3

Materials and methods

3.1 Chemicals and consumables

Diesel oil used in the study was locally collected from the fuel station of Numaligarh Refinery Limited (NRL) located on Lakhra Road, Guwahati, Assam. All the chemicals and consumables were purchased from Rankem^{TM} and Merck India Pvt. Ltd and microbial media were procured from Himedia India Pvt. India.

3.2 Sample collection

Oil (petroleum) logged soil samples were collected from 20 automobile servicing centres located in Guwahati, Assam. The petroleum-contaminated soil samples were brought to the lab in sterile polythene pouches and further processed. Details of the geographical locations of the sampling site are presented in **Table 4.1**.

3.3 Isolation and screening of biosurfactant-producing microorganisms

The soil samples were serially diluted and 1.0 mL from the suitable dilution was individually inoculated on Bushnell and Haas (BH) agar (composition g/L: $MgSO_4$-

0.2, $CaCl_2$-0.02, KH_2PO_4-1.0, K_2HPO_4-1.0, NH_4NO_3-1.0, $FeCl_3$-0.05, agar-agar-20.0, pH-7.0 at 25 ºC) plates supplemented with 200 µL diesel oil as the only carbon source by following spread plating method. These plates were then incubated at 37 ºC for 72 h of incubation period [225 – 227] (Bacteriological Incubator, Optics Technology Pvt. Ltd., India).

All the potential bacterial colonies were characterized based on their shape, size, color, margin, surface, and elevation. Distinct bacterial colonies obtained were individually sub-cultured on nutrient agar (composition g/L: peptone-5.0, agar-15.0, NaCl-5.0, yeast extract-1.50, HM peptone B- 1.50, pH- 7.4 ± 0.2 at 25 ºC) slants to get pure colonies. Each isolate was assigned a unique code before final identification. The glycerol stock of each isolate was prepared and stored at -20ºC for further use.

The best isolate was screened based on their cell density, cell dry biomass, corresponding protein content, and surface tension reduction activity in petroleum-supplemented BH broth [227]. Each bacterial isolate was inoculated in Nutrient Broth and kept in a shaker incubator for 24 h at 37 ºC and 135 rpm. Then, 1 mL of the overnight culture was inoculated in 250 mL of BH broth supplemented with 2% (*v/v*) diesel oil as the only carbon source and kept in a shaker incubator for 7 days at 37ºC and 135 rpm (Remi cold centrifuge, Model no. Neya 16R).

After 7 days of incubation, the cell density of each bacterial culture was determined at 600-620 nm wavelengths. Precisely, 1 ml of the bacterial culture was then centrifuged at 10,000 rpm for 10 min. The supernatant was discarded to obtain the bacterial cell pellet. The pellet was air-dried and weighed to determine the cell dry biomass of each bacterial isolate.

The dried cell pellet was re-suspended in 1 mL distilled water and sonicated for 1 min at 100% amplitude (Rivotek India Pvt. Ltd.). The protein content of each bacterial isolate was determined following Lowry's method [228]. Precisely, 100 mg of BSA (Bovine Serum Albumin) was dissolved in 100 mL of distilled water to obtain a 1 mg/mL concentration of standard stock protein solution. The working solution was prepared by mixing 10 mL of stock solution with 50 mL of distilled water. Standard protein solution of 0.0, 0.2, 1.0 mL was transferred to individual test tubes

and the final volume was made up to 1 mL by adding distilled water. Then, 2.5 mL of protein reagent was added to the test tubes and kept for incubation at room temperature for 20 min. The protein solution was prepared by mixing 49 mL of 2% Na_2CO_3 in 0.1N NaOH with 1 mL of 0.5% $CuSO_4$ in 1% $KNaC_4H_4O_6 \cdot 4H_2O$. After incubation, 0.5 mL of Folin-Ciocalteu reagent was added to the mixture and kept in incubation for 30 min at room temperature. Similar steps were also followed with 1 mL of each test protein solution (lysed bacterial cells). After 30 min of incubation, the absorbance of all the test tubes was taken at 660 nm of wavelength. A standard graph was plotted for the standard protein and the concentration of unknown bacterialisolates was determined from the standard graph.

The surface tension reduction of bacterial treated diesel oil supplementedmedia (test) under the influence of each bacterial isolate was determined by using a tensiometer (KRÜSS force tensiometer Model K20) where surface tension of diesel oil supplemented un-inoculated media was used as control. The surface tension reduction activity of each isolate was determined by using the following formula (**Eq. 3.1**):

$$\text{Surface tension reduction activity (\%)} = 100 - \left[\frac{Surface\ tension\ of\ test}{Surface\ tension\ of\ control} \times 100\right]$$

(**Eq. 3.1**)

3.4 Hemolysis assay of the biosurfactant producing isolate

The ability of the biosurfactant-producing bacterial isolate to lyse blood cells was assessed by inoculating the fresh culture on Blood Agar base No. 2 (composition g/L: Proteose peptone- 15.0, HL extract- 2.5, Yeast extract- 5.0, Sodium chloride-5.0, agar-agar-15.0, pH-7.4±0.2 at 25 ºC) plates containing 5% (*v/v*) defibrinated blood by streak plating method [229, 230]. The plates were then incubated at 37 ºC for 24 h for further observation.

3.5 Drop collapse test

The cell-free extract of the best isolate was initially analyzed for its ability to reduce the surface tension of diesel oil-supplemented media by the drop-collapse method. Bacterial isolate was allowed to grow in 2% (*v/v*) diesel oil supplemented media for 7 days maintained at 37 °C and 135 rpm. The cell-free extract was obtained by centrifuging the culture at 10,000 rpm for 10 min under ice-cold conditions. The supernatant is hereafter referred to as a cell-free extract. A drop of cell-free extract from diesel oil-supplemented media was placed on a hydrophobic surface. The presence of biosurfactant in the cell-free extract was assessed based on its ability to destabilize the drop within 1 min and high-resolution photographs were captured during the process [231]. The presence of biosurfactant in the drops makes the drop collapse but drops of non-surfactant compounds remain stable [231].

3.6 Determination of the emulsification index of the cell-free extract

The emulsification index of bacterial culture was determined by rigorously vortexing 2.5 mL of diesel with equal volume of bacterial cell-free extract for 60 sec followed by incubation at 37°C for 24 h. The emulsification index (E_{24}) was calculated using the following equation (**Eq. 3.2**) [232]:

$$\text{Emulsification Index } (E_{24}) = \frac{Height\ of\ the\ emulsion\ layer\ (mm)}{Total\ height\ of\ the\ solution\ (mm)} \times 100 \qquad (\textbf{Eq. 3.2})$$

3.7 Identification of the best isolate

3.7.1 Microscopic identification

A bacterial smear was prepared on glass slides by inoculating the pure culture in a drop of water and then heat fixed by passing through the flame of a Bunsen burner. The smear was flooded with crystal violet for 30 seconds. After gently washing under slowly running tap water, the slide was flooded with Gram's iodine (mordant) for 60 seconds. Again, the slide was washed with ethanol and counter-stained with safranin for 30 seconds [229, 233]. The slide was then gently washed with water and allowedto air dry. The dried slide was observed under the 100X magnification of a light microscope using immersion oil.

3.7.2 Molecular identification

The genomic DNA from the best isolate was extracted by detergent lysis method [234]. About, 25 mL of overnight bacterial broth was centrifuged at 6,000 rpm for 10 min. The supernatant was discarded and the pellet was re-suspended in 5 mL of lysis buffer (composition g/L: 50 mM Tris HCl- 0.79, 20 mM EDTA- 0.744, SDS-1.25, distilled water-100 mL, pH-8.2) and gently vortexed. The solution is kept in incubator for 30 min at 65 °C. Then, 3 mL of 3M $C_2H_3NaO_2$ is added to the mixture and kept in incubator for 30 min under ice cold solution. The mixture is centrifuged at 10,000 rpmfor 10 min and to the supernatant, double volume of chilled acetone was added. Following which, the mixture was incubated for 1 h under ice cold condition. After incubation, the mixture is centrifuged at 12,000 rpm for 10 min and the pellet is air dried. The pellet is then dissolved in 100 µL of 1X TE buffer. The DNA precipitation was observed after the addition of chilled ethanol [234]. Briefly, molecular identification of the best isolate was done by 16S rDNA gene sequencing using P3- forward (5´-AGA GTT TGA TCA TGG CTC AG-3´) and P13-reverse (5´-GGT TAC CTT GTT ACG ACT T-3´) primers. The consensus sequence thus generated was analyzed with the help of the NCBI-BLASTn tool and the phylogenetic tree was constructed by using the neighbor-joining method in MEGAX^{TM} software setting the bootstrap value at 1000 by aligning the top 10 hits retrieved from the NCBI database [97]. The consensus sequence was then submitted to NCBI GenBank database to obtain the GenBank accession number.

3.8 Extraction and characterization of crude biosurfactant

3.8.1 Extraction of crude biosurfactant

The cell-free media obtained from the best isolate was used for the extraction of crude biosurfactant using a pre-standardized cold acetone precipitation method [236]. Briefly, the cell-free extract from the 7-day-old bacterial culture was mixed with double the volume of chilled acetone and kept overnight with constant stirring [237, 238]. After overnight stirring, the layer formed due to the precipitation of biosurfactant at the bottom was recovered by centrifuging at 10,000 rpm for 10 min and the pellet was air-dried [227, 237] for further use.

3.8.2 Biochemical characterization of the crude biosurfactant

Initially, the crude biosurfactant was subjected to anthrone and ninhydrin tests to estimate the presence of carbohydrate and amino acid moieties respectively [97, 235]. A small amount of crude biosurfactant was taken and mixed with 2 mL of anthrone reagent. The solution was thoroughly mixed and observed for color change from light yellow to bluish-green to confirm the presence of carbohydrates in the crude biosurfactant. Similarly, a few drops of ninhydrin solution were mixed with crude biosurfactant solution and the mixture was kept in a boiling water bath for 5 min. Formation of the purple or yellow-colored complex indicates the presence of amino acid moieties and specifically proline respectively in the crude biosurfactant.

3.8.3 FTIR analysis of the crude biosurfactant

The FTIR spectrometric analysis of the biosurfactant was done within the range of 4000-500 cm^{-1} to determine the presence of various functional groups. The sample was prepared by grinding about 5-10 mg of crude biosurfactant and KBr [196]. The mixture is pressed properly until it becomes translucent. The biosurfactant and KBr mixed pellet were used for FTIR analysis [239] (Brucker Corporation, USA).

3.8.4 SEM analysis of the crude biosurfactant

The crude biosurfactant was observed by using high and low-magnification scanning electron microscopes to analyze the surface morphology (Model JEOL JSM-IT300 Japan Ltd.) [240].

3.9 Synthesis of Ag and ZnO NPs:

The Ag NPs were synthesized by reducing 10 mL (1 mM) $AgNO_3$ solution with 50 mg of bacterial biosurfactant under 121 ºC, and 15 lbs pressure for 15 min using an autoclave. The solution was centrifuged at 10,000 rpm for 10 min after being brought back to room temperature to settle down the Ag NPs [97, 241].

Similarly, the ZnO NPs were synthesized by reducing 10 mL (1 mM) $ZnCl_2$ solution with 50 mg of bacterial biosurfactant following the above-mentioned steps.

The pellets of both the NPs were then dried into powder at 100 °C using a hot plate and kept for further use [242].

3.10 Characterization of the NPs

The Ag NPs and ZnO NPs were characterized and studied by using various analytical techniques such as FTIR (within the spectral range of a 4000-500 cm^{-1}), SEM-EDX, HR-TEM (JEM-2100 Plus Electron Microscope, Japan Ltd.), and XRD (ULTIMA IV, Rigaku Japan Ltd.) [243]. The thermal stability of the Ag NPs was analyzed by differential thermal analysis (DTA) and thermo-gravimetric analyzer (TGA) (NETZSCH STA 44F3 Gmbh, Germany).

3.11 Metal NPs mediated seed germination and plant growth assay

3.11.1 Nanopriming of seeds

Healthy chickpea (*Cicer arietinum*) seeds (n = 50) were surface sterilized with 70% ethanol for 2 min followed by washing with distilled water for 2 min. The sterilized seeds were then primed with different concentrations of Ag NPs (10, 20, 30, and 40 mg/L) for 3 h. Seeds treated with distilled water, and different concentrations of AgNO3 (10, 20, 30, and 40 mg/L) were taken as control. After priming, the seeds were then placed on moistened cotton beds and kept in dark condition for 3 days and the growth of the total length of seedlings was observed every day [244]. Similarly, healthy rice (*Oryza sativa*) seeds (n = 50) were primed for 3 days and kept in dark condition for another 6 days.

Similar steps were also followed to prime chickpea and rice seeds (n = 50) with ZnO NPs. The treated seeds were analyzed for seed water uptake, length of the germinated seedlings, germination rate, alpha-amylase activity, and total soluble sugar content.

3.11.2 Seed water uptake assay

Seeds were weighed before and after priming. Water uptake by chickpea and rice seeds was observed by measuring the difference in weight of the seeds before and after priming which was calculated by the following formula (**Eq. 3.3**) [245]:

$$\text{Water uptake } (\%) = \frac{Weight\ of\ seeds\ after\ priming - Weight\ of\ seeds\ before\ priming}{Weight\ of\ seeds\ before\ priming} \times 100$$

(**Eq. 3.3**)

3.11.3 Assessment of germination percentage and the length of the germinated seedlings

Nanoprimed chickpea and rice seeds were assessed for the determination of their germination percentage after 3rd & 6th day of treatment respectively and were determined by the following formula (**Eq. 3.4**) [246]:

$$\text{Germination rate } (\%) = \frac{No.of\ the\ seeds\ germinated}{Total\ no.of\ seeds} \times 100 \qquad \textbf{(Eq. 3.4)}$$

The length of the germinated chickpea seedlings was measured on each day of incubation till the 3rd day. Similarly, the primed rice seeds were checked for the length of the germinated seedlings on each day of incubation till the 6th day.

3.11.4 Alpha-amylase activity assay

The concentration of α-amylase in terms of maltose content in the treated seeds was determined using dinitrosalicylic acid (DNS) reagent by following the method described by [244]. The α-amylase extraction was done by homogenizing one gram of primed seeds with 6 mL of chilled $CaCl_2$ (1M) under ice-cold conditions followed by centrifugation at 12,000 rpm for 25 min. Precisely 100 µL of the supernatant (enzyme extract) was mixed with 900 µL of distilled water and 1000 µL of 1% starch solution was added to it. The mixture was then incubated at 37 ºC for 15 min and 1000 µL of DNS reagent was added to it. The solution was then kept at 90 ºC in a water bath for 5 min. After cooling, the total volume of solution was made up to 10 mL using distilled water and absorbance was taken at 540 nm [244]. The α-amylase activity exerted in

terms of maltose content was evaluated by comparing it with the standard graph [247].

3.11.5 Total soluble sugar content

One gram of seeds was homogenized in presence of 5 ml of 95% ethanol and centrifuged at 10,000 rpm for 10 min for the determination of soluble sugar (glucose) in the primed seeds [244]. From the above, 1000 µL of supernatant was taken and 5 mL of anthrone was added to it. The mixture was incubated at 90 ℃ for 17 min and the absorbance was recorded at 620 nm [244, 247]. The amount of soluble sugar present in the primed seeds was calculated by comparing the data with the standard graph prepared from glucose.

3.12 Toxicity assay of the NPs

3.12.1 Cytotoxicity assay of the NPs

Cytotoxicity assay was performed on red blood cells. Precisely, 1000 µL of defibrinated sheep blood was taken and centrifuged at 1,000 rpm for 5 min at 4 ℃. The pellet was then washed with 2 mL PBS (phosphate buffer saline) and thencentrifuged at 1,000 rpm for 5 min at 4 ℃ three times. The RBC sample mixture was obtained by adding 20 mL of PBS to the centrifuged pellet and thoroughly mixing. Precisely, 180 µL of RBC sample was incubated at 37 ℃ in the presence of 20 µL of different Ag NPs and ZnO NPs concentrations (10, 20, 30, and 40 mg/L) for 3 h. The tubes were placed on ice for 5 min to stop the reaction after incubation and agitation for 10 min. Finally, the mixture was centrifuged at 2,000 rpm for 5 min maintaining 4 ℃ and 100 µL of the supernatant was used to obtain the absorbance spectrophotometrically at 540 nm. The degree of hemolysis was calculated using the following formula (**Eq. 3.5**) [94, 248-250]:

$$\text{Hemolysis (\%)} = \frac{\text{OD540 of sample} - \text{OD540 of negative control}}{\text{OD540 of positive control} - \text{OD540 of negative control at 540 nm}} \times 100$$

(**Eq. 3.5**)

3.12.2 Environmental toxicity assay of NPs

An environmental toxicity assay was performed by taking earthworms as a bio-indicator. Twenty earthworms (*Eudrilus eugeniae*) were allowed to interact for 1 h with 10 mL of Ag NPs and ZnO NPs (40 mg/L concentration each). One set of controls was treated with distilled water maintaining the same conditions. After 1 h of incubation, each set of earthworms was placed in a separate beaker with their feeding material and observed for 6 days. The treated earthworms were observed for any type of phenotypic changes such as color change, behavioral change, and survival rate. One earthworm from each test and control was taken and washed with 0.9% NaCl and 10% formalin. The gut region was dissected by using sterile blades and kept in 10% formalin for 24 hr. The tissue material was then washed properly in running water overnight followed by processing with different grades of alcohol (10-100%) each for 15 min with 2 changes and cleaning with xylene for 10 min with 2 changes. The tissues were then kept in half xylene and half paraffin for 30 min in a water bath followed by complete paraffin at least for 8 h [251]. Tissues from each set of samples were then fixed by paraffin block preparation and the blocks were kept in water overnight. The fixed blocks were then sliced into thin sections using microtomy and subjected to hematoxylin and eosin staining. After the staining process was done, the tissue slides were visualized under 100X magnification of a light microscope [251].

3.13 Antimicrobial activity of Ag NPs and ZnO NPs

The Ag NPs and ZnO NPs were tested for their antimicrobial activity against *Ralstonia solanacearum* F1C1 and *Fusarium oxysporum* f. sp. *pisi* (van Hall) Synder & Hansen strain 4814 by using well diffusion method. Muller-Hinton (MH) agar (composition g/L: HM infusion B from beef infusion: 300.0, acicase- 17.5, starch- 1.5, agar- 17.0, final pH- 7.3±0.1 at 25 ºC) plates and PDA (Potato Dextrose Agar) (composition g/L: potatoes, infusion from- 200.0, dextrose- 20.0, agar-agar- 15.0) plates were prepared respectively for *Ralstonia solanacearum* F1C1 and *Fusarium oxysporum* f. sp. *pisi* (van Hall) Synder & Hansen strain 4814 and 100 µL of overnight culture of each test microbe was spread plated after adjusting the overnight culture with 1.0 McFarlends standard. Three wells were made in each plate using a cork borer and each well was loaded with 50 µL of 1 mg/mL of NPs (*i.e.*, Ag

NPs and ZnO NPs), 1 mM of their respective salts (*i.e*., $AgNO_3$, and $ZnCl_2$), 5 mg/mL of biosurfactant solution. These were then incubated at 37 °C for 24 h. A clear zone around the well represents a positive antimicrobial result after incubation and the diameter of the zone of inhibition was also tabulated [252]. The efficiency of antimicrobial potential of Ag NPs and ZnO NPs were also checked in terms of MIC (minimal inhibitory concentration) and MBC (minimal bactericidal concentration) or MFC (Minimum Fungicidal Concentration) using a 96 well broth microdilution method [253]. According to the National Library of Medicine, MIC is defined as the minimum concentration of any antimicrobial substance that completely inhibits the visible growth of the test strain [254]. MBC or MFC is defined as the minimum concentration at which the antimicrobial substance kills 99.9% of the test strain [255]. The MIC, MBC and MFC values of Ag NPs and ZnO NPs against *Ralstonia solanacearum* F1C1 and *Fusarium oxysporum* f. sp. *pisi* (van Hall) Synder & Hansen strain 4814 were determined by standard broth microdilution method in 96-well plate by using MTT (3-[4,5-dimethylthiazol-2yl]-2,5 diphenyl tetrazolium bromide) [256]. Each well was loaded with 40 µL of fresh sterile nutrient and potato dextrose broth individually for *Ralstonia solanacearum* F1C1 and *Fusarium oxysporum* f. sp. *pisi* (van Hall) Synder & Hansen strain 4814 respectively. Precisely, 10 µL of inoculum, Ag NPs and ZnO NPs solution was added by maintaining the concentration 0.01, 0.02,....0.1 mg/mL. Broth inoculated with the test organism was taken as the control solution and kept in incubation for 24 hr at 37 °C for *Ralstonia solanacearum* F1C1 and 5 days incubation for *Fusarium oxysporum* f. sp. *pisi* (van Hall) Synder & Hansen strain 4814 at 30 °C. After incubation, 20 µL of MTT dye was added to each well. The change in color from yellow to black shows the presence of live cells. The lowest concentration of NPs which showed a significant level of inhibition of growth was considered as MIC and the concentration of NPs which showed 99.9% prevention of growth was considered as MBC and MFC value [256, 257].

3.14 Statistical analysis

All the results are expressed in mean ± S.D. as an outcome of triplicately done experiments and the statistical significance of the results was analyzed by performing student's t-test using Graphpad™ online software.