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Discussion

Agriculture is one of the major aspects that fulfill the needs of the human population all over the world. The tremendous increase in population has recently raised the demand for food mostly in developing countries. Currently, the population of the world is growing tremendously at a speed of about 1.13% per year which is estimated to change at around 80 million per year [260]. According to UN records, the average population of the world is estimated to increase from 7.4 billion to 8.1 billion within 9 years, *i.e.*, from 2016 to 2025, and by 2050, the population will show a rise up to 9.6 billion, mostly around developing countries [260, 261]. Therefore, it is very pertinentto focus on exploring the agricultural sectors with the recently developing science and technology. Even though the farming sector has already opted for some smart agricultural techniques, there is a need for further improvement to meet the challenges faced by agriculture in increasing the rate of crop production and thus providingproper diets to the increasing population [262]. However, industrialization has greatly affected the fertility of the soil which reduces the rate of crop production in the world [263]. Soil pollution has become a serious threat to the agricultural sectors. To mitigate the problem of soil pollution and increase fertility, farmers are using various chemical fertilizers [264]. Although, its use in the agricultural field improves the rateof crop production, but regular use of chemicals in the soil is a matter of concern

[264]. Chemical fertilizers are hazardous to health as well as the environment. The constant use of chemical fertilizers can change the pH of the soil, pollute the air and water, reduce the quality of the soil, and improper growth of the plants [265]. Recently, biological fertilizers have been in huge demand due to their eco-friendly nature and low cost [266]. Researchers are still working to modernize agricultural techniques that can enhance agricultural production without causing harm to the environment [266].

On the other hand, nanotechnology has brought significant change in various fields by developing innovative techniques of scientific improvements replacing the traditional methods [267]. The exploration of agricultural areas with nanotechnology is mostly theoretical even though a handful of literature reports the use of nanomaterials for improving production [262]. Therefore, there is an immediate need to work on the hypothetical concept so that the farmers can utilize its potential in field application.

Rice is considered a primary food and a major source of calories for half of the world, especially for people living in Asian countries [268]. The rice market value is estimated to annually grow by about 6.99% from 2023-2028 [76]. Similarly, among other pulses, chickpea is used as the highest energy source of food and hence it has a huge demand in countries like India [269]. Even though India is the largest producer of chickpeas, it needs more production to meet the demands of the people [270]. The annual market value of chickpeas is estimated to grow at a rate of about 5.4% between 2022 and 2032 [271]. Furthermore, rice being the staple food requires huge production to meet the needs of the world during the food crisis. Considering these facts, rice and chickpeas were selected for this research study.

In recent times, bacterial biosurfactants have been explored for their wide range of applications in the industrial sector from petroleum exploration, and pharmaceutics to cosmetics [272]. However, in the field of nanotechnology, considering the cost and amount of toxicity released by chemical methods of synthesis of NPs, biological materials such as biosurfactants are believed to be cost-effective and more eco- friendly ways for nano-research [273]. Moreover, biosurfactants are known for their stabilizing property which is an important aspect of the proper synthesis of metal NPs

[274]. Therefore, the use of bacterial biosurfactants for the synthesis of metal NPs has been considered a better as well as cheaper option [105, 275]. Among all the metal NPs, Ag NPs are the most demanding metal NPs due to their successful implicationsin various industrial, biological as well as environmental applications [276]. SilverNPs also show good antimicrobial and catalytic properties and hence may be used in food and biomedical industries [277]. On the other hand, ZnO NPs are known for their beneficial role in various biological applications such as pharmaceutical, biomedicine, cosmetic, food, and agricultural sectors, *etc.* as the cost of their respective salt is comparatively very low [278]. Moreover, ZnO being a nutritional component for living cells has recently attracted researches to utilize ZnO NPs in medical science [279].

In this study, a total of 54 isolates with unique colony characteristics were screened on diesel oil-supplemented BH broth. In the current study, a novel strain of *Klebsiella sp*. RGUDBI03 (GenBank accession: ON945613.1) based on 16S rDNA gene sequencing was screened as the best isolate for its ability to produce biosurfactant most efficiently. The isolate could grow in BH broth where diesel was used as the only source of carbon indicating its capability to degrade petroleum with increasing cell density and corresponding protein content [94, 281]. Studies have also demonstrated that hydrocarbon-degrading microbes utilize hydrocarbon as a source of energy for their proliferation which may be assessed in terms of their cell density, dry cell biomass, and protein concentration [227, 282]. On the other hand, biosurfactant production is an important characteristic of hydrocarbon-degrading bacteria. Biosurfactants are surface-active compounds secreted by microbes that can reduce the surface tension of oily substances thus making it suitable for the microbial cells to utilize it as an energy source for further metabolic processes [283]. In support of the above, the best isolate *i.e.*, *Klebsiella sp*. RGUDBI03 could reduce the surface tension of oil-supplemented medium by 30% within just 3 days of incubation as compared to that of control indicating its ability to produce biosurfactant. The crude biosurfactant extracted from the cell-free extract of the isolate was confirmed by various biochemical tests, drop collapse, emulsification index, FTIR, SEM analysis, and hemolysis. Positive results of ninhydrin and anthrone tests assured the presence of amino acid moieties especially proline and carbohydrate moieties in the biosurfactant extract [94, 235]. Hemolysis is often considered the common criterion for screening biosurfactant-producing microorganisms [284]. Most of the researchers have used the hemolysis assay as an important parameter to test the potential to produce biosurfactants [284]. The best isolate *i.e.*, *Klebsiella sp*. RGUDBI03 in this study exhibited α-hemolysis on blood agar which is also supported by other contemporary research findings for their ability to produce biosurfactant [285 – 287].

Drop collapse test on the cell-free extract of the isolate showed that the drop lost its surface tension and completely collapsed just after 45 sec which may be due to the production of biosurfactant by the isolate in diesel supplemented medium [230], [288, 289]. The emulsification index (E_{24}) is another important factor for the characterization of biosurfactant-producing microbes [290]. The isolate which shows the formation of an emulsion layer is considered as a potential biosurfactant-producingisolate [290]. In this study, the isolate *Klebsiella sp*. RGUDBI03 showed E_{24} value of 35.71% after 24 h against diesel which is somewhat following the findings describedby other researchers [227, 230, 290-292].

The results of the FTIR spectrum confirmed the presence of O-H (bend), O-H (stretch), CH_2 (asymmetrical stretch), C=C (stretch), C-H (bend), C-H in-plane bend, and C=C-H (bends) in the crude biosurfactant produced by the isolate which is also supported by other contemporary findings [97, 293, 294]. Besides, the SEM images also show the surface morphology of the crude biosurfactant extracted from the isolate [295]. Several pieces of literature have also advocated the production of rhamnolipid- based biosurfactant by various strains of *Klebsiella* sp. and their effective *in- vitro* and *in-vivo* roles in petroleum degradation, pharmaceutical, and agricultural use [296 – 299]. Various species belonging to the genus *Klebsiella* are known to produce biosurfactants with excellent emulsifying activity and are employed for hydrocarbon degradation [296, 300]. Moreover, such surfactants are known to remain stable under harsh and extreme environmental conditions which makes them commercially useful [296, 300].

The synthesis of biosurfactant-mediated Ag NPs was initially confirmed by the change in color from white to reddish brown [301]. Similarly, the synthesis of biosurfactant-mediated ZnO NPs were initially confirmed by the formation of a white precipitate [302]. The SEM analysis confirmed the successful formation of Ag and ZnO NPs. In addition to this, EDX analysis shows prominent characteristic peaks confirming the formation of Ag NPs and ZnO NPs. The EDX spectrum showed elemental peaks of silver in the Ag NPs sample at around 3eV [303]. The EDX spectrum of the ZnO NPs sample showed elemental peaks of zinc and oxygen at around 1eV and 9eV [304]. The analysis of Ag NPs through TEM shows the formation of discrete and well-dispersed NPs with an average size range of 10-40 nm. Moreover, the analysis of ZnO NPs through TEM shows the formation of discrete and well-dispersed NPs with an average size range of 2-10 nm. The XRD peaks were matched by using a standard JCPDS card. The XRD peaks represent the presence of

(111) and (200) lattice points for the face-centered cubic crystal of Ag NPs. The XRD peaks represent the presence of (200), (100), and (002) lattice points for the face-centered cubic crystal of ZnO NPs. Similar results were also reported by other literature [304], [305]. The presence of sharp Bragg peaks may have resulted due to the biosurfactant which acts as a capping agent that stabilizes the nanoparticle [305]. The appearance of some additional peaks may be due to the presence of organic compounds used during the synthesis of Ag NPs and ZnO NPs. Similar results were obtained and reported by other literature [305].

The FTIR spectra of the biosynthesized Ag NPs show the presence of C=C-H, C-H (stretch), CH_2, C=C (stretch), C-H (bend), C-H (bend overtone), C=C-H (bends) and C-C (stretches) in the sample showing similarity with the peaks obtained for the biosurfactant confirming their positive role in synthesizing the NPs. Similarly, the FTIR spectra of the biosynthesized ZnO NPs show the presence of C=C-H, C=C (stretch), C-H (bend), and C=C (stretch). Both samples showed a similar presence of the peaks for the biosurfactant which confirms the positive role in synthesizing the NPs.

The thermal stability and the mass loss of the sample were evaluated by TGA and DTA analysis showing thermal dissociation in terms of gravimetric loss of samples due to the evaporation of water and organic compounds above 100 ºC indicating its long-lasting stability profile to withstand high temperature [306, 307]. The DTA- TGA analysis results of ZnO NPs sample showed similar weight loss of the material above 100 ºC. There is almost no weight loss of the AgNPs and ZnO NPs sample above 500 ºC.

Recently, nano-priming has hypothetically proved to show promising activity in replacing conventional priming agents to increase agricultural yield. The fundamental mechanism of nano-priming and its potential to stimulate seed germination is still vague [308]. One of the mechanisms proposed by available literature suggests that Ag NPs and ZnO NPs, when used for seed treatment, generate OH- radicals, which loosen the seed coat and increase water uptake. It is also pertinent to mention that dehydrogenase activity in seeds is often associated with the fast consumption of water by aged seeds [309, 310]. An increase in water absorption rate followed by enhanced growth rate in the seeds with the increase in the concentration of NPs in the priming solution was observed. The maximum water uptake in rice and chickpea seeds increased up to 86% and 88% respectively at a dose of 30 mg/L of Ag NPs. Similarly, ZnO NPs primed seeds showed the highest water uptake of about 89% and 93% for chickpea and rice seeds respectively at 30 mg/L concentrations of ZnO NPs. It was also observed that soluble sugar content was more in nano-primed seeds as comparedto untreated seeds which have increased with its increase in the dose of Ag NPs and ZnO NPs. From the findings presented in the current work, it has been assumed that priming of seeds with NPs increases the water uptake capacity which may catalyze the enzymatic activity of α-amylase which is a major enzyme required in the enhancement of seed germination [21, 311, 312]. Various shreds of evidence show an increased starch hydrolysis rate in nano-primed seeds thereby improving seed germination rate [313]. A handful of contemporary research also demonstrates the catalysis of α-amylase activity leading to the water uptake capacity of NPs treated rice, chickpea, and bean seeds thereby enhancing the starch degradation and germination rate [21, 314, 315]. The stimulation of α-amylase increases the hydrolysis of starchinto maltose and other soluble sugar content which gradually improves the growth rate of the seedlings [21, 316].

Further, to ensure the animal and environmental safety for commercial exploitation of the biosurfactant-mediated Ag NPs and ZnO NPs, the cytotoxicity was assessed on blood cells and earthworms. Sheep blood cells exhibited no sign of hemolysis within the range of 10, 20,...40 mg/L concentration of Ag NPs and ZnO NPs as compared to the positive (triton X) and negative (PBS) control exhibiting 100% and no hemolysis respectively. Thus, it was considered that the Ag NPs and ZnO NPs are non-cytotoxic and thus non-harmful to human health [250, 317]. The substances with less than 2% hemolytic behavior are generally considered non- cytotoxic [318].

Recently, the world has been facing the problem of severe soil pollution caused by various industrial as well as anthropogenic activities [319]. Soil pollutants are detected over several years by using earthworms as indicators [320]. When earthworms come in contact with pollutants, their body responds to the stress condition eventually affecting the normal behavior of earthworms [319]. Hence, they are considered as one of the bio-indicators of soil pollution [321]. The reproductive activity and the population of earthworms are also strongly affected by the presence of toxic substances in soil [321].

Since earthworms are very sensitive to any soil toxicity, no change in earthworm activity depicts that the synthesized Ag NPs and ZnO NPs are environment and soil-friendly in nature which increases their scope for futuristic application in the agricultural sector. Earthworms are one of the most sensitive organisms that are unable to tolerate any environmental hazard [322]. The survival rate of earthworms in the presence of any chemical gives a brief idea of the environmental safety assessment of any material [323]. The Ag NPs and ZnO NPs were tested on earthworms (*Eudrilus eugeniae*) with the maximum concentration of Ag NPs and ZnO NPs (*i.e*., 40 mg/L) which is used for seed germination assay in this study and treatment with distilled water was considered as control. Observation after 6 days of the process shows earthworms with no physiological, behavioral, or color change. Additionally,histological staining of gut tissues shows a healthy gut with villi (V) advocating the safe nature of the Ag NPs and ZnO NPs for field trials in the future [324]. The presence of a normal epithelial layer with intestinal villi in the treated earthworms indicated healthy gut tissues and the non-ecotoxic and cytotoxic nature of the Ag NPs and ZnO NPs [324].

Metal NPs are known for their potential antimicrobial activity against various pathogens such as *E. coli, Enterococcus faecalis, Pseudomonas aeruginosa, Trichosporon asahii, Bacilus subtilis, Staphylococcus aureus, Salmonella typhi, Listeria monocytogenes, Acinetobacter baumannii, Micrococcus luteus, Staphylococcus epidermidis, Yersinia pestis, Staphylococcus haemolyticus, Salmonella enteritidis, Penicillium brevicompactum, Phytophthora infestans and Phytophthora capsici, etc* [325, 326]. In contrast, only a handful of literature is available on the antimicrobial role of metal NPs on plant pathogens such as *Xanthomonas axonopodis pv. Punicae, Ralstonia solanacearum Alternaria alternata, Fusarium oxysporum, Pythiumultimum, and Aspergillus niger, etc* [327 − 329]. In this study, Ag NPs exhibited antimicrobial potential against both bacterial as well as fungal plant pathogens. The Ag NPs showed antimicrobial activity against *Ralstonia solanacearum* F1C1 with a MIC and MBC value of 0.07, and 0.1 mg/mL respectively. Similarly, Ag NPs exhibited antimicrobial activity against *Fusarium oxysporum* f. sp. *pisi* (van Hall) Synder & Hansen strain 4814 with MIC and MFC values of 0.04, and 0.09 mg/mL respectively. It may also be noted that these values are much higher as compared to the maximum dose *i.e.*, 40 mg/L used for the seed germination assay. On the other hand, ZnO NPs showed no antimicrobial activity against both pathogens. However, Zn and ZnO NPs are known for plant growth-promoting activity [330 −332]. In this study, even though both the NPs proved to have excellent seed germination properties with no cytotoxic effect, but ZnO NPs showed somewhat better efficacy in seed germination which advocated their futuristic potential in field trials.