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Biosurfactant-Mediated Green Synthesis of Nanoparticles from Medicinal Endophytic Bacteria: A Comparative Evaluation of Antimicrobial and Anti-Biofilm Efficacy

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ABSTRACT: Biosurfactants, surface-active compounds made by bacteria, have drawn interest in the synthesis of nanoparticles. Using biosurfactants isolated from endophytic bacteria, nanoparticles made were looked for their antibacterial and antibiofilm abilities. UV-Vis spectrophotometry was used to confirm the synthesis and stability of nanoparticles. The nanoparticles showed inhibition that were similar to those of standard antibiotics when tested against bacterial strains of *S. aureus* and *P. aeruginosa*, indicating it to be a potential antibacterial. Additionally, they were found to be successful in preventing preformed biofilms, which is important because biofilms are a contributing factor to antibiotic resistance and chronic infections and thus can be a suitable biomedical application. These results demonstrate the potential of nanoparticles produced from biosurfactants as an alternative to antibacterial drugs. They are suitable for biological applications because of their ability to inhibit bacterial and biofilm growth. More investigation needs to be carried out to evaluate their toxicity, stability, and biocompatibility. Testing on in vivo models and cell cultures is crucial to determine their efficacy and safety in medical applications. Biosurfactant-based nanoparticles may provide a new and environmentally friendly method of creating an antibiotic in light of the growing prevalence of antibiotic resistance. By understanding the need for more research into bio-based options for infection management, this work adds to the expanding area of nanotechnology.

KEYWORDS: Antimicrobial resistance, biosurfactants, biofilms, biomedical devices, medicinal plants, nanotechnology, silver nanoparticles.

INTRODUCTION

Plants used in medicine to treat and prevent illnesses are referred to as medicinal plants. In addition to being utilised in medicine, all or a portion of medicinal plants are also utilised as raw materials for the pharmaceutical industry, which has a variety of both medical and commercial applications. Particularly in the area of traditional medicine, which can be seen by Indian folk medicine and traditional Chinese medicine, where medicinal plants are primary source of natural medications that offer crucial health care services to the people of underdeveloped nations (Wang et al.,2023).

Endophytes are bacteria that are found inside medicinal plant tissues and they produce antimicrobial chemicals, compete with pathogens for resources and space, bring about systemic resistance, and change plant immune responses as part of plant defence systems. The genera *Bacillus, Pseudomonas,* and *Serratia* are common examples of endophytic bacteria, which are present in several phyla, including Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria (Hnamte et al.,2024). Recent studies have shown where an endophyte from Nile Papyrus leaf showed antibiofilm property on catheters that has biofilm formation of A. baumannii (Amer et al.,2023). Lipopeptides have shown antimicrobial properties from vetiver roots (Munakata et al,.2022). Rhamnolipid made by Pseudomonas aeruginosa has shown anti- cancer properties and anti-fungal properties (Thakur et al.,2021). Chemically synthesized surfactants are used by pharmaceutical, in environmental remediation and medicine but are harmful for the environment as well as human beings. It causes skin irritation; allergic reaction and it's also known to cause damage to fish gills and reduce dissolved oxygen in water bodies (Arora et al.,2022). Therefore, Biosurfactant was a game changer because given their biodegradability, low toxicity, and broad-spectrum antimicrobial activity, they hold great potential for biomedical applications such as wound healing, biofilm disruption, and targeted drug delivery (Fadiji et al.,2022).

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The integration of nanotechnology into antimicrobial research has opened new avenues for enhancing the efficacy of bioactive compounds. Silver nanoparticles (AgNPs) are known to be strong antimicrobial agents due to their unique physicochemical properties and multiple modes of action against microbial cells (Zhang et al.,2018). It has been shown that the aggregation and stabilisation processes can be greatly benefited from the biosurfactants that are made by microorganism. As a result, using biosurfactants has become a greener way to improve the synthesis and stabilisation of nanoparticles (Płaza et al., 2014).

It is commonly known that implanted medical devices are extensively used in the healthcare sector and have significantly benefited patient quality of life and the treatment of several diseases. They may, however, provide an ideal environment for the adsorption and formation of biofilms, which are a major contributor to healthcare infections. Implants quickly develop a layer that is made up of proteins, other organic compounds, and ions that attract bacteria when they come in contact with bodily fluids. Microbial cells attach to surfaces after deposition, creating small colonies that develop into communities in an exopolysaccharide matrix. Therefore, using biosurfactants as coating agents to stop biofilm development on medical equipment is one of its most interesting applications. They have several advantages, including improved wetting and decreased surface tension, which restricts microbial adherence. They may be used to build thin, consistent coatings on a variety of surfaces. Surface coating approaches (physical adsorption, bulk incorporation, or covalent grafting) have been developed and used for a variety of materials as a result of constant research and improvements in biosurfactant synthesis and formulation procedures. Additionally, biosurfactant-based coatings serve as good substitute for traditional synthetic coatings due to their superior biocompatibility and biodegradability (Ceresa et al.,2023).

Hence, we aim to isolate endophytic bacteria from four different medicinal plants that have the ability to produce biosurfactants along with green synthesis of silver nanoparticles and look for its anti-biofilm and antibacterial property, which can be a potential biomedical application.

MATERIALS AND METHODS

1. Chemicals and Reagents:

Throughout our laboratory experiment, chemicals were used from LobaChemie and Sisco Research Laboratories Pvt Ltd.

2. Collection of Plants

Leaf samples from three different plants namely Amla (*Phyllanthus acidus*), Brahmi (*Bacopa monnieri*) and Ajwain (*Trachyspermum ammi*) were collected from the suburban region of Mumbai and looked for biosurfactant-producing bacterial endophytes (Figure 1). For isolation of endophytes, fresh and healthy leaf samples of each plant were collected, stored in plastic containers, and taken to the laboratory on the same day of collection for further processing.

Fig 1A: Amla Leaf

Fig 1: Collection of Medicinal Plant

Fig 1C: Ajwain Leaf

3. Isolation of Endophytes

Two methods were used for the isolation of endophytes, firstly, leaf segments were directly placed on the media plate and secondly, they were homogenized and streaked onto the media plate.

In the first method, samples were surface sterilized, and leaf segments were placed directly on the media described by Chaudhari and Powar, 2023. Briefly, the leaf samples were thoroughly washed under tap water followed by distilled water, and air dried inside the laminar. All washed leaves were cut into 5 cm segments that were surface sterilized by sequential immersion in 70% ethanol

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(v/v) for 30 sec, 0.1% Mercuric chloride (v/v) for 30 seconds, and then 70% ethanol (v/v) for an additional 30 sec. Finally, they were washed twice in sterile distilled water to remove residual sterilant and then left to dry under the laminar airflow cabinet until dry. The plant segment was aseptically cut using sterile surgical scalpels and was placed on Trypticase Soy Agar (TSA) plates, ensuring direct contact of the cut edges with the culture media.

In the second method described by Yu et al., 2022, after surface sterilization, 2g of leaf sample were homogenized with 5 ml of sterile distilled water. Serial dilution was performed from 10^{-1}, 10^{-2}, and 10^{-3}. Then, 0.1 ml of diluted homogenate was spread-plated on the TSA medium. Once both methods were performed, the Petri plates were incubated at Room Temperature for 3 days. Biochemical Tests were performed to identify the bacteria.

4. Screening for Biosurfactant Production

Endophytes obtained were screened for biosurfactant production after the preparation of cell-free supernatant (CFS) using the methods described by *Amer et al*., 2023. Flasks were then incubated at Room Temperature for 4 days. CFS was prepared by centrifuging at 5,000 rpm for 40 min. They were then screened for their biosurfactant activity using the drop collapse, oil displacement, and emulsification assays described below.

4.1 Drop Collapse Assay

First, a drop of CFS (20µl) was placed on an oil-layered slide. The spread or collapse of the drop is observed within 15 mins.

4.2 Oil Displacement Test

Approximately, 25ml of distilled water was added in a petri plate followed by which, a thin layer of castor oil was added. CFS was then added at the centre of the oil. Uninoculated TSB was used as a negative control. A clear zone after 30 secs indicates oil displacement activity.

4.3 Emulsification Test

Castor Oil and CFS were combined in 1:1 ratio and then vortexed for 2 minutes. It was then left to stand for 24 hours. The emulsification activity (%EI24) was calculated according to the following formula:

$$\%EI24 = \frac{(\text{Height of the Emulsion})}{(\text{Total height of Solution})} \times 100$$

5. Extraction of Crude Biosurfactant from Endophytic Bacteria

As described by Desai et al.,2022, with some modifications, extraction of biosurfactant was performed. Endophytes obtained were grown in 10 ml of TSB broth and were incubated under static conditions for 5 days. The culture broth was then centrifuged at 5000 rpm for 40 mins. The supernatant obtained was then acidified using 1N HCL and was adjusted to pH2 and kept overnight at 4°C. Next day, 3 ml of chloroform: methanol (2:1), was added to the supernatant and kept for 30 mins for phase separation, followed by centrifugation at 5000 rpm for 40 mins. The supernatant was collected and kept for evaporation. It was then used for further experimentation.

6. Synthesis of Biosurfactant-stabilized Nanoparticles

Nanoparticles were synthesized as described in Guzman et al., 2020 with some modification. 2 ml of 0.6mM Ascorbic acid and 2ml of Biosurfactant extracted was added and pH was set to 9 with 0.1M NaOH with continuous stirring. Next, 0.4 ml of 1mM of silver nitrate (AgNO3) was added and kept on heating at 60-65°C for 1 hour till colour develops.

7. Characterization of BS-AgNPs

The formation and stability of nanoparticles will be observed through UV-vis Spectrophotometer from 200-600nm to obtain λmax.

8. Anti Biofilm Assay

The efficacy of the biosurfactant mediated synthesis of nanoparticles in eradicating established biofilms was performed with slight modifications, described by *Amer et al*.,2023. 100µl of overnight culture of *Pseudomonas aeruginosa* were added to 96-well microtiter plates to form biofilms. After overnight incubation at 37°C, cells were removed by washing the wells thrice with PBS. The biofilm formed were then treated with 200µl of BS-AgNPs. Sterile PBS was used as the negative control. The 96 well plate was then incubated at 37°C for 24 h, after which the supernatants were discarded, and wells were washed thrice with PBS. The residual biofilms were quantified using Crystal Violet (CV) staining as described by O'Toole G. A. (2011). 125µl of 0.1% crystal

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violet solution was added in each test well and incubated at room temperature for 15-20 mins. The plate was then washed thrice with distilled water and was blot dried using tissue paper to get rid of excess cells and any dye present. The plate was then turned upside down and was allowed to dry for 45 mins. Once the plate was dry, 125µl of 30% acetic acid was added to each well to solubilize the dye. The plates were incubated for 10-15 mins and 125µl of solubilized crystal violet was transferred to a new 96 well plate. The absorbance was then taken at 630nm (Aka and Haji, 2015). The calculation for percent eradication is as follows,

$$\textbf{Biofilm Eradication Percentage (\%)} = \frac{(\text{ODCV Control} - \text{ODCV Test})}{\text{ODCV Control}} \times 100$$

Where, $ODCV_{control}$ is the control reading and $ODCV_{test}$ is the absorbance of the treated biofilm.

9. Antibacterial Activity

The BS-AgNPs prepared were also looked for its antimicrobial property and its modified protocol of Hossain et al.,2022 well diffusion agar assay was used. Two bacteria were chosen, Pseudomonas aeruginosa and Staphylococcus aureus which are Gram negative and Gram positive respectively. The bacterial culture was grown in LB broth with an $OD_{.600}$ of 0.5, which was in accordance to McFarland Standard. 100µL of culture was added to sterile MH Agar to create a lawn of bacteria using spread plate method. Four well were created using 9mm cork borer in which 110µL BS-NPs were added along with Azithromycin as positive control and distilled water as a negative control. The plates were than incubated at 37 °C overnight. The inhibition zones around the well were then measured.

RESULTS

1. Isolation of Endophytes and its Biochemical Tests

Four leaf samples were collected from the Suburban region of Mumbai and five bacterial isolates were obtained. One isolate was found from the Amla tree leaf, three isolates from the Brahmi leaf, and one from Ajwain (Figure 2.). Morphological and cultural characteristics were documented as follows, mentioned in Table 1. Pure cultures were obtained by subculturing them on TSA plates which were kept at 4°C for further tests. After screening for the production of biosurfactants, biochemical tests were performed to identify the bacterial isolates. Through partial identification, the most probable organism for AB and AjB is *Bacillus sp*. while for BY and BW, the most probable organism is *Staphylococcus saprophyticus* and *Staphylococcus epidermidis* respectively with reference to Bergey's Manual of Systematic Bacteriology (Table 2,3,4, and 5).

Fig 2A: Amla plate

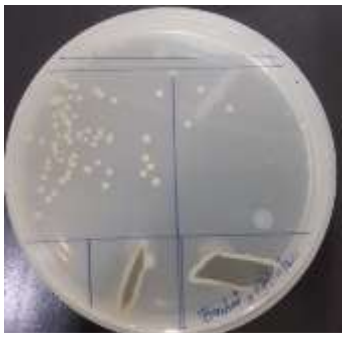

Fig 2B: Brahmi plate

Fig 2C: Ajwain plate

Fig 2. Isolation of Endophytic

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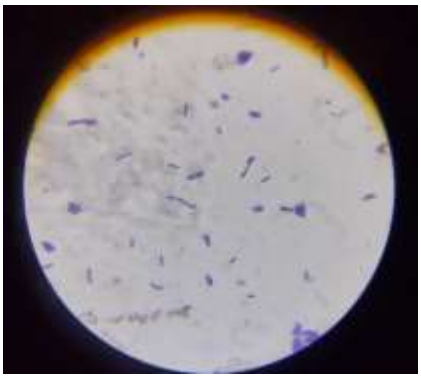
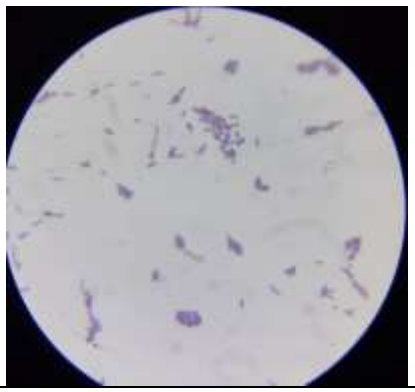
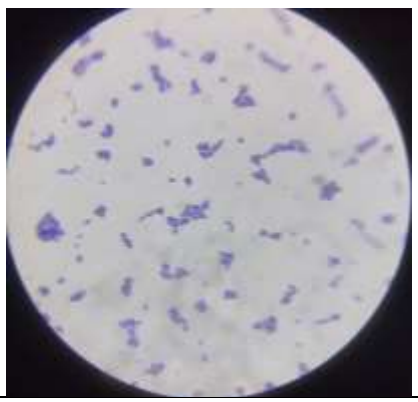
Table 1. Morphological and Cultural Characteristics

Isolates and code name.	Colony Characteristics on TSA	Morphological Characteristics	Picture
Amla Leaf "AB"	Round, entire, flat, Smooth, Translucent, pale colonies	Gram positive short rods in pairs and chain.	
Brahmi Leaf "BB"	Round, entire, flat, Smooth, Translucent, pale colonies.	Gram positive short rods in pairs and clusters.	
Brahmi Leaf "BY"	Round, Entire, Smooth, pin point yellow colonies.	Gram positive cocci in clusters.	

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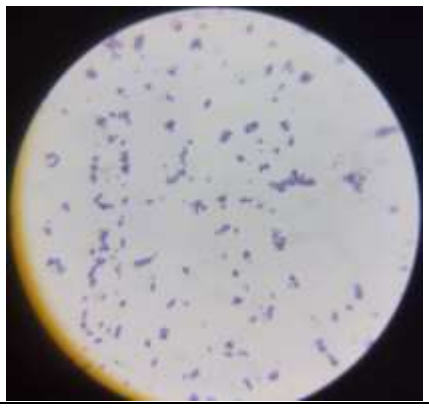
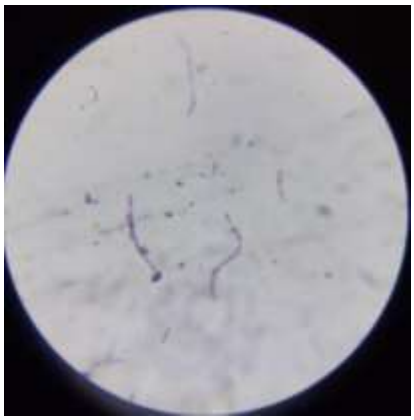
Brahmi Leaf "BW"	Round, Entire, Smooth, white colonies.	Gram positive cocci in singles and clusters.	
Ajwain Leaf "AjB"	Round, Entire, dry, opaque.	Gram positive bacilli in chains showing terminal ends.	

Table: 2

Biochemical Test	Results
Catalase	+
Indole	-
Methyl Red	-
Voges-Proskauer	+
Citrate	+
Urease	-
Gelatinase	+
Glucose	+
Sorbitol	+

Table 2: shows biochemical tests for AB bacilli.

Table: 3

Biochemical Test	Results
Catalase	+
Indole	-
Methyl Red	-
Voges-Proskauer	+
Citrate	+
Urease	-
Gelatinase	+
Glucose	+
Sorbitol	+

Table 3: shows biochemical tests for AjB bacilli.

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Table: 4

Biochemical Tests	Results
Catalase	+
Coagulase	-
Indole	-
Methyl Red	-
Voges-Proskauer	+
Citrate	+
Urease	-
Gelatinase	-
Glucose	+
Mannitol	+

Table 4: shows biochemical tests for BY cocci.

Table: 5

Biochemical Tests	Results
Catalase	+
Coagulase	-
Indole	-
Methyl Red	-
Voges-Proskauer	+
Citrate	-
Urease	-
Gelatinase	+
Glucose	+
Mannitol	-

Table 5: shows biochemical tests for BW cocci.

Key: (+) = Positive Test, (-) = Negative Test

2. Screening Test of Endophyte

From the five CFS, four of them namely AB, BY, BW and AJB were found to be positive when the drop collapsed within 5 mins (Figure 2). Out of all the isolates, BY showed the highest displacement activity (Figure 3). The emulsification index showed positive for all test isolates with AB showing the highest emulsifying activity (Figure 4, Table 6).

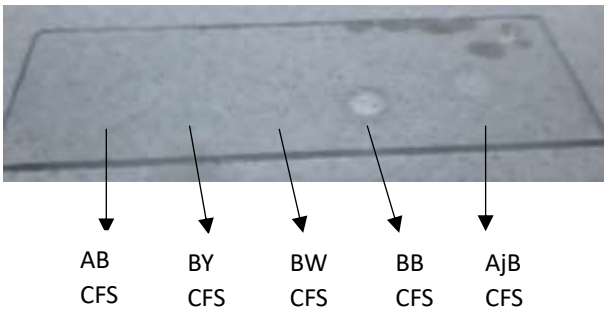

Fig. 2 shows the drop collapse test. Out of five samples, four were positive.

Fig.3 shows Oil Displacement Test for all five endophytes.

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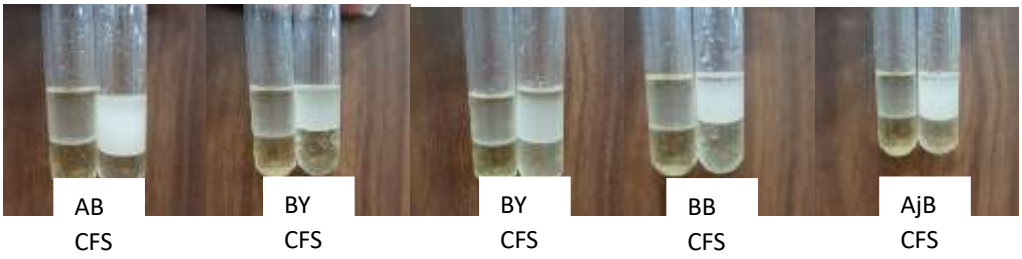

Fig.4 shows the Emulsification Test.

Table 6: shows the Emulsification Index.

Emulsification Layer	Total Solution	%EI24
1.8	2.8	64.285
1.4	3.5	40
1.5	3.4	44.117
1.3	3.3	39.393
1.5	3	50

3. Synthesis of nanoparticles and UV- Visible Spectrophotometry Analysis

A colour change confirms the synthesis of nanoparticles (Figure 5). The synthesis can be confirmed by the maximum absorbance peak between 400 and 500 nm due to surface Plasmon resonance (Tyagi et al.,2020). The maximum peaks observed were 420 nm for AB-AgNPs, BY-AgNPs, and 400 nm for BW-AgNPs and AjB-AgNPs (Figure 6).

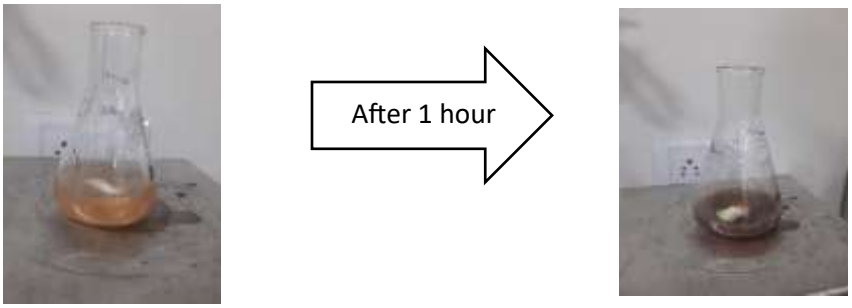

Fig.5 shows the synthesis of nanoparticles where at 5 mins (5A) showed reddish color and after 1 hour showed dark brown color (5B).

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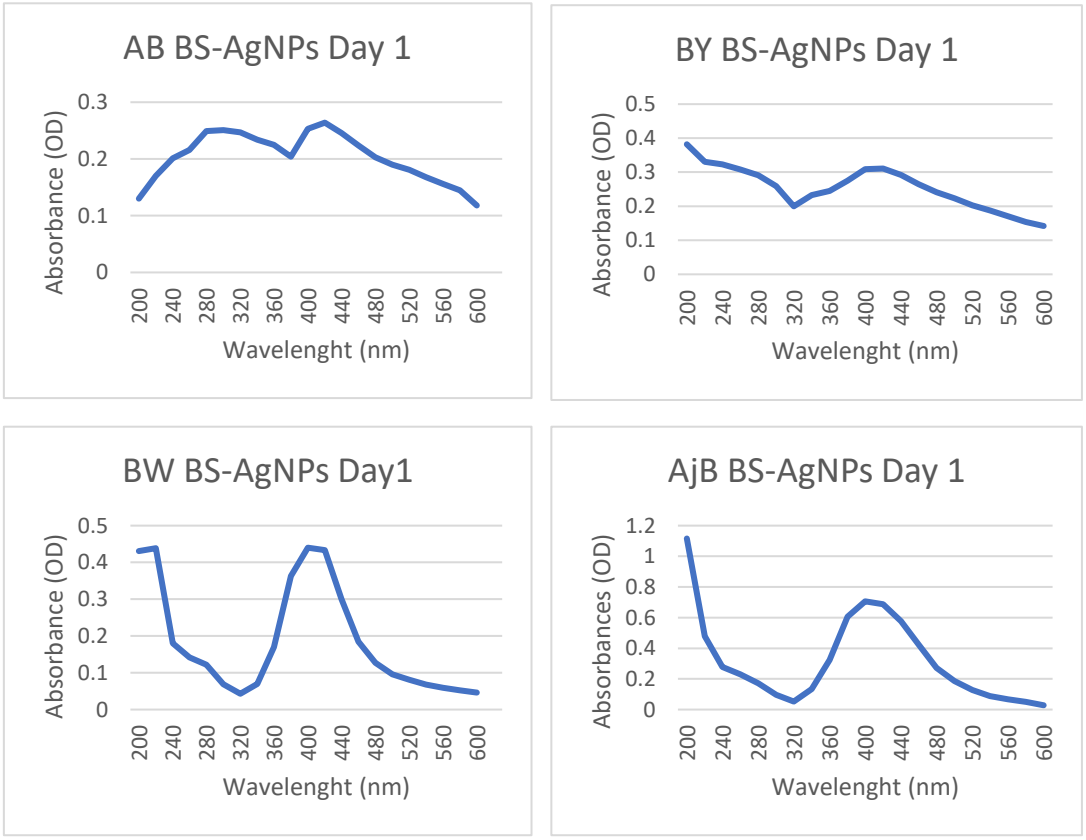

Fig. 6 shows the synthesis of nanoparticles on Day 1 with its absorbance peaks.

A broad and widening of peak shows that the nanoparticles are polydispersed (Ashraf et al., 2016). The initial high optical density reading might be due to precursor reducing molecules or proteins and peptides that might be present with biosurfactants extracted (Tyagi et al.,2020). The stability of the nanoparticles was observed for 14 days. The highest stability was seen in AjB BS-AgNPs, while others were fairly stable, as seen in Figure 7.

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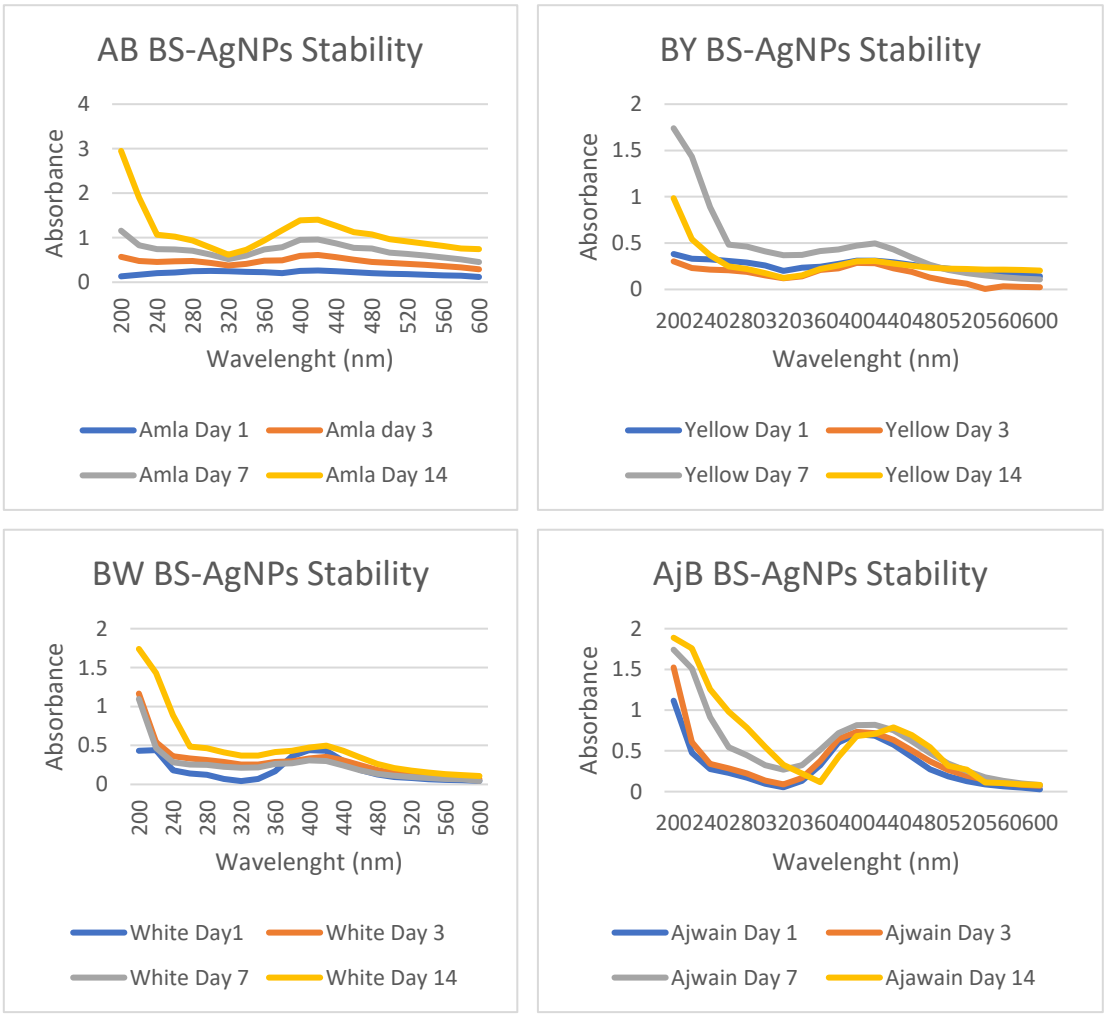

Fig.7 shows the stability of four BS-Ag nanoparticles across fourteen days.

4. Antibiofilm Analysis

The four BS-AgNPs were subjected to an antibiofilm test to determine their efficacy in the ability to eradicate biofilms secreted by test organisms. All four synthesized nanoparticles showed a 40% to 60% reduction in biofilm formation. BY-made BS-AgNPs showed the highest antibiofilm activity, at 63% (Figure 8).

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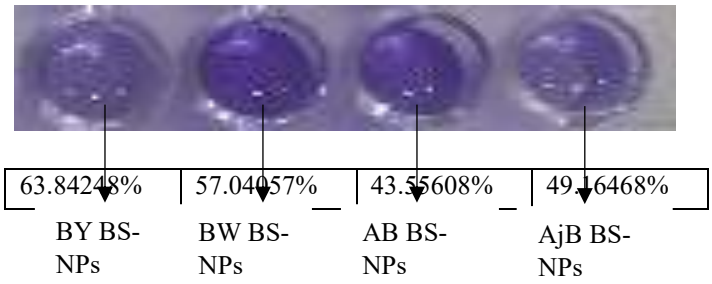
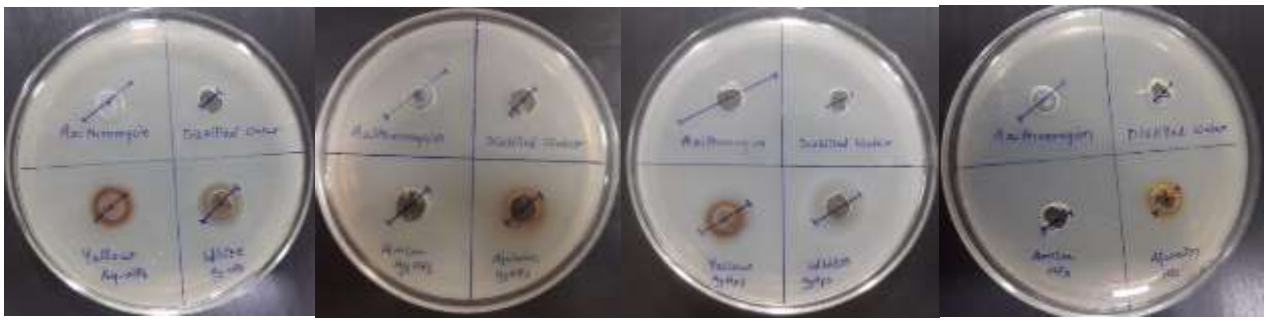


Fig.8 shows the crystal violet staining and Inhibition of Biofilm (%)

5. Antibacterial Analysis

The four BS-AgNPs were tested against *Staphylococcus aureus* Gram-positive bacteria and *Pseudomonas aeruginosa,* Gram-negative bacteria (Figure 9). The highest inhibition of 12 mm and 18 mm against Gram-positive and Gram-negative was seen by BY synthesized nanoparticles, followed by BW, and similar results were seen for AjB and AB. Refer to Table 7.



Against *Pseudomonas aeruginosa* Against *Staphylococcus aureus*

Fig.9 Plate Results of Antibacterial Activity of BS-AgNPs.

Table.7 Results of Antibacterial Activity

Test Organism	Zone of Inhibition (mm)					
	Azithromycin	Distilled water	Yellow BS-Ag	White BS-Ag	Amla BS-Ag	Ajwain BS-Ag
Pseudomonas aeruginosa	29mm	9mm	18mm	17mm	16mm	14mm
Staphylococcus aureus	18mm	9mm	12mm	16mm	9mm	12mm

CONCLUSION

Biosurfactants made by four endophytic bacteria have shown promising antimicrobial properties, particularly against bacterial biofilms. The endophyte "BY" showed the highest effectiveness, followed by "AjB". The incorporation of nanoparticles offers potential new approaches to antimicrobial strategies. While these results are encouraging, further research is required in order to optimise their production, assess their safety, such as the level of toxicity, conduct in vivo and ex vivo studies, and explore their potential applications in biomedical and pharmaceutical industry.

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DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article.

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