# Characterization and Application of Biosurfactant producing Bacteria and Nanoparticle Synthesis for Bioremediation and Plant Growth Promotion

**Sowbaranika U.[1], Ashokkumar K.[1]*, Abirami G.[1], Miller Samson S.[2] and Prakash Balu[1]**
1. Department of Biotechnology, Vels Institute of Science, Technology and Advanced Studies, Pallavaram, Chennai – 117, TN, INDIA
2. Department of Microbiology, Srinivasan College of Arts and Science, Perambalur 621212, INDIA
*bioashok2002@gmail.com

***Abstract***

*Biosurfactants are surface-active biomolecules with significant applications in bioremediation, particularly for the breakdown of hydrocarbon pollutants. This study investigates the isolation and characterization of biosurfactant-producing bacterial strains from oil-contaminated soils in Chennai, India, with a focus on Enterobacter cloacae. Screening assays including hemolysis, oil spreading and emulsification, identified Enterobacter cloacae as a promising candidate. Optimal production conditions: 37°C, pH 7, glucose and casein as carbon and nitrogen sources, yielded a high-performance biosurfactant. This biosurfactant facilitated the synthesis of silver nanoparticles (AgNPs), confirmed by UV-visible, FTIR, SEM and XRD analyses, demonstrating antimicrobial properties. Bioremediation potential was validated by applying the biosurfactant and its AgNP composite to oil-contaminated soils, showing significant hydrocarbon degradation.*

*Additionally, Enterobacter cloacae exhibited plant growth-promoting traits such as nitrogen fixation and indole acetic acid production. These findings suggest that biosurfactants and biosurfactant-AgNP composites are effective agents for environmental remediation and sustainable agriculture.*

**Keywords:** Biosurfactant, *Enterobacter cloacae*, Bioremediation, Hydrocarbon degradation, Nanoparticles, Plant growth promotion.

## Introduction

Hydrocarbon contamination is a persistent environmental problem, largely stemming from industrial spills, vehicle workshops and other anthropogenic activities that release harmful pollutants into ecosystems. These hydrocarbons are not only toxic to plant and animal life but also pose severe risks to human health, potentially contaminating soil and water sources. Traditional chemical surfactants are often used in cleaning up hydrocarbon spills; however, they present issues like poor biodegradability and toxicity to non-target organisms[1].

Biosurfactants, microbial metabolites with surface-active properties, are emerging as promising alternatives due to their eco-friendly profile, high efficiency and lower environmental impact. These compounds are biodegradable and can efficiently reduce surface and interfacial tensions, enhancing the bioavailability of hydrocarbons for microbial degradation[2]. Biosurfactants are produced by various microorganisms including bacteria, yeast and fungi which makes them renewable and sustainable for large-scale applications. Their structure varies widely including glycolipids, lipopeptides and phospholipids, each with specific properties that make them suitable for applications in bioremediation and microbial enhanced oil recovery[3].

This study investigates the potential of *Enterobacter cloacae*, a bacterium isolated from oil-contaminated soils, in producing biosurfactants. Screening assays were performed to evaluate their biosurfactant production capabilities, while optimization tests were conducted to identify the most favorable production conditions. Biosurfactants produced by *Enterobacter cloacae* was also used to synthesize silver nanoparticles (AgNPs), which hold considerable interest for their antimicrobial properties[4]. Studies have shown that silver nanoparticles, when combined with biosurfactants, not only enhance microbial activity but also increase hydrocarbon degradation, thereby amplifying bioremediation potential[5].

In addition to its environmental applications, *Enterobacter cloacae* was tested for plant growth-promoting traits, such as nitrogen fixation and indole acetic acid production, highlighting its dual application in both bioremediation and sustainable agriculture[6]. The present study aims to optimize biosurfactant production from *Enterobacter cloacae*, to synthesize AgNPs for antimicrobial testing and to assess the bacterium's potential in hydrocarbon degradation and plant growth promotion, supporting its use in environmental management and agriculture.

## Material and Methods

We have successfully isolated biosurfactant-producing bacterial strains earlier from hydrocarbon-contaminated soils collected from vehicle workshops in Chennai, Tamil Nadu, India. The isolates demonstrated significant crude oil degradation and biosurfactant production under optimized conditions.

**Purification of biosurfactant:** In a column with 1.5 x 60 cm, the partial purified biosurfactant obtained from solvent extraction was filled with silica gel (60 -120 mesh). Silica gel powder was activated for 18hr in the oven at 100 °C

before use. It was packed tightly by a continuous flow of equal volume of methanol: chloroform and washed with the same solvent mixture. The biosurfactant was then loaded into the column until the overall solution was absorbed. Chloroform and methanol were then used to elude the column (1:1 v/v). Three ml from each fraction of the eluted extract flowing at a rate of 20 ml per hour, was collected. All eluted fractions were gathered and dried at 40–45°C. The purified powder was stored in a clean vial at 4 ˚C for the remaining experiment[12].

**Synthesis of nanoparticles with biosurfactant:** 1 mM aqueous solution of silver nitrate was added to a pure solution of biosurfactant (1 g) while stirring magnetically. The mixture was then heated to 60 °C, facilitating the reduction of silver ions (Ag+) to metallic silver (Ag0). This reduction process can be monitored by the noticeable color change from colorless to dark brown, indicating the completion of the reaction[7].

**Characterization of AgNps of biosurfactant**

**UV-visible spectrophotometer analysis:** The absorption spectrum for synthesized AgNPs was recorded on UV-visible spectrophotometer. The solution was filled in 1 cm optical path quartz cuvette and the spectra were recorded in the wavelength range of 300 to 800 nm.

**Fourier Transform Infrared Spectroscopy (FTIR) analysis:** To conduct FTIR analysis, first grind the biosurfactant sample thoroughly with potassium bromide (KBr) salt in a clean mortar and pestle to create a homogeneous powder mixture. Transfer this powdered mixture into a mechanical press and apply sufficient pressure to compress it into a translucent pellet. Next, place the pellet into the sample holder of the Shimadzu spectrometer, setting the device to scan within the range of 500–4000 $cm^{-1}$. Record the FTIR spectrum, ensuring that the beam of light passes through the pellet for a clear spectrum. Finally, analyze the obtained spectrum to identify the biomolecules involved in the synthesis and stabilization of nanoparticles by examining the characteristic absorption peaks.

**X-Ray Diffraction (XRD) analysis:** X-ray diffraction (XRD) is used to determine the atomic and molecular structure of silver nanoparticles. This method was carried out for irradiating of the material with incident X-rays and to measure the intensities and scattering angles the X-rays scattered by the nano- materials. The X-ray beam was diffracted by the sample and detected at various angles. The XRD (RIGAKU miniflex-600, Japan) was performed using an X-ray diffractometer–Cu, K$\alpha$ radiation $\lambda$ 1.54 nm in the 2$\theta$ range of 30-80 operated data voltage of 40kV and a current of 30 mA. The graph was detected between 2$\theta$ on x-axis and intensity on y-axis with different peaks corresponding to different planes of the crystal.

**Scanning electron microscope analysis (SEM):** The size and morphological topographies of synthesized AgNPs were further confirmed by SEM photographs. For disaggregation of nanoparticles, the solution was sonicated on a probe sonicator. The homogeneous solution was applied on cleaned and grease-free glass discs of 0.3-0.5 cm diameter. Discs were air-dried and utilized for Field Emission SEM photography. Field Emission (FE)-SEM was equipped with energy dispersive spectroscopy [energy dispersive X-ray spectroscopy (EDX) by Bruker].

**Application of biosurfactant on soil sample:** The effectiveness of biosurfactants in remediating heavy engine-oil polluted soil was evaluated. Twenty grams of de-moisturized soil were contaminated with 10% oil in a conical flask. The soil-remediating solutions included 60 mL of distilled water with 10 and 40 mg/L (w/v) concentrations of the biosurfactant (test setup). In comparison, positive and negative controls contained 10 and 40 mg/L of the chemical surfactant sodium dodecyl sulfate (SDS) and distilled water respectively. All flasks were incubated in a shaking environment at 130 rpm for 24 hours at 28 °C. After incubation, samples were centrifuged at 5000 rpm for 15 minutes and the supernatants were extracted with n-hexane. The residual oil was then measured gravimetrically following solvent evaporation[8].

**Indole acetic acid (IAA):** The potential isolate was screened for IAA production. The isolates were grown in Nutrient broth containing 0.2% L-tryptophan and were incubated at 28°C with continuous shaking at 125 rpm for 7 days. After the isolate were grown, they were centrifuged at 10,000 rpm for 20 min. Then 1 ml of the supernatant was mixed with 2 ml of Salkowski reagent. The mixture was incubated at dark room for 30 min and the production of IAA was observed by the development of pink color.

**Ammonia ($NH_3$):** Bacterial isolate was tested for the production of $NH_3$ in peptone water. Freshly grown cultures were inoculated in 10ml peptone water in each tube and incubated for 48-78 hr at 28°C. Nessler's reagent (0.5 ml) was added to each tube. Development of brown to yellow color indicated a positive test for $NH_3$ production.

**Hydrogen cyanide (HCN):** The potential was screened out for the formation of HCN[11]. Isolate streaked on NA plates amended with glycine (1.4 g/L). Whatmann no. 1 filter paper strips were soaked in 0.5% picric acid followed by 2% sodium carbonate and were placed in the lids of each Petri plates. Plates were sealed and incubated at 28°C for 4 days. Plates were examined for changes in filter paper colour from yellow (-) to light brown (++) to brown (+++) and dark brown (++++).

**Phosphate solubilization (PS):** Determination of insoluble phosphate was carried out by spotting the bacterial culture on Pikovskaya's agar plates[9,10] containing 2% tri-calcium phosphate incubated at 28°C for 7 days. The phosphate solubilization was evidenced by the zone of clearance surrounding the bacteria.

**Nitrogen fixing capacity (NF):** Nitrogen fixation of the bacterial isolate was qualitatively checked on Norris glucose nitrogen free medium (NGNFM) with BTB as indicator. The bacterial isolate was inoculated on prepared agar plates and incubated at 28°C for 7 days. The change in the color of the bacterial colonies was observed.

## Results and Discussion

**Isolation and Screening:** Of the 42 isolates screened from oil-contaminated soils, *Enterobacter cloacae* demonstrated superior biosurfactant-producing abilities, showing positive results across multiple assays. The screening involved various tests such as the drop collapse, emulsification and CTAB plate assay to detect anionic biosurfactant production[3,7]. In the drop collapse test, *Enterobacter cloacae* caused a noticeable optical distortion, confirming surfactant activity[11] (Figure 1, Table 1). The emulsification test also highlighted its efficiency with an initial emulsification index of 28.5% that increased to 35.8% after purification. This index which measures the stability of the emulsion formed by the biosurfactant in oil-water mixtures, indicates the bacterium's capability to produce a stable biosurfactant suitable for hydrocarbon degradation applications.[3,7]

**Optimization of Biosurfactant Production:** To enhance the yield and effectiveness of biosurfactant production, an optimization process was conducted. Among the various conditions tested, the study found that glucose and casein were the most effective carbon and nitrogen sources respectively[5]. Optimal production occurred at a neutral pH of 7 and a temperature of 37°C[12]. Under these optimized conditions, *Enterobacter cloacae* achieved the highest biosurfactant production levels, enhancing the hydrocarbon degradation potential of the biosurfactant. At 37°C, the biosurfactant exhibited a 25.1% hydrocarbon degradation efficiency, suggesting that these specific growth parameters could maximize the practical applications of *Enterobacter cloacae* for environmental bioremediation.

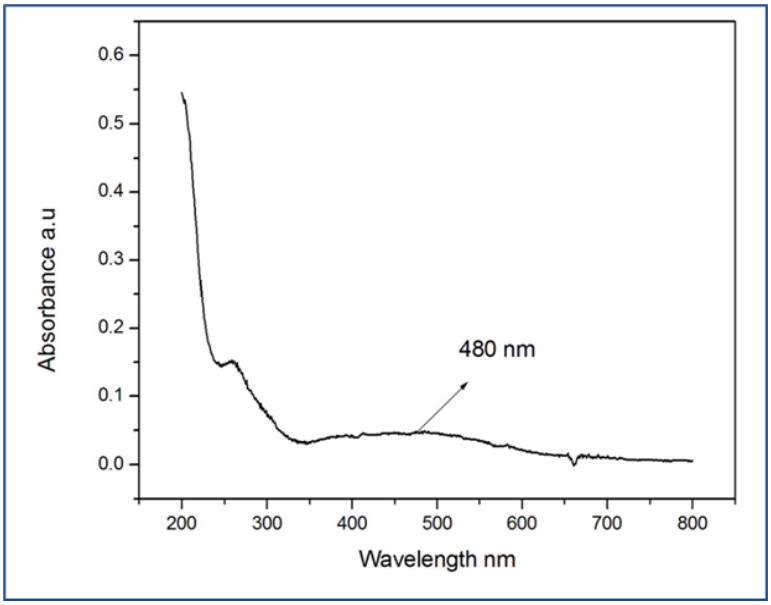


**Figure 1: UV-visible spectroscopy results of synthesized AgNPs, showing peaks at 480 nm**

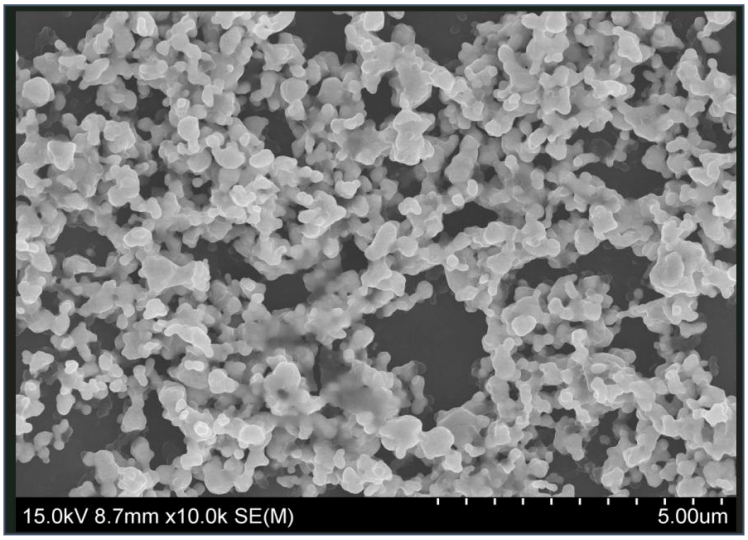


**Figure 2: SEM images of AgNPs, displaying particle morphology and crystalline structure**

**Table 1**
**Screening of biosurfactant producing isolates by emulsification index**

| S.N. | Isolates name | Emulsification result (%) |
|---|---|---|
| 1. | Isolate 2 | 28.5 |
| 2. | Isolate 16 | 11.5 |
| 3. | Isolate 19 | 24.4 |
| 4. | Isolate 35 | 27.0 |
| 5. | Isolate 39 | 11.8 |

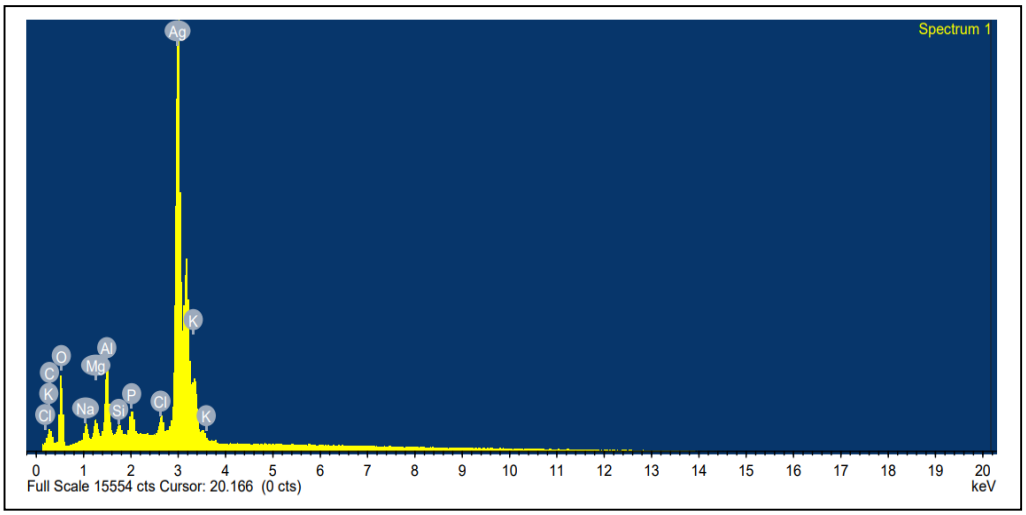


**Figure 3: XRD analysis of AgNPs, displaying particle morphology and crystalline structure**

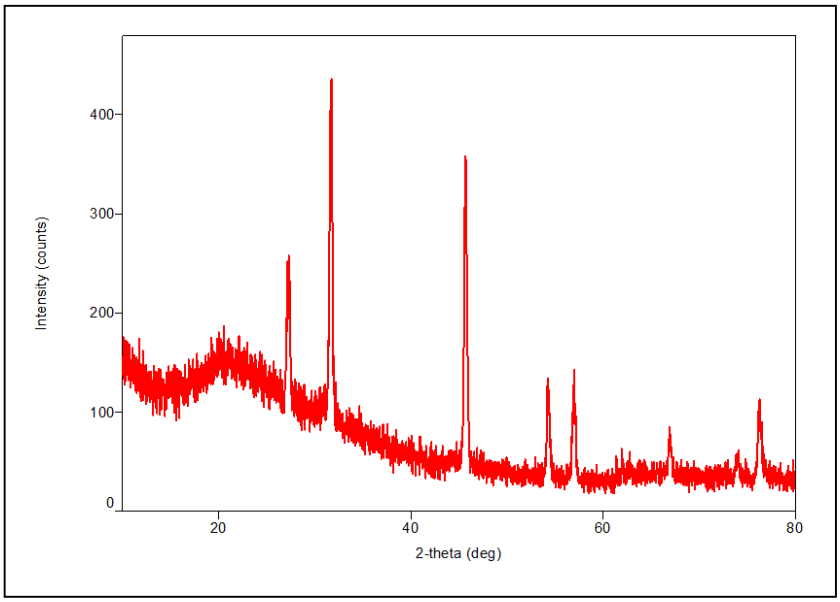


**Figure 4: XRD analysis of AgNps of biosurfactant**

**Nanoparticle Synthesis and Characterization:** Following biosurfactant production, the biosurfactant was used to synthesize silver nanoparticles (AgNPs), capitalizing on the biosurfactant's unique properties to facilitate nanoparticle formation. UV-visible spectroscopy of the synthesized AgNPs revealed a distinct peak at 480 nm, corresponding to the surface plasmon resonance of silver, confirming successful nanoparticle formation[11]. Fourier-transform infrared (FTIR) analysis further identified functional groups such as amines and carboxyls which contribute to nanoparticle stability by reducing and capping silver ions[11]. Scanning electron microscopy (SEM) images illustrated that the particles were of varied shapes and sizes with an average particle size between 82.2 and 110 nm (Figures 2 and 3). These nanoparticles also displayed a face-centered cubic (FCC) crystal structure, as confirmed by X-ray diffraction (XRD) analysis, indicating the crystalline nature of the synthesized AgNPs[6].

**Table 2**
**Optimal Conditions for Biosurfactant Production and Hydrocarbon Degradation**

| Parameter | Optimal Condition | Biosurfactant Production (g) | Crude Oil Degradation (%) |
|---|---|---|---|
| Carbon Source | Glucose | 0.613 | 27.7 |
| | Sucrose | 0.588 | 24.0 |
| | Dextrose | 0.597 | 24.9 |
| Nitrogen Source | Casein | 0.617 | 27.7 |
| | Urea | 0.611 | 26.7 |
| | Peptone | 0.587 | 24.5 |
| pH | 7 | 0.655 | 25.9 |
| | 6 | 0.517 | 17.0 |
| Temperature (°C) | 37°C | 0.658 | 25.1 |
| | 27°C | 0.537 | 17.8 |

**Table 3**
**Bioremediation effectiveness of biosurfactant and AgNP-biosurfactant composite in oil-contaminated soils.**

| Treatment | Biosurfactant Concentration (g/kg soil) | AgNP-Biosurfactant Concentration (g/kg soil) | Oil Degradation (%) | Time Required (Days) |
|---|---|---|---|---|
| Control (No Treatment) | - | - | 8.5 | 30 |
| Biosurfactant Alone | 0.5 | - | 45.3 | 20 |
| Biosurfactant Alone | 1.0 | - | 58.7 | 20 |
| AgNP-Biosurfactant Composite | - | 0.5 | 62.4 | 15 |
| AgNP-Biosurfactant Composite | - | 1.0 | 78.9 | 15 |

**Table 4**
**Summary of plant growth-promoting traits confirmed for *Enterobacter cloacae*.**

| Isolate | Indole acetic acid | Ammonia production | Nitrogen fixation | Phosphate solubilization | HCN production |
|---|---|---|---|---|---|
| *Enterobacter cloacae* | + | + | + | + | + |

**Bioremediation Application**: The biosurfactant synthesized by *Enterobacter cloacae* demonstrated remarkable potential in bioremediation applications, particularly for hydrocarbon degradation in oil-contaminated soils. In a test using oil-polluted samples, the biosurfactant alone reduced oil content by 0.814 grams from an initial 2 grams. However, when the biosurfactant was combined with AgNPs, the degradation rate increased significantly, demonstrating a synergistic effect between the biosurfactant and nanoparticles[1]. This finding suggests that biosurfactant-AgNP composites could serve as efficient agents for hydrocarbon degradation, potentially outperforming traditional chemical surfactants in environmental cleanup efforts (Table 3). The AgNP-biosurfactant composite's enhanced degradation rate further underscores the advantages of this innovative approach to bioremediation, which may provide more efficient and sustainable solutions for hydrocarbon-contaminated environments[6,12].

**Plant Growth Promotion:** Apart from its applications in hydrocarbon degradation, *Enterobacter cloacae* displayed several plant growth-promoting characteristics, indicating its dual utility in environmental and agricultural biotechnology. Tests confirmed the bacterium's ability to produce indole acetic acid (IAA), an essential plant hormone that stimulates root growth and cell division, which could improve plant resilience and growth in contaminated soils[5,14] (Figure 4, Table 4). Additionally, *Enterobacter cloacae* exhibited ammonia production and nitrogen fixation capabilities, essential for soil fertility as well as phosphate solubilization, which enhance nutrient availability for plants. The production of these compounds makes *Enterobacter cloacae* an effective biofertilizer, supporting plant health and facilitating sustainable agriculture.

## Conclusion

This study highlights the promising capabilities of *Enterobacter cloacae* in producing biosurfactants which offer effective applications in hydrocarbon bioremediation and agriculture. The biosurfactant demonstrated robust degradation potential in oil-contaminated soils, particularly when combined with synthesized silver nanoparticles (AgNPs), enhancing their efficiency through a synergistic

effect. The biosurfactant-AgNP composite not only degraded hydrocarbons but also showed potential as an antimicrobial agent, indicating versatility in environmental remediation.

Additionally, *Enterobacter cloacae* exhibited multiple plant growth-promoting traits such as indole acetic acid production and nitrogen fixation, making it suitable as a biofertilizer. These dual applications environmental cleanup and agricultural support put *Enterobacter cloacae* as a valuable asset in sustainable biotechnology. Future studies should focus on scaling up biosurfactant and AgNP production, optimizing formulations for field applications and testing these composites in diverse environmental conditions to fully realize their potential in large-scale, real-world remediation and agricultural enhancement efforts.

## References

1. Das A., Das N., Rajkumari J., Pandey P. and Pandey P., Exploring the Bioremediation Potential of Bacillus spp. for Sustainable Mitigation of Hydrocarbon Contaminants, *Environ. Sustain*., **7**, 1-22 **(2024)**

2. Felix A.K.N., Martins J.J., Almeida J.G.L., Giro M.E.A., Cavalcante K.F., Melo V.M.M. and de Santiago Aguiar R.S., Purification and Characterization of a Biosurfactant Produced by Bacillus subtilis in Cashew Apple Juice and its Application in the Remediation of Oil-Contaminated Soil, *Colloids Surf. B Biointerfaces*, **175**, 256-263 **(2019)**

3. Gómez-Graña S., Perez-Ameneiro M., Vecino X., Pastoriza-Santos I., Perez-Juste J., Cruz J.M. and Moldes A.B., Biogenic Synthesis of Metal Nanoparticles Using a Biosurfactant Extracted from Corn and their Antimicrobial Properties, *Nanomaterials*, **7(6)**, 139 **(2017)**

4. Hala M.A. and Haider N.H., The Antibacterial Activity of Glycolipopeptide Produced from Lactococcus lactis HN21 Against Some Clinical Pathogens in Combined with Some Standard Antibiotics, *Iraqi J. Agric. Sci*., **55(Special Issue)**, 12-24 **(2024)**

5. Jiang C.Y., Sheng X.F., Qian M. and Wang Q.Y., Isolation and Characterization of a Heavy Metal-Resistant Burkholderia sp. from Heavy Metal-Contaminated Paddy Field Soil and its Potential in Promoting Plant Growth and Heavy Metal Accumulation in Metal-Polluted Soil, *Chemosphere*, **72(2)**, 157-164 **(2008)**

6. Kumar V., Sharma N. and Kansal S., Characterization of Potential PGPR's Isolated from Rhizosphere of Wheat from Trans-Himalayas and their Efficacy on Seed Germination and Growth Promotion of Wheat Under Net House Conditions, *J. Plant Dev. Sci*., **11(3)**, 121-131 **(2019)**

7. Mahanty R., Saha I., Halder J., Rajwar T.K., Dash P., Das C. and Rath G., Biosurfactant-Based Silver Nanoparticles: Recent Advances and their Applications in Antimicrobial Drug Resistance, *J. Drug Deliv. Sci. Technol*., **92(2)**, 105382 **(2024)**

8. Ng Y.J., Chan S.S., Khoo K.S., Munawaroh H.S.H., Lim H.R., Chew K.W. and Show P.L., Recent Advances and Discoveries of Microbial-Based Glycolipids: Prospective Alternative for Remediation Activities, *Biotechnol. Adv*., **68**, 108198 **(2023)**

9. Pikovskaya R.I., Mobilization of Phosphorus in Soil Connection with the Vital Activity of Some Microbial Species, *Microbiologiya*, **17**, 362-370 **(1948)**

10. Priya S.D., Moovendhan M., Sundaram T., Punniyakotti P., Vinayagam S. and Rajamohan R., Environmental Fate and Ecotoxicological Behaviour of Pesticides and Insecticides in Non-Target Environments: Nanotechnology-Based Mitigation Strategies, *J. Environ. Chem. Eng*., **12(5)**, 113349 **(2024)**

11. Sharma C., Sharma P., Kumar A., Walia Y., Kumar R., Umar A. and Baskoutas S., A Review on Ecology Implications and Pesticide Degradation Using Nitrogen Fixing Bacteria Under Biotic and Abiotic Stress Conditions, *Chem. Ecol*., **39(7)**, 753-774 **(2023)**

12. Singh S., Gupta G., Khare E., Behal K.K. and Arora N.K., Phosphate Solubilizing Rhizobia Promote the Growth of Chickpea Under Buffering Conditions, *Int. J. Pure Appl. Biosci*., **2(5)**, 97-106 **(2014)**

13. Yesankar P.J., Pal M., Patil A. and Qureshi A., Microbial Exopolymeric Substances and Biosurfactants as 'Bioavailability Enhancers' for Polycyclic Aromatic Hydrocarbons Biodegradation, *Int. J. Environ. Sci. Technol*., **20(5)**, 5823-5844 **(2023)**

14. Rao C.N.R., Chemical Applications of IR Spectroscopy, Academic Press, London, 250 **(1963)**.

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