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Oil-Contaminated Soil Bacteria: Biosurfactant-Mediated ZnO NP Synthesis, Characterization and Biological Activity

Ashitha K.* and Vanitha N.

Department of Microbiology, Hindusthan College of Arts & Science, Coimbatore 641028, Tamil Nadu, India

**Corresponding Author*

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Abstract: The study focuses on the synthesis of ZnO nanoparticles using a biosurfactant extract from *Pseudomonas stutzeri*, a microorganism that produces biosurfactants from soil contaminated with oil. The isolated microorganisms were identified through molecular identification of 16s rRNA gene sequencing and characterization, and their biosurfactant production was determined using various assays such as hemolysis techniques, emulsification index, oil spreading tests, and drop-collapsing assays. The biosurfactant-synthesized ZnO nanoparticles were characterized using various biophysical techniques, including UV-Vis, FTIR, XRD, SEM, and EDAX. The biosurfactant-synthesized ZnO were found to be effective against bacterial and fungal pathogens, including *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Aspergillus niger*, *Aspergillus flavus*, and *Fusarium oxysporum*. Moreover, these nanoparticles showed *in vitro* anticancer efficacy against lung cancer cells (A549) and breast cancer (MCF-7). Based on the dose-dependent suppression of cancer cell growth, the MTT experiment demonstrated that biosurfactant-synthesized ZnO NPs cause toxicity. It was discovered that treating lung and breast cancer cells with increasing concentrations of biosurfactant-synthesized ZnO NPs inhibited their proliferation and migration. Furthermore, biosurfactant-synthesized ZnO NPs caused significant nuclear alterations as well as a reduction in mitochondrial membrane potential in lung and breast cancer cells. The study concludes that the use of biosurfactant-synthesized ZnO NPs in suitable materials is safer for the environment and improves antimicrobial and anti-cancer activity at low concentrations.

Keywords: *Pseudomonas stutzeri*, Biosurfactant, Zinc nanoparticles, Antimicrobial activity; *In-vitro* cytotoxicity

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Introduction

Nanoparticles have attracted considerable attention due to their distinctive physiochemical properties, permitting them to develop a combination with other groups owing to their charged surface (Randive *et al.*, 2023; Abdelrahman *et al.*, 2024). Zinc nanoparticles (ZnONPs) have gained significant attention in recent years due to their distinct properties,

including their small size and broad surface area. These characteristics make them highly suitable for a wide range of applications in fields such as biomedicine, electronics, and agriculture (Gomaa, 2022). ZnO nanoparticles have been employed in a wide range of applications, including drugs, cosmetics, photocatalysis, and animal dietary supplements (Ong *et al.*, 2018).

Zinc oxide nanoparticles (ZnO NPs) may be produced using chemical, physical, and biological techniques. The chemical techniques used include microemulsion, reduction, sol-gel hydrothermal, and precipitation. The physical approaches mentioned in the study by Król *et al.* (2017) include spray pyrolysis, vapor deposition, plasma, and ultrasonic irradiation. However, these approaches often involve the use of toxic substances, high temperatures and pressures, and expensive equipments (Agarwal Menon *et al.*, 2018). Therefore, it is essential to use a cost-efficient and eco-friendly approach to produce nanoparticles.

The biological method has great potential as an alternative to nanoparticle production. This approach is preferable to other strategies because of its safety, non-toxicity, eco-friendliness, biocompatibility, and cost-effectiveness. The process entails the use of biologically active components, either from microorganisms or extracted from plant parts, to carry out ZnO NPs (Khalid *et al.*, 2017). Microbes have a greater advantage than plants in terms of their ability to reproduce, microbes being more suitable for synthesizing ZnO-NPs. Recently the use of microorganisms such as bacteria, actinomycetes, fungi, and yeast has attracted significant attention in the synthesis of ZnO-NPs (Murali *et al.*, 2023).

There has been a significant increase in the number of research studies on biosurfactants over the past decade. There is an increasing enthusiasm for biosurfactants due to the growing demand from industries for more eco-friendly processes and products. Plants and microorganisms commonly produce biosurfactants, also known as biologically active chemicals, natural surfactants, and renewable surfactants. They have proven to be highly effective in a wide range of applications (Kamalesh, 2024). Microorganisms such as bacteria, yeast, and fungus produce biosurfactants, also known as microbial surfactants, as secondary metabolites during biotechnological processes (Vecino *et al.*, 2021). The use of surfactants in liquid solutions aids in the solubilization and mobilization of contaminants, thereby facilitating bacterial recovery and reuse of these substances (Kumari *et al.*, 2018). Biosurfactants are highly valued for their ionic character, non-toxicity, high emulsifying ability, interfacial properties, and environmental compatibility, which makes them good choices for a lot of different uses (Pşaza and Achal, 2020).

As nanotechnology advances, biosurfactants have the potential to be a key factor in promoting responsible and sustainable use. Additionally, their ability to act as stabilizing agents has led to their recognition as an environmentally friendly alternative for nanoparticle synthesis and stabilization. Biosurfactants have greatly enhanced the production of high-performance nanomaterials. These substances have the unique ability to easily form different liquids in aqueous solutions (Chandana *et al.*, 2017; Kamalesh, 2024). According to researchers, there have been reports of a biological approach that involves using biosurfactants, specifically rhamnolipids derived from *Pseudomonas aeruginosa*. Researchers conducted their analysis and found that zinc nanoparticles mediated by biosurfactants exhibit remarkable antioxidant properties, making them highly promising and preferred in the field. The method of production described in this study relies on an economical and eco-friendly approach for synthesizing nanoparticles (Singh *et al.*, 2014).

Hazra *et al.* (2013) demonstrated the use of rhamnolipid biosurfactant caps in the modification of zinc sulfide (ZnS) nanoparticles. They also explained their structural composition, biological effects, effectiveness against cancer, and capability as nanophotocatalysts for degrading textile azo dyes. The manufacture of zinc nanoparticles used

pure rhamnolipid as a capping ingredient. ZnS nanoparticles significantly removed brown textile colors, and encapsulation with biosurfactants reduced their toxicity. Biosurfactants made by *Pseudomonas spp.* are different from biosurfactants made by other bacteria because they have better physicochemical properties, are less toxic, break down quickly, and have a high yield (Soberón-Chávez *et al.*, 2021). Biosurfactants generated by *P. aeruginosa* have many biological activities, such as antiviral, anticancer, and antibiotic effects (Kashif *et al.*, 2022; Chauhan *et al.*, 2023). These findings suggest its potential use as a preventative and/or therapeutic adjuvant. Narayanan *et al.* (2010) used water-soluble rhamnolipids generated by *Pseudomonas spp.* to encapsulate ZnS NP. They specifically focused on the metal affinity, shape, and optical characteristics. These substances exhibited excellent stability and high solubility in water, and they were easily accessible in an aqueous solution without the need for any organic solvent. Scientists have determined that the coating of zinc nanoparticles with biosurfactants holds significant promise for the development of the next generation of nanoparticles (Kamalesh, 2024).

The present investigation largely focuses on a bacterial strain found in soil samples that can produce biosurfactants. Furthermore, we attempted to develop and evaluate a biosurfactant by an environmentally friendly method that relies on ZnO nanoparticles. This research aims to investigate the prospective applications of biopotentials, specifically focusing on studying their antibacterial and anticancer actions.

Materials and Methods

Sample Collection:

Oil-contaminated (petrol and diesel) soil samples were collected from a petrol pump station in Coimbatore, Tamil Nādu, India. The sample was meticulously collected using a sterile spatula and placed in an air-tight container. After being transported to the laboratory, the sample was subjected to further analysis.

Isolation of bacterial strains:

The ability of microorganisms to degrade and utilize hydrocarbons as a carbon source has been well-documented. The stock solution was prepared by adding 1 g of oil spilled soil to 10 ml of 0.85% sterile saline (Bordoloi and Konwar, 2008). 0.1 ml of the solution was removed from the stock and added to 0.9 ml of sterile distilled water, resulting in $1/10^{th}$ of the initial concentration of microorganisms (Rabah *et al.*, 2010). This process of aliquoting and resuspension continued until the final tube was reached, diluting the stock concentration by a factor of 10. 0.1 ml from $10^{-2,}$ $10^{-3,}$ and 10^{-4} serial dilution tubes were transferred to nutrient agar plates with 2% crude oil and incubated for 24 h at 37 °C. After confirming the growth, distinct colonies were taken from plates for each sample and enriched in Mineral Salt Medium (MSM) at 37°C in a shaker incubator (200 rpm) for 7 days. After incubation, the culture broth from each sample was centrifuged at 6000 rpm at 4 °C for 15 min and the supernatant was filtered using Millipore filter paper. The Cell-free supernatants were screened for the production of biosurfactants (Nayarisseri *et al.*, 2018)

Screening of biosurfactant (*BS*) *producers*:

The bacterial strains were evaluated using blood hemolysis techniques followed by Rajesh *et al.* (2017). An analysis was conducted to determine the strain's capacity to produce biosurfactants. Other screening methods were employed, such as the emulsification index, oil spreading test, and drop collapsing test. The experiments were carried out on agar plates coated with oil, as detailed in the corresponding studies (Sohail *et al.*, 2019; Bami *et al.*, 2020)

Identification of bacterial isolates:

The amplification of the 16S rRNA gene from bacterial genomic DNA was accomplished by using the widely used primers 27F and 1492R. Following a short PCR run at a temperature of 94°C, a sequence of 35 cycles consisting of denaturation, annealing, and extension steps were

performed. The specimen was maintained at a temperature of 4°C. A 50 µl solution was created for PCR amplification, and the molecular weights of a particular product were assessed using 1% agarose electrophoresis. The amplified products were examined using a biosystem 3500 Genetic analyzer for sequencing. Subsequently, the obtained sequence was cross-referenced with the GenBank database to ascertain the specific organism of origin (Rivas *et al.*, 2007). This research used NCBI Blast to examine the nucleotide sequence and establish the principal analysis. We use the BioEdit Sequence Alignment Editor and MEGA11 software to validate and rearrange the sequences. The bacterial phylogenetic study was performed using the MEGA 11 program. MEGA's latest iteration, Version 11, offers a wide range of approaches and tools to cater to the changing needs of academics. The inclusion of evolutionary dating techniques in MEGA streamlines the process of estimating the periods at which species and strains diverged. Enhancing the accuracy of acquired information may be achieved by meticulous calibration of the nodes and fine-tuning the sample timings (Tamura *et al.*, 2021).

Biosurfactant (*BS*) *extraction*:

Through the process of bacterial species exploration, the culture broth was carefully collected and subsequently subjected to centrifugation. This allowed for the extraction of a cell-free supernatant, which is known to contain a valuable biosurfactant. The supernatant was acidified to pH 2 using 0.1M HCl and left undisturbed overnight at 4°C. The supernatant underwent centrifugation at 10,000 rpm at 4°C to separate the biosurfactant which precipitated by acid, resulting in a pellet. The crude biosurfactant has been treated using a specific ratio of Chloroform and Methanol (2:1). Following the treatment, the biosurfactant underwent another round of centrifugation to obtain a purified biosurfactant precipitate. This process has been described by Tabatabaee *et al.* (2005), Batool *et al.* (2017), and Abu-Ruwaida *et al.* (1991). The biosurfactant was dried and stored in a phosphate buffer solution (PBS) (Sohail *et al*., 2019).

Synthesis of zinc oxide nanoparticles using biosurfactant:

A two-step approach was employed to synthesize ZnO nanoparticles. In brief, a bacterial biosurfactant was mixed with a 0.1 M aqueous solution of Zinc acetate. The mixture was stirred constantly for 10 min at room temperature and then subjected to autoclaving at 121°C under 15 lbs pressure for 30 min. The transformation from a clear state to a pale white particle indicates the formation of ZnO NPs. The mixture was processed by centrifugation at 10,000 rpm for 10 min at room temperature. The supernatant was discarded and the pale white particle was dried in an oven set at 60°C.

Biosynthesized n-ZnO characterization:

Through the analysis of the reaction mixture, the morphological and physicochemical attributes of the ZnO NPs synthesized by the biosurfactant were confirmed using UV-visible spectra. Additional analysis was carried out utilizing techniques such as FESEM and EDAX. The phase purity of the ZnO nanoparticles synthesized using biosurfactant was determined through X-ray diffraction (XRD) analysis. Furthermore, a previous study (Ramimoghadam *et al.*, 2013) employed FTIR spectroscopy to examine the particle size and the functional groups that were present.

Antibacterial effect of ZnO NPs:

We assessed the antibacterial properties of the ZnO nanoparticles facilitated by the biosurfactant. The agar well-diffusion method was used to evaluate the efficacy of ZnONPs against different bacterial strains, such as *E. coli*, *K. pneumonia*, and *Salmonella typhi*. To conduct the antibacterial assay, we utilized pure cultures of each bacterium which was cultivated in a nutrient broth medium. After collecting these cultures, they were meticulously spread onto various agar plates using a sterile glass L-shaped rod. Precise holes, with a diameter of 5 mm, were meticulously formed in

nutrient agar plates using a borer. A biological synthesis method was used to incorporate 20 µl of zinc oxide nanoparticles (ZnO NPs) into the wells of all media plates. After 24 h of incubation at a temperature of 28±2°C, it was observed for bacterial growth inhibition zone (Azam *et al.*, 2012). To make comparisons, a disc with Amoxyclav antibiotic as a positive control and DMSO was used as a negative control, in addition to our synthesized nanomaterials.

Antifungal activity:

The antifungal study was evaluated using the well-diffusion method. To prepare the Petri plate, malt extract agar was poured into it. After solidification, 80 µl of fungal spores from *Aspergillus niger, Aspergillus flavus*, and *Fusarium oxysporum* were evenly spread using a sterile cotton swab. Wells were formed using a cork borer, and the addition of ZnO NPs was facilitated by the biosurfactant. An antibiotic (F-Fluconazole) was used as a positive control, while DMSO was used as a negative control. The plates were placed in an incubator at 30°C for 3-5 days. After incubation, the measurement of the inhibition zone was recorded in millimeters.

Anticancer activity of biosurfactant-mediated ZnO NPs as a potential tool against human cancer cell line:

Cell culture:

The human cancer cells (A549-Lung cancer and MCF-7- Breast cancer) were obtained from the National Center for Cell Sciences (NCCS), Pune, India. The cancer cells used in the study were cultured in a specific medium called Dulbecco's modified eagles' medium (DMEM). This medium was supplemented with various components including l-glutamine, balanced salt solution (BSS), Na_2CO_3, nonessential amino acids, sodium pyruvate, glucose, HEPES, and fetal bovine serum. These components were carefully adjusted to create an optimal environment for the cancer cells to grow and thrive. Penicillin and streptomycin were adjusted to a concentration of 100 IU/100 µg per 1 ml. The cells were kept at 37°C in a CO_2-rich environment with proper humidity.

Evaluation of cytotoxicity:

An MTT assay was done to evaluate the inhibitory concentration (IC50) value. The A549-Lung cancer and MCF-7-Breast cancer cells were cultured in a 96-well plate with a density of 1×10^4 cells per well. The cells were allowed to grow for 48 h until they reached 80% confluence. A fresh medium was used to replace the existing one, which contained serially diluted biosurfactant-mediated ZnO NPs. The cells were then incubated for an additional 48 h. The culture medium was removed, and 100 µl of the MTT solution was added to each well and incubated at 37°C for 4 h. Following the removal of the supernatant, a 50 µl volume of DMSO was introduced to each well and allowed to incubate for 10 min, effectively dissolving the formazan crystals. The optical density was measured at 620 nm using an ELISA multiwell plate reader from Thermo Multiskan EX, USA. The OD value was used to calculate the percentage of viability using the following formula.

% of viability = OD of experimental sample/OD of experimental control ×100

Morphological Study:

The Human cancer cells chosen for the experiment were cultivated on coverslips at a density of 1×10^5 cells per slip. They were exposed to biosurfactant-mediated ZnONPs at varying concentrations (10 µg/ml, 25 µg/ml, 50 µg/ml) for 24 h. Following this, the cells were fixed using an ethanol: acetic acid solution in a ratio of 3:1 (v/v). The coverslips were carefully placed on glass slides for the morphometric analysis. Micrographs were taken of three monolayers in each experimental group. The cells' morphological changes were analyzed using Nikon's bright field inverted light microscopy at a magnification of 10x.

Fluorescence microscopic analysis of apoptotic cell death:

1 µl of the dye mixture, consisting of acridine orange and ethidium bromide dissolved in distilled water, was combined with a cell

suspension on microscope coverslips. The cancer cells were treated with various concentrations of ZnO nanoparticles, which were facilitated by a biosurfactant. After treatment, the cells were collected, washed with phosphate-buffered saline (pH 7.2), and stained with AO/EtBr. Following a 2-min incubation period, the cells were washed twice with PBS before being observed using a fluorescence microscope (Nikon Eclipse, Inc., Japan) at a magnification of 40x. The visualization was done using a 580 nm excitation filter. In addition, the cells were carefully positioned on a glass coverslip within a six-well plate and exposed to varying concentrations of biosurfactant-mediated ZnO NPs for 24 h. The cells were treated with 0.2% triton X-100 (50µl) to make them permeable. This was done for 10 min at room temperature. Afterwards, a coverslip was placed over the cells and 10 µl of DAPI was added. The coverslip helped to ensure that the stain spread evenly. The cells were observed using a fluorescent microscope from Nikon Eclipse Inc. Japan.

Results and Discussion

Isolation, screening, and identification studies for selected strains:

The results obtained indicate that a pure strain was successfully isolated from soil contaminated with oil (Fig. 1). The production capacity of biosurfactants by the isolated bacterial strain was evaluated using well-established screening methods. These methods included hemolysis, emulsification index, oil spreading test, and drop collapsing test. The selected bacterial strains exhibited favorable outcomes for all the above-mentioned tests, as shown in Table 1.

Determining hemolytic activity is a widely accepted method for screening biosurfactant production (Nogueira *et al.*, 2011). Similarly, a study was carried out by Abouseoud (2007) to investigate the biosurfactant activity of *Pseudomonas fluorescence*. Various tests, such as the emulsification assay, emulsification index, and hydrocarbon layered assay, can provide valuable insights into the presence of biosurfactants. It is worth mentioning that these tests may not always align with surface activity. The collapse and spreading assay rely on the destabilization of droplets due to reduced interfacial tension in the presence of surfactants and surfactant activity (Walter *et al.*, 2010). Previous studies conducted by Shoeb *et al.* (2015) and Rath *et al.* (2016) have also observed the presence of the genus *Pseudomonas* in petroleum-contaminated environments, demonstrating its impressive emulsifying activity, foam forming activity, and excellent surface tension activity. Sanuj and Vanitha (2024) discovered comparable findings. They were able to identify *Pseudomonas stutzeri*, bacteria that produce biosurfactants, from soil samples that were contaminated with petrol and diesel.

Molecular Identification of Isolated Bacteria:

The bacterial isolate was identified using 16s rRNA sequencing. One of the most effective methods for identifying species and determining evolutionary relationships is 16s rRNA sequencing. 16s rRNA contains both conserved and variable regions. The variable sections are particularly intriguing in terms of identification, while the conserved portions play a crucial role in determining the evolutionary history of the species. A common method to determine the identity of an organism is by analyzing its small subunit ribosomal ribonucleic acid (ribosomal SSU). This component is found in all bacteria and exhibits unique variations that are specific to each bacterial species. The bacteria were identified as *Pseudomonas stutzeri* strain AVMB2 via sequencing. The strain *P. stutzeri* was identified as the most efficient producer of biosurfactants among the strains tested. According to the phylogenetic tree, it appears that the 16s rRNA sequence is closely related to *Pseudomonas stutzeri* strain AVMB2 (Fig. 2). The results were analyzed using the BLAST tool and the final alignment was carefully checked and aligned using MEGA11 software. Shekhar *et al.* (2019) identified *Pseudomonas stutzeri* as the sources of biosurfactants. The identification was performed

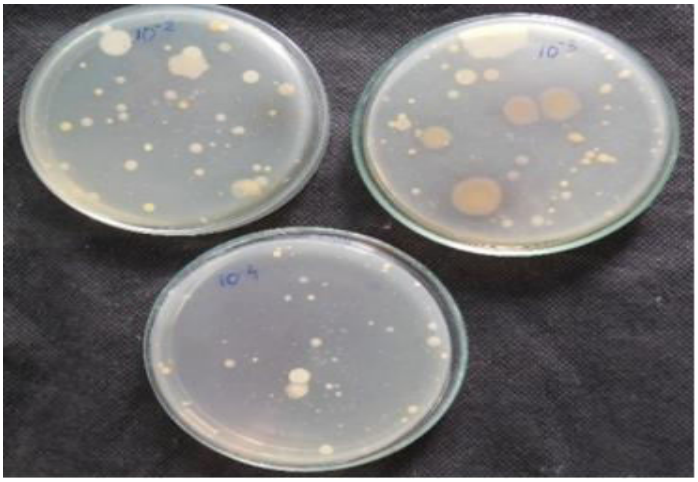


Fig. 1: Cultivation of bacterial strain on nutrient Agar Plates.

Table 1: Preliminary test for screening of isolated bacteria for biosurfactant production ability

Strain	Hemolysis activity	Emulsification index (EI)	Oil spreading test (CM)	Drop collapsing test
Bacterial strain	+	6.5	3.5	+

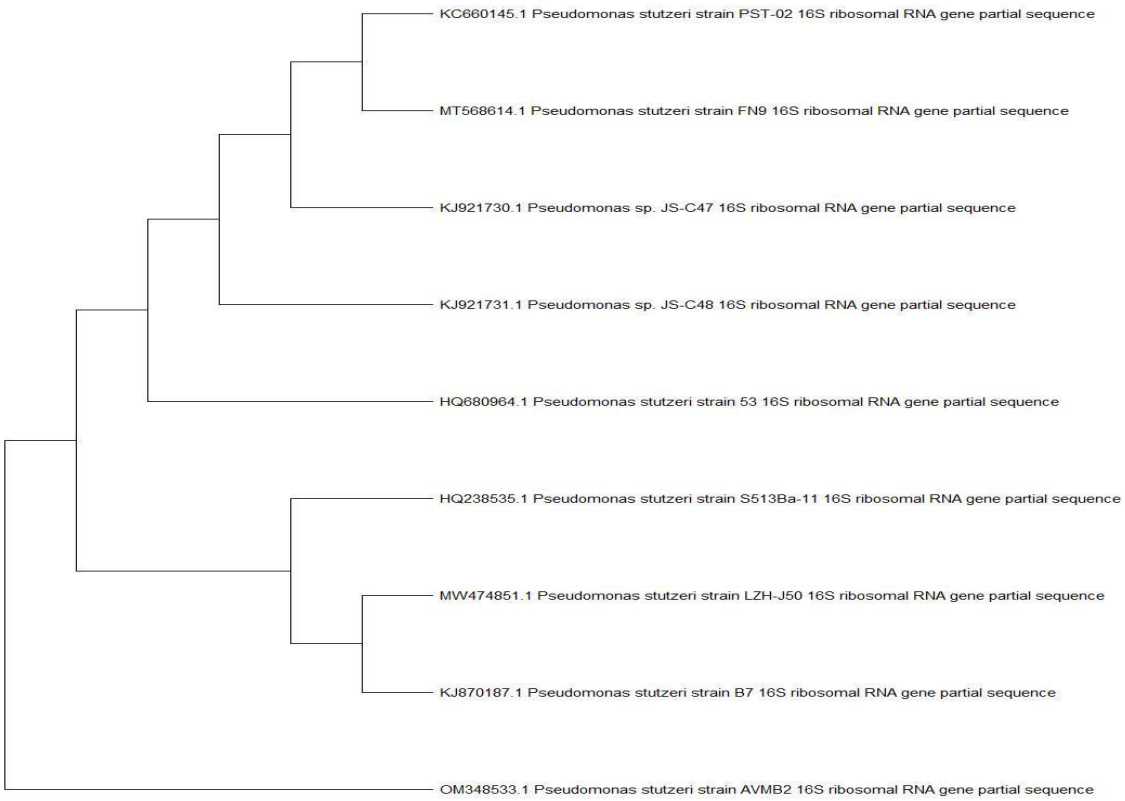
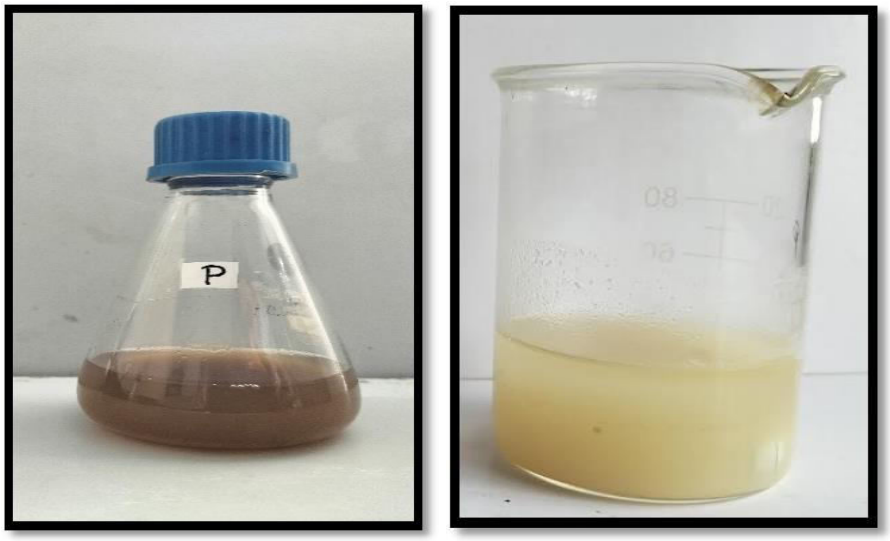


Fig. 2: The phylogenetic tree of Pseudomonas Stutzeri and related strains based on 16S rRNA sequences, was constructed by the neighbor-joining method using MEGA11.



(3) (4)

Fig. 3: Extraction of Biosurfactant using *Pseudomonas stutzeri.*

Fig. 4: Extraction of biosurfactant synthesized ZnO NPs.

using 16S rRNA gene sequencing. In previous studies, researchers such as Mohd Kamil *et al.* (2012) and Yalaoui *et al.* (2018) discovered a strain of *Pseudomonas stutzeri* (HS-D36) (PS2) in petroleum sludge and crude oil-contaminated soil. This particular strain was found to have the ability to produce biosurfactant, which can effectively enhance the biodegradation of phenanthrene.

Extraction of Biosurfactant using Pseudomonas stutzeri:

Investigating Biosurfactant Production:

To produce biosurfactants, we used the bacterial strain *Pseudomonas stutzeri* into a broth with 0.5% crude oil as the sole carbon source. The soup was placed on a rotatory shaker for incubation. After the centrifugation process, the liquid remaining above was collected. To assess its surface activity, the pH of the liquid was adjusted to 2.0. Afterwards, an equal amount of a 2:1 ratio of chloroform to methanol was introduced into the mixture. The colored biosurfactant was clearly visible (Fig. 3). The biosurfactant produced was subsequently dried and stored for future use.

Synthesis of zinc oxide nanoparticles using biosurfactant Characterization:

The characterization of the extraction of ZnO nanoparticles synthesized by biosurfactant was conducted using a range of techniques including UV-Vis FTIR, XRD, SEM, and EDAX analysis. The process included the bio-reduction of zinc acetate using a biosurfactant derived from the *Pseudomonas stutzeri* bacterial strain. Figure 4 shows an apparent change in the color of the substance, turning it into a pale-yellow white shade. The observed color change clearly indicated the formation of ZnO nanoparticles, providing a visual demonstration of their biogenesis.

UV–Visible Spectroscopy:

Using a UV-vis spectrophotometer, a peak at approximately 340 nm was observed during the analysis of biosurfactant-synthesized ZnO nanoparticles. Based on the symmetrical structure of the band, it can be inferred that the particles are

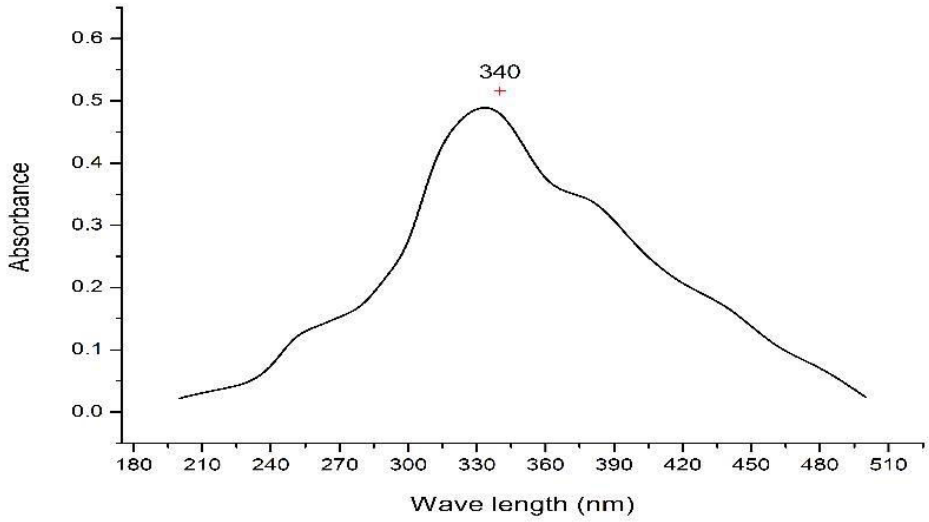


Fig. 5: UV-visible spectrum **of** biosurfactant synthesized ZnO nanoparticles.

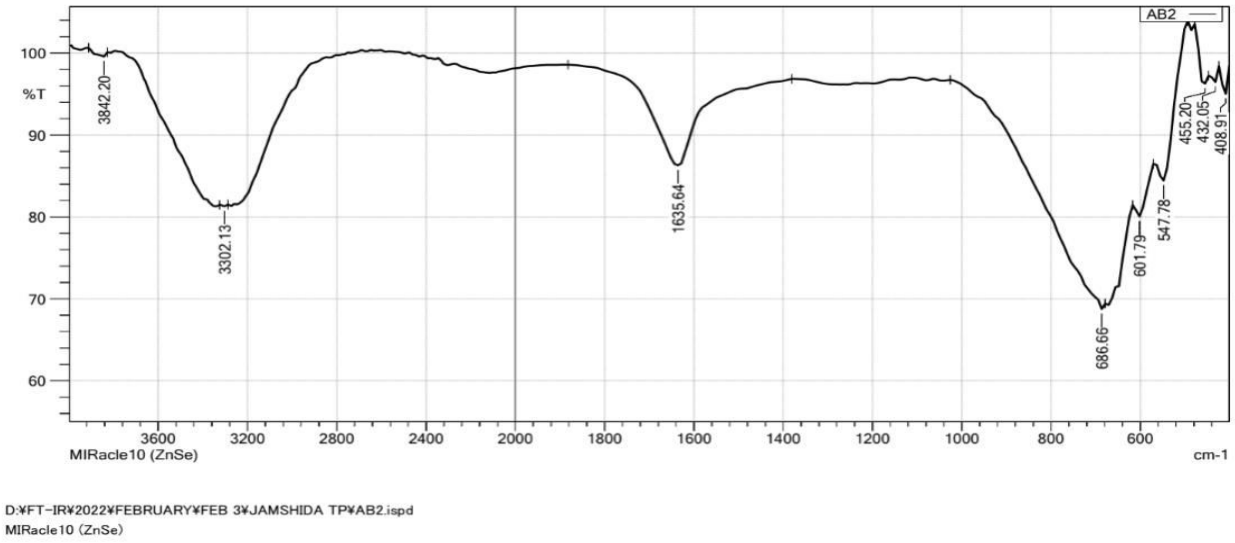


Fig. 6: Fourier transform infrared spectroscopy analysis of biosurfactant synthesized ZnO nanoparticles.

evenly distributed, providing evidence for their existence and stability (Fig. 5). This aligns well with the findings of previous studies conducted by Selvarajan and Mohanasrinivasan (2013), as well as Jayaseelan *et al.* (2012). A study by Samanta and Chaudhuri (2011) showed the absorption peak was observed to shift towards a shorter wavelength in comparison to the bulk ZnO, where the absorption was maximum at 373 nm. In a previous study, Narayanan *et al.* (2010) discovered a significant absorption peak at a wavelength of 340 nm for ZnS particles that had been coated with a biosurfactant.

FTIR studies:

The FTIR transmittance spectra of biosurfactant-synthesized ZnO NPs was shown in Figure 6. The exhibit displays distinct bands within the range of 600-4000 cm^{-1}, each with its specific peaks. The

FTIR values observed 3302.13 cm^{-1}, 1635.34 cm^{-1}, and 686.66 cm^{-1}. The values are 1,601.79 cm^{-1}, 547.78 cm^{-1}, 455.20 cm^{-1}, 432.05 cm^{-1}, and 408.91 cm^{-1}. The FTIR spectrum of synthesized ZnO NPs shows a range of bands from 4000-600 cm^{-1}, which provide information about the functional groups present in ZnO NPs (Das *et al.*, 2012; Rajiv *et al.*, 2013). As an example, a significant and wide range is observed at 3302.13 cm^{-1}. These indicate the presence of alcohol compounds. The 1635.34 cm^{-1} and 686.66 cm^{-1} peaks are indicative of the stretching of alkene C=C bonds, with the former being medium and the latter being strong. The compound obtained in biosurfactant-synthesized ZnO NPs shows a peak at 601.79 cm^{-1} and 547.78 cm^{-1}, suggesting the presence of C-I and C-Br stretch. The peaks observed in this study exhibited a unique combination of properties, highlighting the significance of functional groups in the biosynthesis of ZnO nanoparticles (Kundu *et al.*, 2014). Achieving the fabrication of inorganic nanoparticles on biological scaffolds, whether metallic or semiconducting, has been made possible by selectively reducing metal ions to a metallic form in the presence of biomolecules (Fang *et al.*, 2011).

X-ray diffraction studies:

Through X-ray diffraction analysis, the biosurfactant-synthesized ZnO NPs were carefully examined to determine their phase and crystalline structure. Interestingly, a distinct and characteristic diffraction pattern was observed, as shown in Figure 7. The ZnO nanoparticles synthesized using biosurfactant displayed distinct peaks at specific 2θ values. These values corresponded to various (hkl) diffraction planes, including 100, 002, 101, 102, 110, 103, 200, 112, 201, 004, and 202. The XRD peaks showed a strong resemblance to the hexagonal phase (wurtzite structure) when compared to the data from JCPDS card No. 89-7102 (Kundu *et al.*, 2014). The XRD pattern revealed distinct diffraction line peaks, suggesting the excellent crystallinity of the biosynthesized ZnO NPs.

SEM Analysis:

Scanning Electron Microscopy (SEM) was used to examine the morphological features of ZnO nanoparticles synthesized by biosurfactants and identify their precise shape. The SEM analysis shows the formation of semi-spherical nano-particles with sizes ranging from 30-50 nm. The particles appear to be highly agglomerated, as shown in Figure 8. Rajaboopathi and Thambidurai (2019) observed the formation of agglomerated spherical structures during the synthesis of Ag/ZnO nanoparticles using bio-surfactants. The SEM analysis supports the observation that the ZnO nanoparticles have a spherical shape with smooth edges, which aligns with earlier research (Elumala *et al.*, 2015; Gandhi *et al.*, 2017).

Energy Dispersive X-ray Analysis (*EDX*) *Spectrum of ZnO NPs*:

A study was conducted to determine the identification and quantification of elements in the biosurfactant-synthesized ZnO nanoparticles. This analysis relied on energy-dispersive X-ray Spectroscopy, which detects the specific X-ray energies emitted by these elements. Based on the analysis, it was found that the ZnO nanoparticles contained zinc (Zn) and oxygen (O) atoms, as shown in Figure 9. These findings align with a previously published study that examined the composition of biosynthesized ZnO-NPs and found correlations between O and Zn composition (Zhang *et al.*, 2002; Dutta *et al.*, 2012; Vimala *et al.*, 2014).

Antibacterial assay:

Nanoparticles of ZnO were synthesized using a biosurfactant and then subjected to testing against *E. coli*, *K. pneumoniae*, and *S. typhi* on a nutrient agar medium at specific doses. After conducting comprehensive studies, it was discovered that the use of 20 µl concentrations resulted in significantly stronger antibacterial effects. Figure 10 presents the results of the study, showing the presence of a zone of inhibition against *E. coli, K. pneumoniae*, and *S. typhi*. The measured inhibitory zones for *E. coli*, *S. typhi*, and *K. pneumoniae* were 28 mm, 28 mm, and 26 mm, respectively (Table 2).

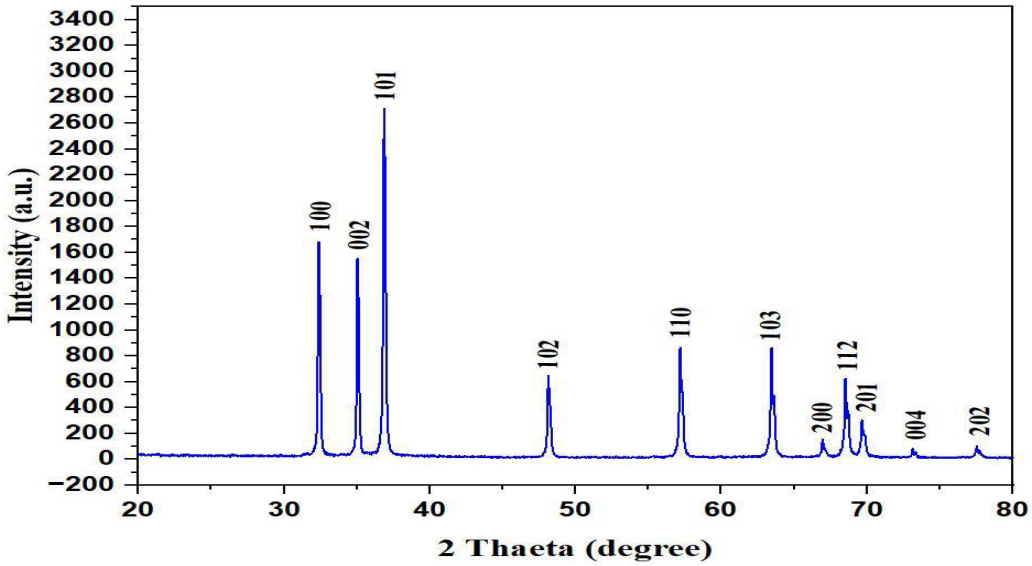


Fig. 7: XRD analysis of biosurfactant synthesized ZnO nanoparticles.

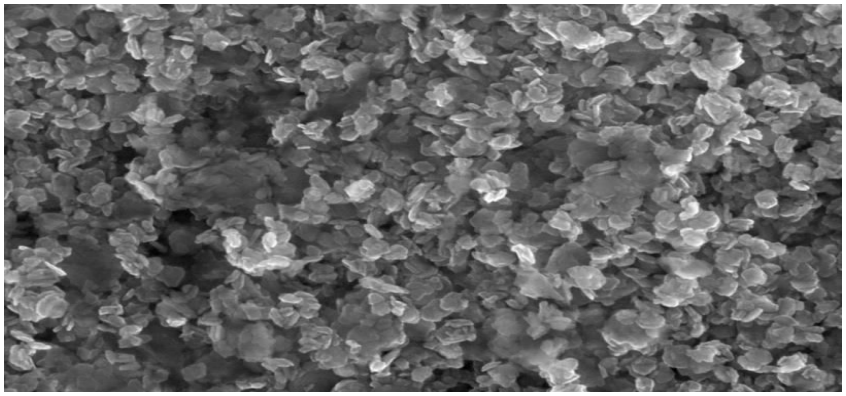


Fig. 8: Scanning electron microscopy of biosurfactant **s**ynthesized ZnO nanoparticles.

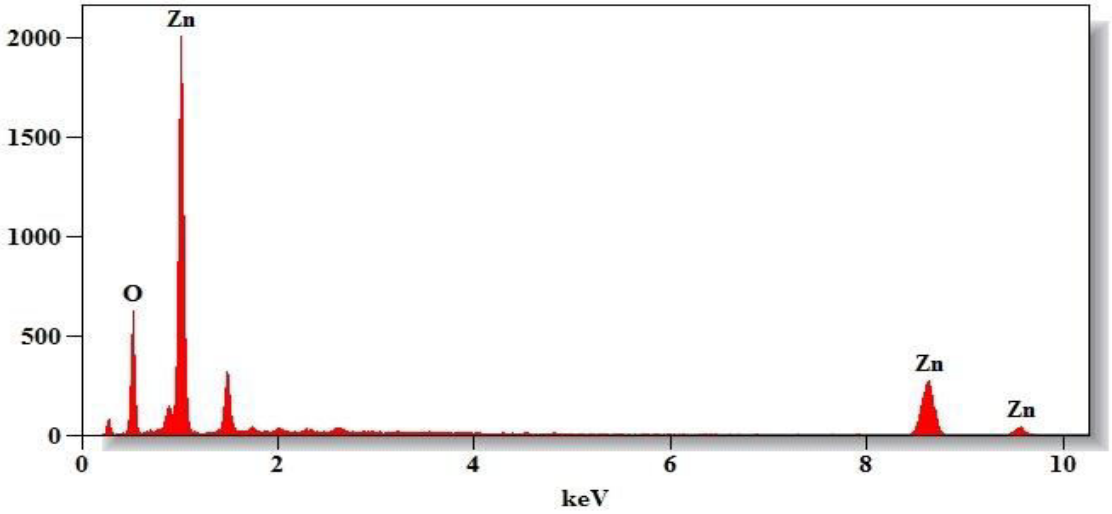


Fig. 9: Energy dispersive X-ray analysis of biosurfactant synthesized ZnO NPs.

Table 2: Inhibition zone generated by Biosurfactant synthesized ZnO nanoparticles against the bacteria *E. coli, K. pneumoniae,* and S. *typhi*

Name of the Organism	Zone of Inhibition (mm)		
	PZnP	Negative Control	Antibiotic Disc
Escherichia coli	28	Nil	16
Klebsiella pneumoniae	26	Nil	10
Salmonella typhi	28	Nil	11

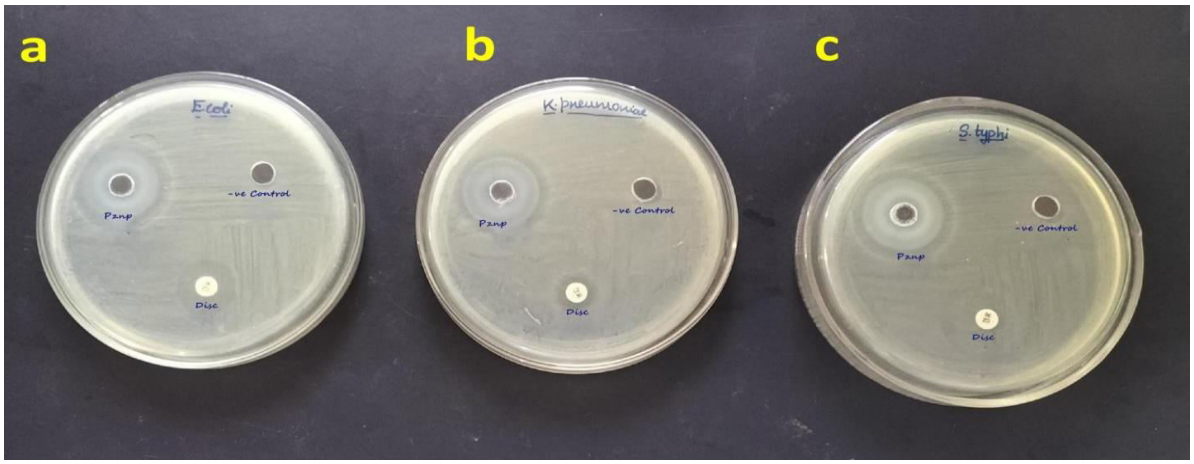


Fig. 10: Antibacterial activity of Biosurfactant synthesized ZnO nanoparticles against the tested microbial species (a) *E. coli,* (b) *K. pneumoniae,* (c) S. *typhi.*

Albukhaty (2020) discovered that ZnO nanoparticles exhibited greater efficacy against *E. coli* compared to *S. aureus*. Emami-Karvani and Chehrazi (2012) used disk diffusion and well diffusion agar methods to carry out their research. Yamamoto (2001) and Brayner *et al.* (2006) found that these substances have a strong ability to inhibit the growth of bacteria and prevent the cells from adhering to surfaces. As a result, they also play a role in preventing the formation of biofilm. In a recent study conducted by Malakar *et al.* (2021), it was found that at a concentration of 250 µg/ml, the use of rhamnolipid-coated zinc oxide nanoparticles resulted in a significant 80% inhibition of pathogen growth. This finding highlights the potential of these nanoparticles in combating pathogens. In addition, the findings of this study suggest that the antibacterial properties of ZnO nanoparticles synthesized using biosurfactant are enhanced against *E. coli*, *S. typhi*, and *K. pneumoniae* bacteria (Sanuj and Vanitha, 2024).

Antifungal assay:

The ZnO nanoparticles derived from biosurfactants demonstrated potent antifungal properties against various strains of fungi, including *A. niger*, *A. flavus*, and *F. oxysporum*. Based on the *in vitro* examination conducted in this study, it was found that disrupting the integrity of the membrane enables the test sample to be used as an antifungal agent. Figure 11 demonstrates the effectiveness of the test samples against *A. niger*, *A. flavus*, and *F. oxysporum*. From the results, ZnO nanoparticles produced through biosurfactant synthesis demonstrated promising antifungal properties against fungi that cause damage to cell membranes, including *A. niger*, *A. flavus*, and *F. oxysporum*. The inhibition zones observed were 18 mm, 15 mm, and 16 mm,

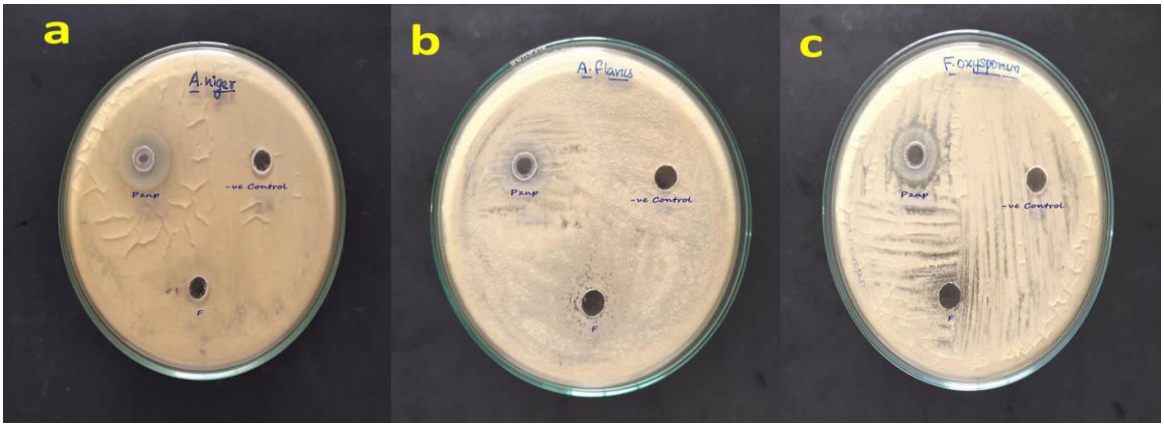


Fig. 11: Anti-fungal activity against Test Pathogens. (A) *Aspergillus niger*, (B) *Aspergillus flavus*, and (C) *Fusarium oxysporum.*

Table 3: Antifungal activity of Biosurfactantsynthesized ZnO nanoparticles against fungal pathogens (Zone of Inhibition)

Name of the Organism	Zone of Inhibition (mm)		
	PZnP	Negative Control	Fluconazole
Aspergillus niger	18	Nil	Nil
Aspergillus flavus	15	Nil	Nil
Fusarium oxysporum	16	Nil	Nil

respectively (Table 3).

Jayaseelan *et al.* (2012) examined the effectiveness of bacteria-assisted ZnO nano-particles in combating *A. flavus*, *A. niger*, and *C. albicans*. The results of the study showed that when the concentration of ZnO nanoparticles was 25 µg ml^{-1}, they were able to inhibit the growth of the mycelia. Sumanth *et al.* (2020), discovered that ZnO-NPs derived from *Xylaria acuta* extract showed promising antifungal properties. These nanoparticles were found to effectively inhibit the growth of *A. flavus*, *Cladosporium cladosporioides*, *Fusarium oxysporum*, and *Phomopsis sp*. The inhibition percentage of fungal mycelia increased with higher doses of the nanoparticles, indicating a dose-dependent relationship. In addition, Suba *et al.* (2021) found that ZnO nanoparticles produced from an extract of *Lactobacillus spp*. demonstrated significant antifungal properties. The nanoparticles exhibited a maximum inhibition zone of 18-21 mm against *A. flavus* and *C. albicans*.

Exploring the anticancer potential of biosurfactant synthesized ZnO NPs against human lung cancer A549 and breast cancer MCF-7 cells:

MTT assay:

We conducted an experiment on the effects of ZnO nanoparticles synthesized using biosurfactant on cells associated with lung and breast cancer. We also evaluated the potential harm caused by ZnO nanoparticles synthesized through biosurfactants. We exposed A-549 lung cancer and breast cancer MCF-7 cells to different concentrations of these nanoparticles (up to 100 µg/ml) for 24 h. To measure the cytotoxic effects, we employed the MTT assay. Based on this experiment, it is evident

Table 4: Cytotoxic activity of Samples (µg/ml)

Complex	A549 cells (IC_{50})	MCF-7 cells (IC_{50})
B-ZnONPs	22 ±0.4	35 ±1.5
Doxorubicin (Standard)	18 ±1.2	17±0.5

IC_{50} – Values of respective Compounds (at 24 h)

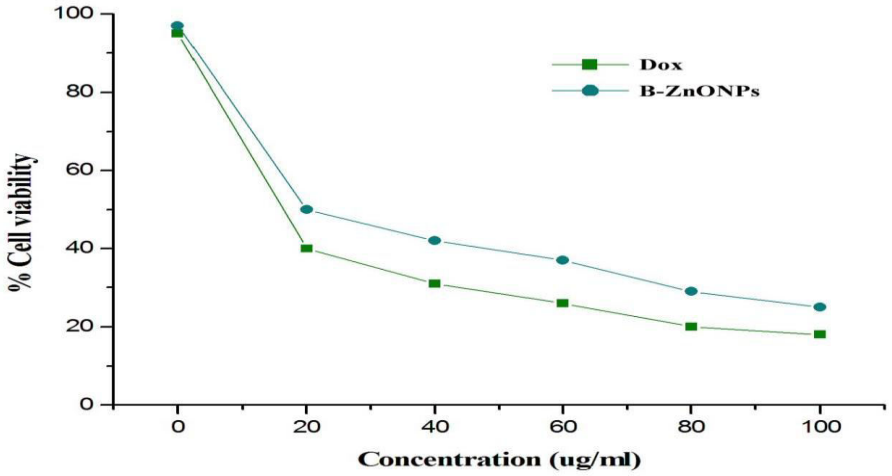


Fig. 12: MTT analysis of biosurfactant synthesized ZnO NPs (A549-lung cancer).

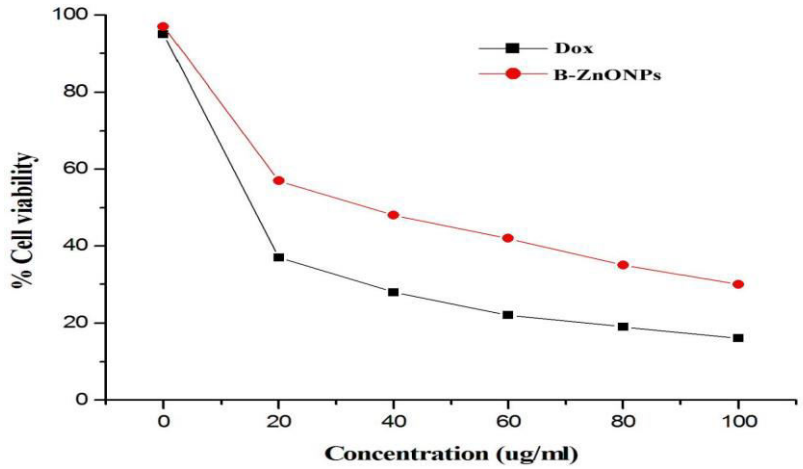


Fig. 13: MTT analysis of biosurfactant synthesized ZnO NPs (MCF-7 breast cancer).

that the biosurfactant-synthesized ZnO NPs (B-ZnONPs) had a significant impact on cell proliferation, with the inhibition being directly proportional to the dosage. The findings, including the IC_{50} value, are displayed in Table 4 and Figures 12 and 13. Figures 12 and 13 demonstrate the significant impact of biosurfactant-synthesized ZnO NPs on reducing the growth of specific lung and breast cancer cells. The IC_{50} values were found to be 22 and 35 µg/ml in lung and breast cancer

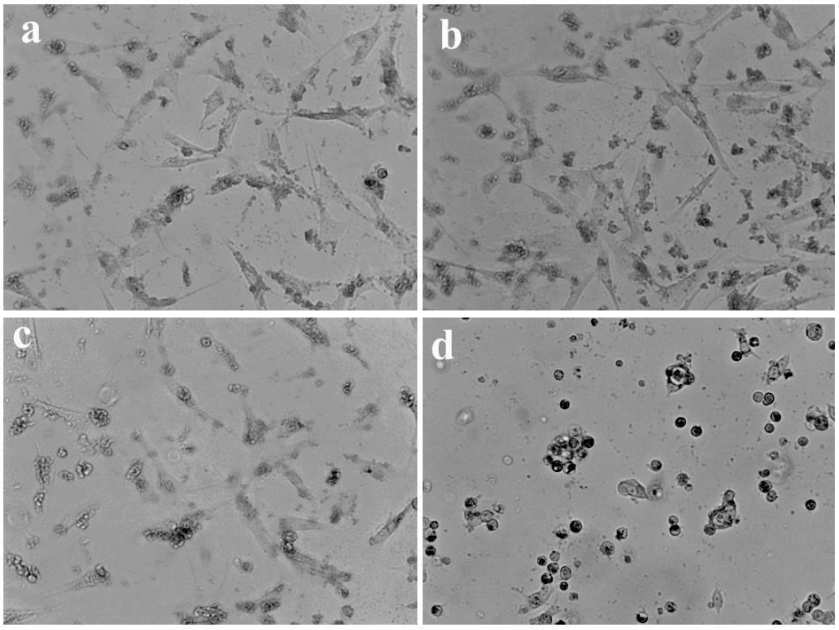


Fig. 14: Morphological analysis of biosurfactant synthesized ZnO NPs treated - A549 cells for 24 h (a) Control (b)10 µg/ml (c) 25 µg/ml (d) 50 µg/ml.

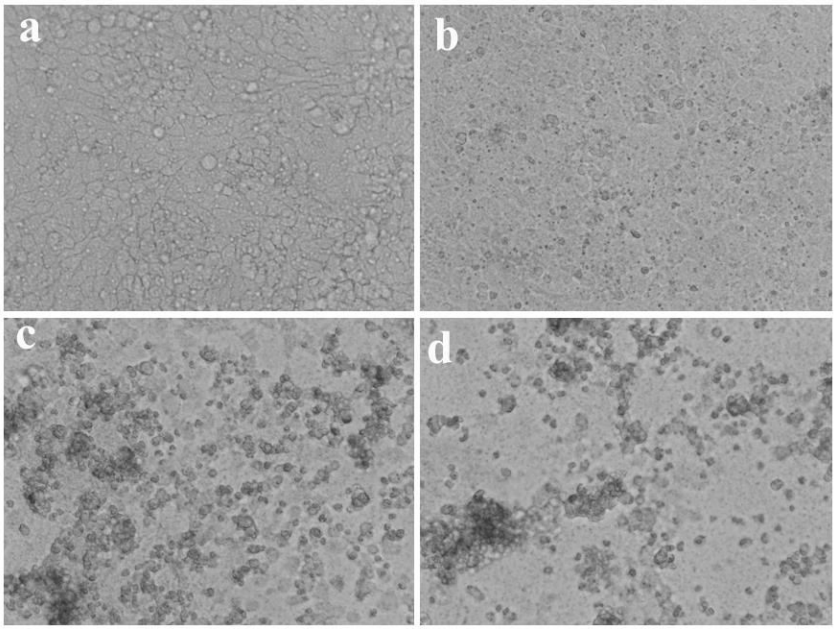


Fig. 15: Morphological analysis of biosurfactant synthesized ZnO NPs -treated MCF-7 cells for 24 h (a) Control (b)10 µg/ml (c) 25 µg/ml (d) 50 µg/ml.

cells, respectively. By contrast, doxorubicin (standard) exhibited IC_{50} values of 18 and 17 µg/ml in lung and breast cancer cells, respectively. The ZnO nanoparticles synthesized using biosurfactants revealed remarkable efficacy against specific cancer cells when compared to conventional treatment methods. Several research studies have provided evidence that ZnO nanoparticles can selectively target cancer cells without harming normal cell lines. This finding has been supported by multiple studies conducted by Umamaheswari *et al.* (2021), Mishra *et al.* (2023),

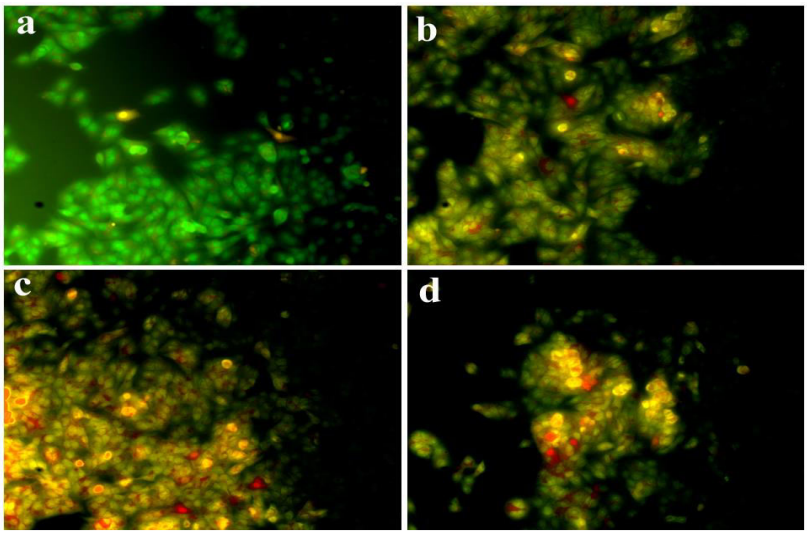


Fig. 16: AO/EtBr staining assay of biosurfactant synthesized ZnO NPs - treated A549 cells (a) Control (b) 10 µg/ml (c) 25 µg/ml (d) 50 µg/ml.

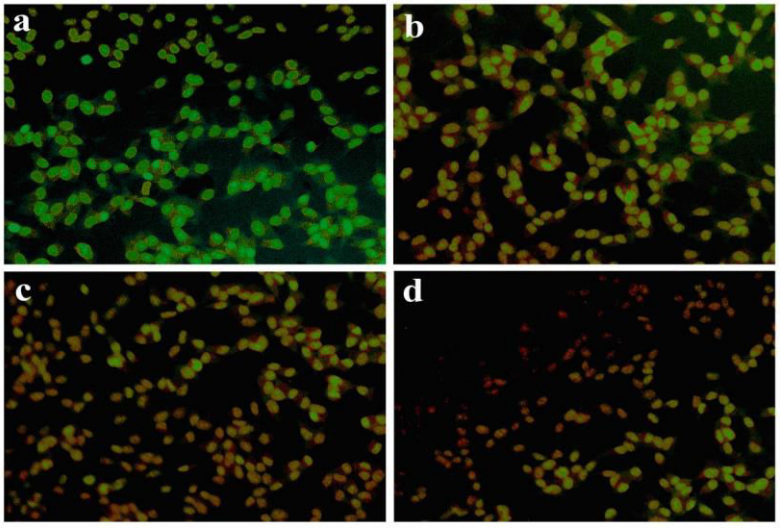


Fig. 17: AO/EtBr staining assay of biosurfactant synthesized ZnO NPs - treated MCF-7 cells (a) Control (b) 10 µg/ml (c) 25 µg/ml (d) 50 µg/ml.

and Nagajyothi *et al.* (2017). It was observed that the cell line's viability decreased as the concentration of NPs and standard drugs increased, which aligns with the findings of Valsalam *et al.* (2019). There is a growing interest in the use of microbe-mediated ZnO NPs as a potential anticancer therapy due to their low toxicity, surpassing chemically synthesized ZnO NPs (Gao *et al.*, 2019). Suba *et al.* (2021) found that the MTT test, which utilized biosynthesized ZnO-NPs from *Lactobacillus spp.* Extract demonstrated phenomenal biocompatibility and showed a potential toxic effect of 54.16 µg ml^{-1} in the human colon cancer (HT-29) cell line.

Morphological Analysis:

The A549 and MCF-7 cancer cells were exposed to the IC_{50} concentration of biosurfactant-synthesized ZnO NPs for 24 h, and demonstrated a significant alteration in their morphology (Figs.

14, 15). There was a noticeable change in the appearance of the treated cells, which varied depending on the dosage. As the concentrations of biosurfactant-synthesized ZnO NPs increased, there was a corresponding increase in cytotoxicity. The extract caused cellular shrinkage, membrane blebbing, reorganization, and a decrease in the total number of viable cells. These findings strongly indicate that the biosurfactant-synthesized ZnO NPs triggered apoptosis in A549 cells, as illustrated in Figures 14b, c, and d. Similarly, the MCF-7 cancer cells are shown in Figures 15b, c, and d. On the other hand, the control cells that were not treated, did not exhibit any negative impacts (Figs. 14a, 15a).

The selectivity of the biosynthesized ZnO NPs towards various cancer cell lines has been demonstrated in multiple studies (Ahamed *et al.*, 2012; Bisht and Rayamajhi, 2016; Rajeshkumar *et al.*, 2018), as our findings have revealed. The biosynthesized ZnO NPs have the potential to disrupt cellular membranes, create vacuoles, and alter the metabolism of cancer cells, thereby triggering the apoptosis pathway and effectively eliminating cancer cells. In addition to the changes in cellular and nuclear morphology, the presence of ZnO NPs leads to the release of intracellular zinc ions and the subsequent production of reactive oxygen species (ROS) (Bisht and Rayamajhi, 2016). In a previous study, Bansal *et al.* (2019) successfully combined a newly developed biosurfactant from *C. parapsilosis* with graphene quantum dots that were adorned with folic acid, serving as a targeting ligand. The bioconjugate demonstrated significantly greater efficacy in inhibiting the growth of MCF-7 breast cancer cells compared to the biosurfactant alone.

Fluorescence microscopic analysis-- AO/EtBr and DAPI staining for nuclear fragmentation:

The apoptogenic effects of biosurfactant-synthesized ZnO NPs were studied on selected cancer cells using fluorescence microscopy. A concentration of biosurfactant-synthesized ZnO NPs was used to treat the A549 and MCF-7 cells. A fluorescence microscopy technique was employed to evaluate the activation of apoptosis by examining the cells that were stained with acridine orange/ethidium bromide (AO/EtBr). Based on the observations, it was found that live cells emitted a vibrant green fluorescence, whereas dead cells emitted a distinct acridine orange fluorescence. Figures 16a and 17a show an unusual abundance of viable cells in the untreated control cells. On the other hand, the A549 and MCF-7 cells treated with ZnO NPs synthesized using biosurfactants indicated an increased presence of cells undergoing apoptosis and the formation of apoptotic bodies. These were distinguishable by their unique characteristics, including nuclear shrinkage, nuclear damage, and the development of blebs, which appeared as bodies emitting an orange/red coloration (Figs. 16, 17b, c, d). It appears that the treatment caused apoptotic changes in both the A549 and MCF-7 cells, resulting in noticeable alterations that suggest apoptosis when examined.

Further, we evaluated the effectiveness of biosurfactant-synthesized ZnO NPs through DAPI staining. Figures 18 and 19 provide a visual representation of the fluorescence microscopic images of cells stained with DAPI. These images were taken after 24 hours, with and without the presence of biosurfactant-synthesized ZnO NPs. It is worth mentioning that the cells treated with biosurfactant-synthesized ZnO NPs exhibited distinct characteristics. In both A549 and MCF-7 cells, bright spots were observed, indicating the presence of clumped chromatins and fragmented nuclei (Figs. 18 b, c, d for A549 cells, and Figs. 19 b, c, d for MCF-7 cells). On the other hand**,** Figures 18a and 19a do not exhibit any significant alterations in the cells. From the fluorescence microscopic analysis, it is evident that the biosurfactant synthesized ZnO NPs show great promise as a therapeutic agent in the treatment of cancer. The study's results reveal a noteworthy suppression of growth in the A549 and MCF-7 cancer cell lines due to the biosurfactant-synthesized ZnO NPs. These findings align with previous research exploring the impact of nanoparticles on cell death in various cell types.

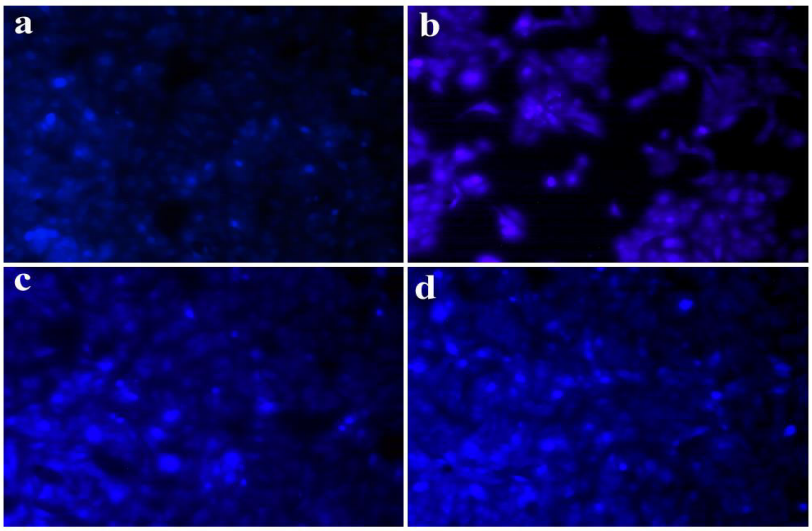


Fig. 18: DAPI staining assay of biosurfactant synthesized ZnO NPs - treated A549 cells (a) Control (b) 10 µg/ml (c) 25 µg/ml (d) 50 µg/ml.

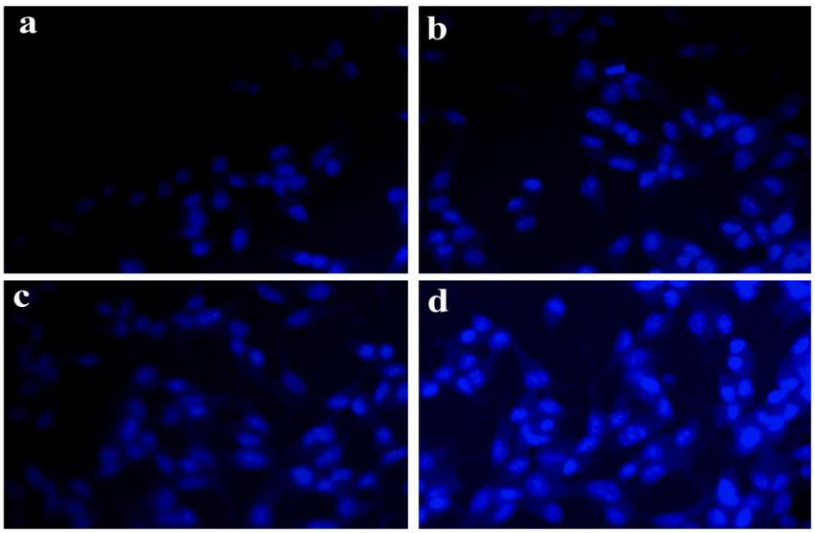


Fig. 19: DAPI staining assay of biosurfactant synthesized ZnO NPs - treated MCF-7 cells (a) Control (b) 10 µg/ml (c) 25 µg/ml (d) 50 µg/ml.

The researchers focused on investigating the process of apoptosis in human cervical cancer cells (HeLa cells) by utilizing copper oxide nanoparticles that were synthesized using green methods (Nagajyothi *et al.*, 2017). Previous research has demonstrated that the activation of MCF-7 cells using biosynthesized silver nanoparticles resulted in cell death (Kulandaivelu and Gothandam, 2016).

Furthermore, the results we discovered are consistent with the findings of Namvar *et al.* (2014), who showed that the presence of iron oxide nanoparticles can lead to cell cycle arrest and death in MCF-7, HepG2, and Jurkat cells. Similarly, research findings revealed that the application of ZnO NPs led to the occurrence of apoptosis in HepG2 (liver cancer) and MCF-7 (breast cancer) cells (Wahab *et al.*, 2014). Recently, ZnO nanoparticles derived from *Solanum procumbens* have shown promising results in damaging lung cancer cells. In the early stages of apoptosis, less robust cells underwent both

survival and death. In the realm of advanced apoptosis, a multitude of cells were undergoing the process of cell death. When the dosage was adjusted to a moderate level, it was observed that the number of viable cells decreased, but there was no noticeable effect on the occurrence of early or late apoptosis. Aziz *et al.* (2022), noticed an increase in apoptotic cells and a decrease in surviving cells as the doses were increased. Ultimately, these findings strongly indicate that the biosurfactant-synthesized ZnO NPs primarily induce cell death through apoptosis.

Conclusion

This study effectively demonstrates the biosynthesis of zinc oxide nanoparticles using biosurfactant extracted from *Pseudomonas stutzeri* bacteria. This presents valuable insights into a unique method for synthesizing nanoparticles. The results obtained in the study were highly encouraging, which suggests the successful synthesis of ZnO NPs using a biosurfactant extract from *P. stutzeri* strain as a capping agent. As a result, small-sized ZnO NPs were successfully produced, exhibiting improved stability. We conducted various analyses, including UV-Vis spectroscopy, FTIR, XRD, SEM, and EDX, to characterize and confirm the synthesized nanoparticles. These analyses provide strong evidence supporting the successful biosynthesis of biosurfactant extracted from *P. stutzeri* mediated ZnO NPs. The biosurfactant synthesized ZnO NPs were observed to effectively inhibit the activity of bacterial and fungal pathogens. In addition, our research has shown that the biosurfactant synthesized ZnO NPs have significant inhibitory effects on the proliferation and migration of human lung cancer (A549) and breast cancer MCF-7 cells. This effectively prevents the growth of cancerous cells. Hence, the biosurfactant synthesized ZnO NPs exhibit strong potential as antimicrobials, effectively inhibiting a wide range of microbial pathogens. Moreover, their ability to be employed in drug preparation for therapeutic purposes, particularly in diseases such as cancer, has great potential.

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