycete *Streptomyces thinghirensis* 7SDS was reported to reduce the surface tension of distilled water to 27.96 mN/m at a concentration of 350 mg/L (Bellebcir et al., 2023). Similarly, rhamnolipid producing *Pseudomonas aeruginosa*, used in liquid detergents, demonstrated a reduction in water surface tension to 30.12 mN/m at a CMC of 70 mg/L (Jadhav et al., 2019). *Pseudomonas aeruginosa* LBI, utilizing soapstock as the sole carbon source for rhamnolipid production, was shown to reduce the surface tension of distilled water to 34.0 mN/m at a CMC of 120 mg/L (Benincasa and Accorsini, 2008). Additionally, A lipopeptide producing *Bacillus subtilis* SPB1, reduced the surface tension of distilled water to 27.96 mN/m at a CMC of 150 mg/L (Bouassida *et al*., 2018).

Rhamnolipid production from *Pseudomonas aeruginosa* MTCC-424 and *Pseudomonas aeruginosa* PTCC-1340 surface tension reduces up to 28.5 mN/m (120mg/L CMC) and 25.8 mN/m (0.09g/L CMC), respectively (Mishra et al., 2021; Li et al., 2022; Safari et al., 2023). A reported rhamnolipid is a mixture of more than 4 to 28 homologous compounds. So, the CMC of rhamnolipid ranges from 1 to 200mg/L. The aggregate size may vary based on the size of the molecule (Rekiel *et al*., 2020).

Chemical characterization

TLC analysis

TLC analysis of crude biosurfactant of MS-05A showed a green spot with R_f value 0.82 which is similar to the standard rhamnolipid. Fig. 6 indicates that the crude biosurfactant of MS-05A may be of mono-rhamnolipid (Chopra *et al*., 2020).

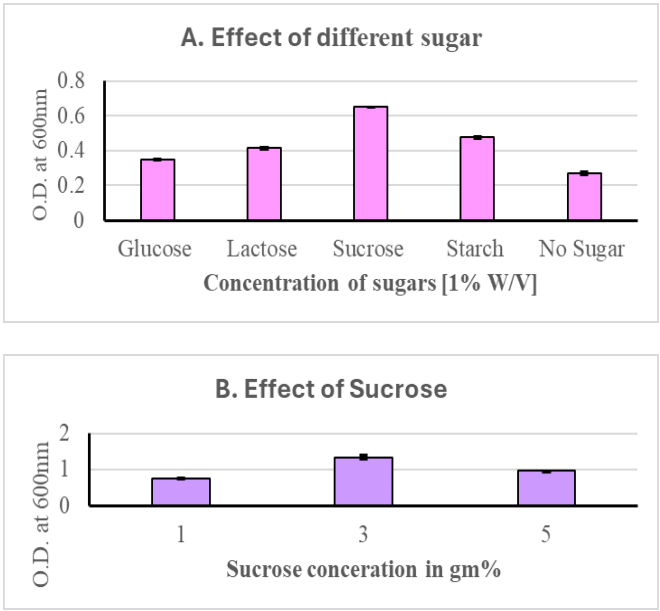

Fig. 4.: A. Effect of different Sugars on the growth of isolate MS-05A and B. Effect of sucrose concentrations on the growth of isolate MS-05A

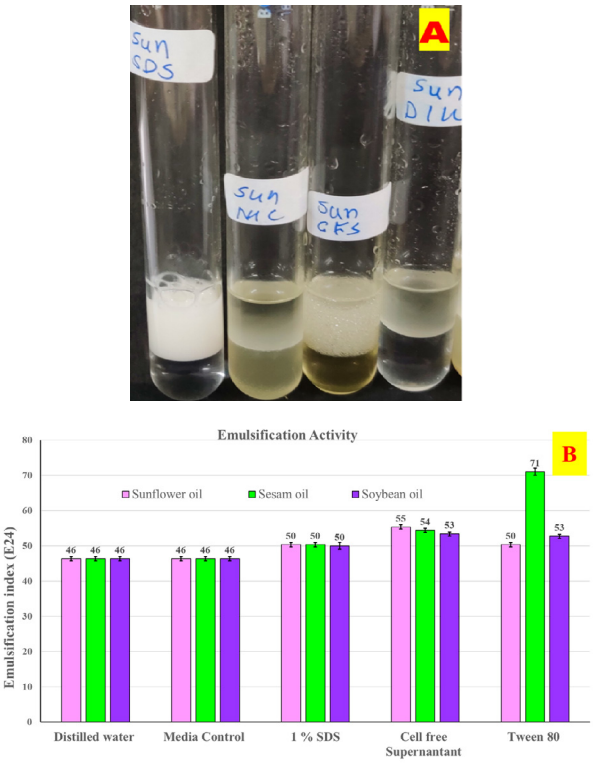

Fig. 5: A) The emulsification activity of MS-05A after 24 hours of incubation at room temperature obtained using sunflower oil. B) Bar chart of emulsification activity of different oils such as sunflower, sesame and soybean oil

FTIR analysis

The FTIR analysis of MS-05A crude biosurfactant (fig. 7). The weak intensity peaks at 3390cm^{-1} and 3361cm^{-1}, corresponding to the hydroxyl (-OH) polysaccharide group and weak intensity peaks at 2987cm^{-1} and 2945cm^{-1}, corresponding to aliphatic CH_2, CH_3 stretching (Chopra *et al*., 2020; Heyd *et al*., 2008; Chooklin et al., 2023). The high-intensity peak at 1755cm^{-1} and 1705cm^{-1} corresponds to C=O stretching of ester functionality and the peaks at 1390cm^{-1} and 1234cm^{-1} correspond to C-O stretching, which conforms to the presence of the lactone group. The peak at 1051cm^{-1} corresponds to the C-O-C stretching of ether linkage in polysaccharides. The absorption peaks at 921cm^{-1} and 883cm^{-1} indicate pyranyl C-C stretching (Singh & Cameotra, 2013). The observed spectra displayed noticeable absorption peaks corresponding to the characteristic functional groups of may be rhamnolipids (Heyd *et al*., 2008; Singh & Cameotra, 2013; Patowary *et al*., 2018; Chopra *et al*., 2020; Chooklin et al., 2023).

Application of Biosurfactant

Washing performance test

The crude biosurfactant of MS-05A showed significant removal of basic fuchsin (0.5% w/v) and tea-stained cotton fabric compaired to distilled water and ethyl acetate. Most of the light staining part on cotton fabric was removed with market detergent and SDS. Also, the impact of bloodstain washing performance was effective compaired with others as shown in fig. 8. The crude biosurfactant effectively removed sunflower and soybean oil, without leaving any yellow spots.

In the present study, the crude biosurfactant of MS-05A effectively removed stains from cotton fabric, without the addition of any additives or peptides. Pendse and Aruna (2020) used a crude biosurfactant of *S. rubidaea* KAP and other conjugates for the washing performance tests. They found that biosurfactant alone showed a good detergent activity. In commercial detergents, other compounds are mixed to enhance washing performance. In another study, biosurfactants and market detergents were used in proportion to enhance washing performance, such as *Bacillus subtilis* SPB1 produces lipopeptide and combination with commercial detergent enhance the washing performance up to 12 to 15% (Bouassida *et al*., 2018).

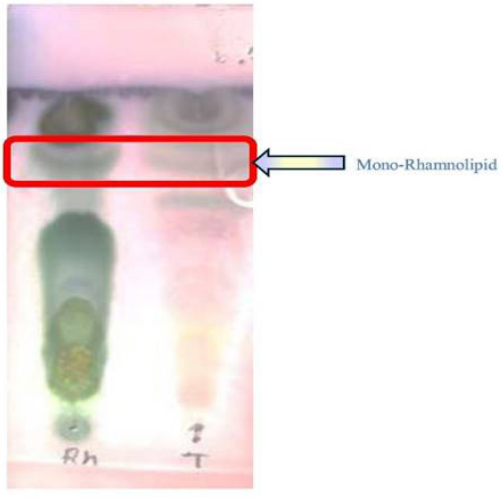

Fig. 6: TLC plate was developed using p- anisaldehyde as a developer for a crude biosurfactant of MS-05A and standard rhamnolipid. Abbreviations: Rh: Standard rhamnolipid T: crude biosurfactant of MS-05A

Antibacterial activity

The standard antibiotics tetracycline and erythromycin exhibited zones of inhibition ranging from 13.0 mm to 26.5 mm against all four bacterial isolates. As per the chart given in Cappuccino and Sherman 10th edition, in case of tetracycline if zone inhibition is less than 14 mm then organism is considered as resistant and if it is greater than 19 mm then organism is considered as susceptible. So, all the four isolates were found to be susceptible to tetracycline as shown in Fig. 9. Erythromycin showed intermediate zone of inhibition for all four isolates (Cappuccino & Sherman, 2013). However, crude biosurfactant of MS-05A showed zone of inhibition against *E. coil* 48 ± 0.8mm, *Bacillus* sp. 38.8 ± 1.16mm, *Pseudomonads* sp. 44.2 ± 1.47mm and *Salmonella* sp. 50.6 ± 1.36mm at 0.4 gm/mL concentration (fig. 10). Intermediate zone of inhibition obtained at 0.04 gm/ml concentration of crude biosurfactant of MS- 05A, such as 27.6 ± 1.03mm *E. coli*, 21± 3.55mm *Bacillus sp.*, 28.6 ± 3.07mm *Pseudomonas* sp. and 29.3 ± 1.63 mm *Salmonella* sp.

Combination of rhamnolipid and nisin reduces its concentration and response time against *L. monocytogenes* (Magalhaes & Nitschke, 2013). However, MS-05A crude biosurfactant showed minimum inhibitory concentration 0.04 gm/mL against *Escherichia coli* (MCC-3099), *Salmonella enterica* (MCC-3910), *Pseudomonas aeruginosa* (MCC- 2265) and *Bacillus subtilis* (MCC-2110).

Antifungal activity

The molecular analysis of fungus isolated from leaf surface of *I. coccinea* plant showed 100% similarity with *Aspegillus fastidious* CBS 121.28 with gene bank accession no NR_163668.1. There were a total of 1094 positions in the final dataset. The hit analysis shown in Table. 2

Fluconazole was not effective against phylloplane fungi (Guideline, 2006). In present study crude biosurfactant of MS-05A was more effective at higher concentration but not effective at lower concertation below 0.004gm/ml against *Aspergillus fastidous* CBS121.28. At 0.4gm/mL concentration of MS-05A crude biosurfactant showed 50 ± 5mm zone of inhibition and 0.04 gm/mL concentration showed a zone of

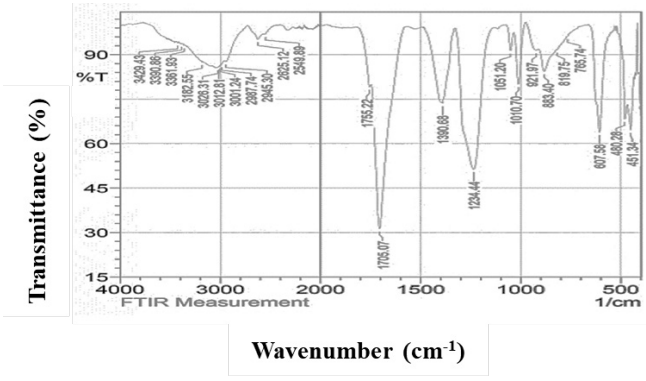

Fig. 7: FTIR analysis of a crude biosurfactant of MS-05A

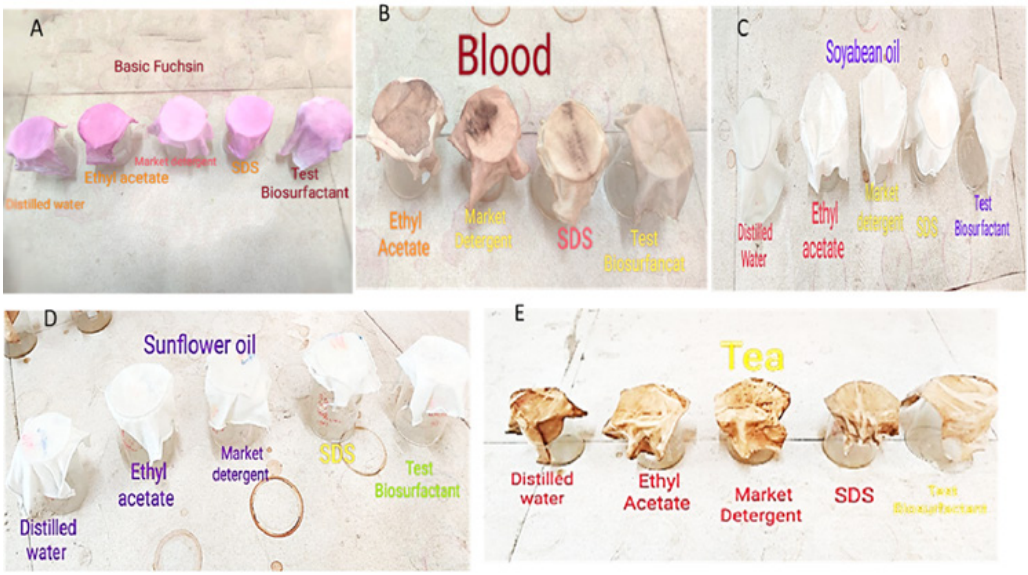

Fig. 8.: Washing performance test for crude biosurfactant of MS-05A compared to market detergent on cotton fabric stained with A: Basic fuchsin; B: Blood; C: Soybean oil D: Sunflower oil; E: Tea

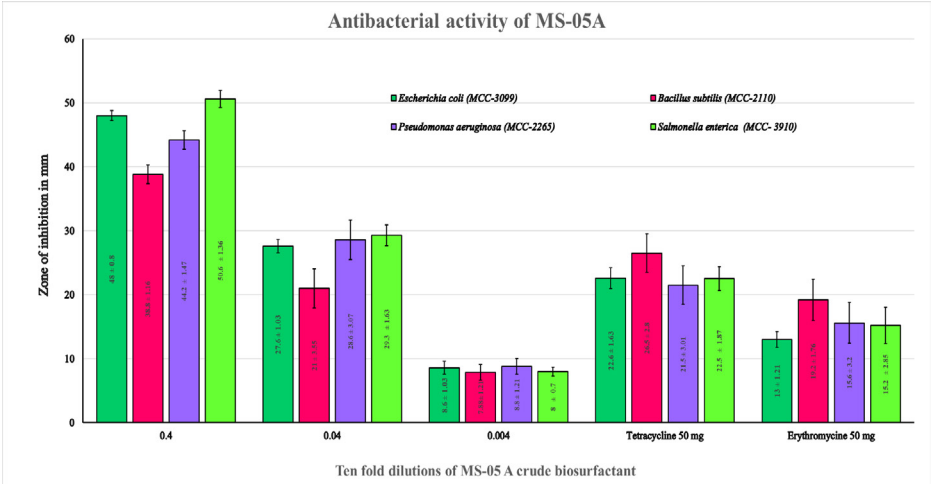

Fig. 9: Graphical representation of antibacterial activity of MS-05A crude biosurfactant against *Pseudomonas aeruginosa* (MCC-2265), *Escherichia coli* (MCC-3099), *Salmonella enterica* (MCC-3910) and *Bacillus subtilis* (MCC-2110)

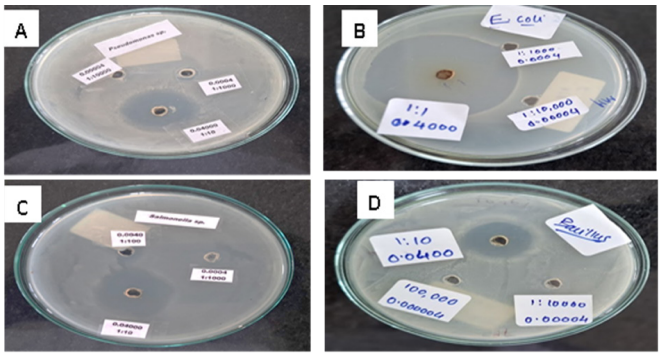

Fig. 10: Antibacterial activity of MS-05A crude biosurfactant against A. *Pseudomonas aeruginosa* (MCC-2265), B. *Escherichia coli* (MCC-3099), C. *Salmonella enterica* (MCC-3910) and D. *Bacillus subtilis* (MCC-2110)

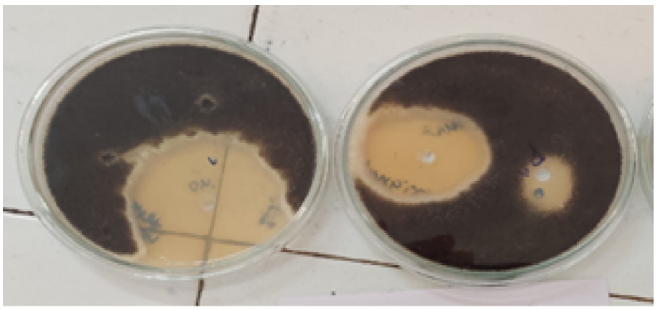

Fig. 11: Antifungal activity of MS-05A crude biosurfactant against plant pathogenic fungi *Aspergillus fastidious* CBS121.28

Table 2: BLASTn analysis of fungal isolated from *I. coccinea* plant leaves

Gene Bank Accession No.	*Description*	*Max Score*	*Total Score*	*Query coverage*	*E value*	*Identity (%)*
NR_163668.1	*Aspergillus foetidus* CBS 121.28	1094	1094	100%	0%	100.00%
AY373852.1	*Aspergillus niger stain* ATCC 16888	1085	1085	99%	0%	100.00%
NR_137513.1	*Aspergillus welwitshiae* CBS 139.54	1085	1085	99%	0%	100.00%
AF138904.1	*Aspergillus niger* ATCC16888	1085	1085	99%	0%	100.00%
MH862988.1	*Aspergillus costaricensis* culture CBS:115574	1077	1077	100%	0%	99.49%

inhibition of 25 ± 2 mm against the black-colored Aspergillus fastidious CBS121.28 as shown in Fig. 11.

A hydrophobic part of biosurfactant mostly lipid chain. This lipophilic character may help in distraction of lipid membrane by hampering the ion transport. A sophorolipid produced from *Metschnikowia churdharensis* CIG6A^T may change in permeability of the fungal cell, hence cell death occurs in food spoiling fungi *F. solani,* (Kumari *et al*., 2021). In this study crude biosurfactant of MS-05A showed 0.04gm/mL minimal inhibitory concentration against *Aspergillus fastidous* CBS121.28. A study conducted on *Pseudomonas aeruginosa* strain B5 demonstrated the production of rhamnolipid B, which exhibited antifungal activity against *Phytophthora capsici* and *Colletotrichum orbiculare* (Kim et al., 2000). In a separate study, rhamnolipid-producing *P. aeruginosa* also showed significant antifungal activity against *Rhizopus oryzae* F5, *Aspergillus flavus* F2, *Cunninghamella bertholletiae* F1, and *Aspergillus niger* F14 (Onlamool et al., 2023).

Conclusion

The potent isolate MS-05A was characterized at the molecular level and showed 99.64% similarity with *Cytobacillus oceanisediminis* H2 (GQ292772). The isolate MS-05A exhibited optimal growth in Zobell marine broth supplemented with 3% sucrose and 5% NaCl, under incubation conditions of 35°C, pH 7.0, with continuous agitation at 120 RPM for 4 days. The cell-free supernatant obtained after incubation demonstrated an emulsification activity of 55% against sunflower oil, indicating its potential as a bioemulsifier-producing strain. The crude biosurfactant produced by the MS-05A isolate showed satisfactory washing efficiency. Furthermore, it exhibited notable antibacterial and antifungal activities. These properties suggest its potential application in the suppression of phytopathogenic fungi on plant surfaces and highlight its importance in sustainable agricultural practices.

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Author Contribution

Kalumbe A. A.: Investigation, writing original draft, data curation, writing – review and editing, validation and visualization.
Pawar S.T.: Writing – review and editing, validation, visualization, supervision and final draft correction.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationship that could appeared to influence the work reported in this paper.

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