Antibacterial efficacy of silver nanoparticles and ethyl acetate's metabolites of the potent halophilic (marine) bacterium, *Bacillus cereus* A30 on multidrug resistant bacteria

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ABSTRACT

Bacteria are generally responsible for the prevalence of several diseases and pathogenic bacteria are showing increasing resistance to different antibacterials. During the present study an extremophilic bacterium-A30 isolated from the marine waters was characterized and evaluated against four multi-drug resistant (MDR) pathogens, viz; Methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*. The sensitivity pattern of the selected pathogens was tested with 31 antibiotics. Among the 47 marine microbial extracts tested on 4-MDR pathogens viz: Methicillin-resistant *Staphylococcus aureus* (MRSA), *E. coli*, *K. pneumoniae* and *P. aeruginosa*, only our strain A30 strain exhibited highest efficacy. This strain was subsequently subjected to 16S rDNA sequencing which confirmed its allocation as *Bacillus cereus*. Silver nanoparticle (AgNPs) synthesis and ethyl acetate extraction were performed using the supernatant of *B. cereus*. The synthesized AgNPs were characterized by UV-Visible, Fourier-transform infra-red (FT-IR), X-ray diffraction (XRD), Field emission-scanning electron microscopy (FE-SEM), energy dispersive X-ray spectroscopy (EDX), and Zeta potential analyses. The presence of functional groups and 13 bioactive components in the ethyl acetate extract were analyzed using FT-IR and gas chromatography-mass spectrometry (GC-MS). The synthesized AgNPs and the ethyl acetate extract showed preponderant activity against *P. aeruginosa* and MRSA, respectively. The effects of AgNPs were significant when compared to ethyl acetate extract. Therefore, the halophilic bacterium, *B. cereus* mediated AgNPs could provide antibacterial applications in the biomedical industries.

KEYWORDS

Extremophilic bacteria; silver nanoparticle; GC-MS; FT-IR; FE-SEM; MDR pathogen; MIC; cytotoxicity assay

1. Introduction

Marine microbes are considered to be a rich natural source for structurally varied bioactive compounds. Among the producers of industrially significant bioactive metabolites, halophilic bacteria have the ability to produce substantial varieties of biologically active secondary metabolites [1]. Halophilic microorganisms have recently been shown to possess advantages for the above mentioned desirable properties [1]. Halophiles are those microorganisms that require salt for their growth, and they can be found in all three domains of microbial life viz., archaea, bacteria and eucarya [2]. Halophiles can be found distributed in various hypersaline environments like saline lakes, salt pans or salt marshes [3]. According to the salt concentration for optimal growth, halophiles can be roughly divided into two groups, viz; moderate and extreme halophiles [4]. A moderate halophile grows at salt concentration of 3–15% (w/v) that can tolerate up to 0–25% (w/v) [5]. A large number of phylogenetic subgroups contain many types of halophilic bacteria, most

of which belong to the family, Halomonadaceae [6]. A decade ago, the microbes of *Bacillus marinus*, *B. subtilis*, *B. pumilus*, *B. licheniformis*, *B. cereus* and *B. mycoides* were reported to be common inhabitants of Pacific Ocean habitats [7]. Several halophiles from water samples were found to have a high GC content, viz; *Arthrobacter*, *Vibrio*, *Bacillus* and *Erythrobacter* [8]. To thrive in the hypersaline environment, halophiles have two main adaptive mechanisms, especially to prevent NaCl from diffusing into the cells. The first mechanism is the accumulation of inorganic ions (mainly KCl) for balancing osmotic pressure. This mechanism is mainly utilized by aerobic and extremely halophilic archaea and some anaerobic halophilic bacteria [6]. In contrast, most halophilic bacteria and eukaryotes accumulate water soluble organic compounds of low molecular weight, which are referred to as compatible solutes or osmolytes, to maintain low intracellular salt concentrations [2,4,9]. Compatible solutes can act as stabilizers for biological structures by allowing the cells to adapt not only to salts but also to heat, desiccation, cold or even freezing conditions [10,11].

Furthermore, the halophiles can grow at a pH of 10 and temperatures up to 50 °C [12]. Many halophilic bacteria accumulate ectoine or hydroxyectoine as the predominant compatible solutes. Other intracellular compatible solutes include amino acids, glycine betaine that are accumulated in small amounts [13].

Nanoparticles (NPs) are of great interest to the scientific community, particularly in the medical field because of their unique properties. In view of their high chemical and thermal stability, low cost, and ecofriendly nature and due to their potential in medical applications, the biological method of synthesis of AgNPs has recently received much attention of researchers. However, there are challenging issues in current nanotechnology that include the development of reliable experimental techniques for the synthesis of nanoparticles of different compositions and sizes along with high monodispersity [14]. Use of microbes such as bacteria, fungi and yeasts for the synthesis of NPs is a new and exciting area of research. Silver is considered to be one of the most essential nanoparticles because of its peculiar physico-chemical properties [15]. The AgNPs have been shown to be more efficient as it has pronounced antimicrobial efficacy against bacteria, viruses and some protists [16]. The bio-reduction of Ag⁺ to colloidal Ag by microbes in aqueous solutions is a stepwise process, where several composites of Ag⁺ are reduced to metallic Ag atoms, followed by the agglomeration of Ag⁰ into oligomeric clusters [17]. Finally, these clusters are converted into AgNPs. Also, microbes might entrap target ions from the reaction mixture, retaining the reduced metal in its elemental state through enzymes produced by microbial activities [18]. In an earlier study, the first halobacterium used for the synthesis of AgNPs was *Halococcus salifodinae* [19]. The present study focusses on the characterization and identification of the most effective marine bacterium against MDR pathogens, to synthesize and characterize AgNPs from that strain, and to evaluate the efficacy of resulting AgNPs against MDR pathogens.

2. Experimental part

2.1. Materials and instruments

Marine water samples were collected from Rameswaram (9.2876° N, 79.3129° E) at 10 m from the coastline and 5 m depth in sterilized containers by Scuba Diving. The collected samples were transported to the laboratory. Silver nitrate (AgNO₃) was purchased from ACROS, (Geel, Belgium). The culture medium and antibiotics used in this study were procured from HiMedia (Mumbai, India). The clinical pathogens Methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* were obtained from SKS hospital, Salem (India). All other reagents and the solvents used were purchased from Merck (Mumbai, India) which were of analytical grade with maximum purity.

2.2. Culture conditions and isolation of halophilic bacteria

Ten microliters of sample were spread over the surface of the Zobell Marine agar medium (55.25 g L⁻¹) with various NaCl concentrations (0–25% w/v) and incubated at 30 °C. The pH was adjusted to 7.6 ± 0.2 before autoclaving. Bacterial colonies were observed after 7 days of incubation. These halophilic bacterial strains were sub-cultured in halophilic agar medium at 30 °C for 5 days; a single colony of each strain was picked up and incubated at 30 °C for two weeks for obtaining heavy growth. Totally, 47 isolates were purified based on the nature of morphology. Axenic cultures were stored at –80 °C in marine broth supplemented with 40% glycerol for further studies. The isolated strains were tested against four clinical pathogens (MRSA, *E. coli*, *K. pneumoniae* and *P. aeruginosa*) by the Sagar well diffusion method. The cell free supernatant (100 µL) of 47 strains was loaded in respective wells (at a diameter of 6 mm) on Mueller Hinton Agar (MHA) plates (38 g L⁻¹) and the zones of inhibition were measured. Among the tested supernatants, the A30 strain showed maximum inhibitory zones in all treated pathogens. Hence, the strain A30 was selected for further analysis. Total genomic DNA from the selected strain was extracted and purified using Thermo Scientific Gene JET Kits (K0721, K0722) according to the manufacturer's protocol. PCR amplifications of 16S rDNA gene fragments were performed using Hi-Chrom PCR Master Mix (2X) kit (Hi-Media, Mumbai, India) according to the supplier's instructions. The primer sets used for amplifying and sequencing the strain A30 were 27F (5' AGAGTTTGATCMTGGCTCAG 3') and 1525R (5' AAGGAGGTGATCCAGCCGCA 3'). The PCR amplification was performed using the thermal cycler (96 Well–Applied Bio-systems) program. An aliquot of 30 µl of PCR reaction products was electrophoresed on a 1.2% agarose gel containing ethidium bromide (10 mg mL⁻¹ in H₂O) and the DNA bands were visualized under UV light. PCR products were purified using QIAquick PCR clean up kit according to the supplier's instructions and then sequenced by an automated sequencer. DNA sequences were determined by the dideoxy chain termination method [20]. The nucleotide sequence data were submitted to the BLAST programs search nucleotide data bases (<http://www.ncbi.nlm.nih.gov>) within the National Center of Biotechnology Information (NCBI), GenBank [21].

2.3. Synthesis of AgNPs and preparation of ethyl acetate extract

The marine bacterium, *B. cereus* A30 strain was grown in 250 ml Erlenmeyer flasks containing 100 mL of nutrient broth prepared in marine water with an optimized pH of 7.2. The 48 h grown bacteria were separated from the culture broth by centrifugation (8000 rpm) for 15 min.

The 40 mL of collected cell-free supernatant from A30 strain was suspended in 60 mL of 5 mM aqueous silver nitrate solution and was kept at room temperature for 7 days at dark condition. After the formation of a brown coloured solution, it was subjected to an ultrasonication bath for 30 min in order to separate nanoparticles from the bio-organic complex with nanoparticles. The filtrate solution was centrifuged for 45 min at 12,000 rpm to collect bio-reductant nanoparticles.

Bioactive compounds extracted by ethyl acetate were recovered from the culture supernatant of the freshly grown halophilic bacterium, *B. cereus* by the solvent extraction method (acid-based extraction method). Equal volumes of culture supernatant of liquid broth and ethyl acetate were mixed and the organic phase was separated by using separating funnels. Then, this was repeated with saturated NaCl (brine solution) instead of culture supernatant of liquid broth. To remove the water content in the organic phase, sodium sulphate anhydrous was added until freely floating. The organic phase was filtered and concentrated by evaporation and the crude extract was sorted for further characterization of bioactive compounds.

2.4. Characterization of AgNPs and ethyl acetate extract

The synthesized aqueous AgNPs were preliminarily measured in a CYBERLAB UV-100, spectrophotometer operated at a resolution of 1 nm. A standard UV-absorbance range (200–800 nm) was used to plot the strong surface plasmon resonance of AgNPs. The filtrate solution of synthesized NPs was dried, powdered and used for XRD analysis. The crystallographic spectrum of the AgNPs was recorded by Rigaku Miniflex II, Japan advance X-ray diffractometer. The analysis of the functional spectrum of the supernatant and AgNPs were done by ART model FT-IR Spectrophotometer (Perkin Elmer RX1, Germany). The KBr pellet technique was used for frequency annotation and the spectrum was focussing on the mid IR region of 400–4000 cm^{-1} which was adopted by Attenuated Total Reflectance (ART) technique beach measurements. The FE-SEM micrographs were acquired by Carl Zeiss, SIGMA instrument (Oberkochen, Germany). X-ray diffraction analysis (Philips X'Pert Pro X-ray diffractometer, Homberg/Nieder-Ofleiden, Germany) was performed by preparing a thin film of powdered SNPs. The average particle size distribution and stability of nanoparticles was measured by Malvern Zeta-sizer (Nano ZS90, Malvern WR14 1XZ, UK) instrument [22]. Similarly, the FTIR spectroscopic analysis of the ethyl acetate extract was done by ART model FT-IR Spectrophotometer. GC-MS analysis was performed to identify the bioactive compounds in

the cell free supernatant of the ethyl acetate extract of *B. cereus*. Identification of compounds was done by injecting 1 μL of sample into a RT x – 5 column (30 $\times$ 0.25 nm) of GC-MS model, Perkin Elmer, Clarus 680 and helium (1 mL min^{-1}) was used as a carrier gas. The following temperature gradient program was used: 60 $^{\circ}\text{C}$ for 2 min followed by an increase from 60 to 300 $^{\circ}\text{C}$ at a rate of 10 $^{\circ}\text{C per min}$ and finally 6 min at 300 $^{\circ}\text{C}$. The m/z peaks representing mass to charge ratio characteristics of the antimicrobial fractions were compared with those in the mass spectrum library of the corresponding organic compounds [23].

2.5. Susceptibility testing

Disc diffusion method: The MHA plates were prepared and seeded with four clinical pathogens (MRSA, *P. aeruginosa*, *E. coli* and *K. pneumoniae*) that were diluted at a standard concentration (approximately 1 to 2×10^8 CFU mL^{-1}), respectively. Commercially prepared antibiotic discs of 31 different antibiotics at a standard concentration were tested against these pathogens. Then each antibiotic disc was evenly dispensed and lightly pressed onto the agar surface. The test antibiotics did immediately begin to diffuse outwards from the discs, creating a gradient of antibiotic concentration in the agar such that the highest concentration was found close to the disc with decreasing concentrations further away from the disc [24,25]. After an overnight incubation at 37 $^{\circ}\text{C}$, the zone of inhibition was measured. The zone of inhibition was compared to a standard interpretation chart used to categorize the isolate as susceptible, intermediate susceptible, or resistant.

2.6. Antibacterial activity of the cell free supernatant, AgNPs and ethyl acetate extract

The antibacterial susceptibility of the cell free supernatant, AgNPs, and ethyl acetate extracts were evaluated using the well diffusion method. The prepared test samples of AgNPs (5 mg mL^{-1}) and the ethyl acetate extract (5 mg mL^{-1}) with different concentrations (25, 50, 75 and 100 μL), respectively, were tested with four clinical pathogens, that were seeded at a concentration of 10^8 CFU mL^{-1} . The selected pathogens, MRSA, *E. coli*, *K. pneumoniae* and *P. aeruginosa* were swabbed evenly on the surface of the medium and wells (6 mm diameter) were cut out from the agar plates using a sterile stainless-steel borer and the test samples were loaded, accordingly. Then, the plates were incubated at 37 $^{\circ}\text{C}$ for 24 h and the next day the zone of inhibition was measured in millimeter (mm). This experiment was carried out in triplicate for the confirmation of results.

2.7. Determination of minimal inhibitory concentration of synthesized AgNPs and ethyl acetate extract

Micro-dilution method: The antimicrobial activity of the AgNPs and ethyl acetate extract of *B. cereus* were tested against the clinical pathogens, *P. aeruginosa*, *K. pneumoniae*, *S. aureus* and *E. coli*. The micro-dilution method was used to identify the minimum inhibitory concentration (MIC) quantitatively. MIC values of test samples were determined based on a micro-well dilution method using a 96-well sterile microtiter plate [26]. The synthesized AgNPs and ethyl acetate at different concentrations (3.15, 6.25, 12.5 and 25 μ l) were selected with reference to the initial concentration (25 μ l) of antibacterial activity by the well diffusion method. From the bacterial suspension containing 10^6 CFU ml⁻¹ bacteria, 50 μ l was inoculated in wells. The bacterial suspension (as control) without test samples served as a CFU growth control. The 96-well microtiter plate was read at 595 nm to measure optical density (OD) after 24 h at 37 °C and the OD of the each well was recorded every 4 h up to 24 h.

2.8. Cytotoxic assay of cell free supernatants, AgNPs and ethyl acetate extracts on *Artemia salina*

Cytotoxic effects of culture supernatants, ethyl acetate extracts, and AgNPs were determined using a brine shrimp lethality assay, following the method of Bibi et al., [27]. Brine shrimp (*Artemia salina*)-eggs were hatched to nauplius larvae in sea water (3% salt solution). Final concentrations of 25, 50, 75, and 100 μ l of test samples were used in this assay. Twenty-five *artemia* nauplii were transferred to vials containing 2.5 ml of sea water using pasteurized pipettes. Then the volume was raised to 2.6 ml with sea water. These vials were allowed to stay for 24 h at 28°C temperature. Surviving and dead nauplii were counted using a magnifying glass and the LD₅₀ was subsequently calculated as following: Mortality (%) = $X - Y/X \times 100$ where X was the survival in the untreated control and Y was the survival in the treated sample.

2.9. Statistical analysis

The data analyses were done using the SPSS Statistical Software Package version 20.0. One-way ANOVA was performed with Tukey's honest significant difference (HSD) test by comparing the antimicrobial activity of the test samples of the bacterial pathogens, respectively. In all analyses, a probability level of $p < 0.05$ was used as the significance of differences between values. The difference between the groups was considered to be significant when the P value was less than 0.05, moderately and highly significant when the P value was less than 0.01, and 0.001, respectively.

3. Results

3.1. Identification of halophiles from marine water samples

Out of the 47 marine isolated strains, only the A30-strain exerted maximum inhibitory zones of the 4 MDR bacterial strains. The pale yellowish colored A30 strain cultivated on marine agar medium were found to be rod shaped gram-positive bacilli (Figure 1(a) and (b)). The amplification and sequencing of the 16S rDNA resulted in a 1252 base pair long nucleotide sequence that was blasted with existing nucleotide sequences in the National Centre for Biotechnology Information (NCBI). The BLAST and pair-wise two sequence alignment analysis revealed 99–100% identity with the sequences available from *Bacillus cereus* (Figure 1(c) and (d)). Our sequence was confirmed and deposited in Genbank, as *Bacillus cereus* A30 and received the accession number, KX369381.

3.2. Synthesis and characterization of AgNPs

The formation of AgNPs from the cell free supernatant of *B. cereus* A30 strain was confirmed by the appearance of pale brown color on the next day of the reaction and further, the complete reaction of brown colored solutions was observed after 6 days (Figure 2(a)). The UV absorbance value of bio-reduced AgNPs was recorded with a strong surface plasmon resonance peak at 420 nm (Figure 2(b)). The recorded XRD spectrum of AgNPs confirmed their crystalline nature by 2θ values of 32.08°, 38.03°, 46.23°, 57.80° and 76.89° that can be indexed to the (1 0 1), (1 1 1), (2 0 0), (2 1 0) and (3 1 1) Bragg's reflections of cubic structure of silver (JCPDS card No. 04-0784), respectively (Figure 3). The formation of nanoparticles was indicated by the broadening of Bragg's peaks. The average size of AgNPs was calculated using Debye-Scherrer's equation by determining the width of the (1 1 1) Bragg's reflection. The crystalline size was calculated from the width of the XRD peaks by using Scherrer's formula; $D = k\lambda/\beta\cos\theta$; where D is the crystallite size, k the Scherrer coefficient, λ the wavelength of X-rays, β the full width half maxima (FWHM), θ Bragg's angle (half of 2θ). The average crystalline size of the synthesized SNPs was approximately 44 nm. And the joint FTIR analysis was employed to identify the functional groups responsible for the bio-reduction of silver into the respective nanoparticles. The frequency peaks obtained at the wave number for the AgNPs (Figure 4(a)), (3403 cm⁻¹: alcohols, phenols (O–H stretch, H-bonded); 2223 cm⁻¹: alkynes–C $\equiv$ C– stretch); 1574 cm⁻¹: nitro compound (N–O asymmetric stretch); 1401 cm⁻¹: aromatics C–C stretch (in-ring); 1486 cm⁻¹: aromatics (C–C stretch); 1123 cm⁻¹: aliphatic amines (C–N stretch); 923 cm⁻¹: alkenes (O–H bend); 831 cm⁻¹: alkyl halides (C–Cl stretch); 763 cm⁻¹: alkenes (=C–H bend); 618 cm⁻¹: alkyl halides (C–Br stretch)), cell free supernatant (Figure 4(b)) (3432 cm⁻¹:

Figure 1. Photograph showing the *B. cereus* A30 (A) Culture plate, (B) Gram staining, (C) Genomic DNA, and (D) PCR amplified products.

alcohols, phenols (O–H stretch, H-bonded); 2989 cm^{-1} : alkanes (C–H stretch); 2613 cm^{-1} : aldehydes (H–C=O: C–H stretch); 2491 cm^{-1} : nitriles (C≡N – stretch); 2093 cm^{-1} : alkynes (C≡C stretch); 1599 cm^{-1} : primary amines (N–H bend); 1377 cm^{-1} : alkenes (C–H rock); 1056 cm^{-1} : aliphatic amines (C–N stretch); 929 cm^{-1} : carboxylic acids (O–H bend); 848 cm^{-1} : alkyl halides (C–C stretch); 784 cm^{-1} : alkyl halides (C–C stretch); 624 cm^{-1} : alkyl halides (C–Br stretch) and ethyl acetate extracts (Figure 4(c)) (3447 cm^{-1} : alcohols, phenols (O–H stretch, H-bonded); 1636 cm^{-1} : amides (NH stretch); 1400 cm^{-1} : carboxylic acids (C–O stretch); 662 cm^{-1} : alkynes (–C≡C–H: C–H bend) represents the corresponding functional groups with their respective stretches. The magnified FESEM confirms that the AgNPs were in well-resolved spherical structure with soft surfaces (Figure 5(a)). FESEM results also revealed morphological characteristics of the synthesized AgNPs that were spherical in shape and the sizes ranged between 24 and 46 nm (Figure 5(b)). EDX profile peaks confirmed the presence of silver (Ag), followed by chlorine (Cl) and oxygen (O) signals. The percentage of different elemental signals was represented as the EDX profile (Figure 5(c)). The size distribution analysis of the capped silver nano-conjugates confirmed that the particles were well dispersed. The Zeta potential measurements of the biosynthesized AgNPs revealed a

sharp peak at –26.3 mV (Figure 5(d)) suggesting that the surface of the NPs was negatively charged that would be dispersed in the medium. The electrostatic repulsive forces between the nanoparticles upon charged negatively may prevent from aggregation that attributed for the stable nature of AgNPs. In GC-MS analysis, the chromatogram of the ethyl acetate extract of *B. cereus* and the peaks representing the chemical structure of the compounds are shown in Figure 6. Totally, 31 peaks were identified after comparing the mass spectra with WILEY and NIST libraries, that indicate the presence of 13 bioactive components (Figure 6). Their active principles with retention time (R_t), molecular formulae, molecular weight and concentration (peak area %) are presented in Table 1. Among 13 major bioactive compounds, 11 are of the compound Hentriacontane with a maximum peak area percentage (87.44% of total) and the rest were of Distearyl thiodipropionate (11.70%) and 1, 2-benzenedicarboxylic acid, mono-(2-ethylhexyl) ester (0.86%).

3.3. Antibiotic susceptibility test

The antibiotic sensitivity pattern of the MDR pathogens due to 31 antibiotics showed varied ranges of inhibition (Table 2). Among the pathogens tested, *E. coli* showed highest resistance to 14 antibiotics, followed by

Figure 2. Cell free extract nanoparticles with AgNO_3 (A) and UV visible spectrum of absorbance (B).

Figure 3. XRD spectrum of synthesized AgNPs.

P. aeruginosa (13), MRSA (2) and *K. pneumoniae* (1). But good sensitivity was exhibited by *K. pneumoniae* MRSA, *E. coli* and *P. aeruginosa* to 19, 14, 7 and 3 antibiotics, respectively. The cell free supernatant of all bacterial isolates was treated against the four selected MDR pathogens, in which strain A30 showed a pronounced activity. Furthermore, the synthesized AgNPs and ethyl acetate extract of strain A30 were used to assess their antibacterial potential at their varied concentrations.

3.4. Antibacterial activity

Antibacterial activity of synthesized AgNPs and ethyl acetate extract was evaluated using well diffusion method against the selected Gram positive and Gram negative clinical pathogens. The antibacterial activity of AgNPs (Figure 7) and ethyl acetate extract (Figure 8) exhibited significant results at the concentration range of $25\text{--}100\ \mu\text{g mL}^{-1}$, respectively. The highest antibacterial activity of AgNPs was found at $100\ \mu\text{g mL}^{-1}$ against *P. aeruginosa*, followed by *K. pneumoniae*, *E. coli*, and MRSA (Figure 9(a)). Similarly, the ethyl acetate exerted a maximum zone of inhibition at a concentration of $100\ \mu\text{g mL}^{-1}$ on MRSA followed by *P. aeruginosa*, *E. coli* and *K. pneumoniae* (Figure 9(b)). The one-way ANOVA of the test samples (AgNPs and ethyl acetate) was performed with Tukey's HSD *post hoc* comparison test. The analyses were made between the concentrations of the samples tested individually against each bacterial pathogen. Significant values were represented in Table 3.

3.6. Minimal inhibitory concentration of AgNPs and Ethyl acetate extract

The cell free supernatant mediated AgNPs had detrimental effects on the growth curve of four tested clinical

Figure 4. FT-IR analysis (A) *B. cereus* A30 mediated AgNPs and (B) Cell free supernatant (C) of ethyl acetate extract.

pathogens (Figure 10). The MIC of the extracellular synthesized AgNPs against clinical pathogens was calculated using the micro dilution method (Table 4). The MIC of

the AgNPs had most significant effects on *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* among the clinical pathogens, whereas the ethyl acetate extract showed

Figure 5. FESEM (A) AgNPs spherical structure, (B) spherical in shapes and the size, (C) EDX profile of AgNPs revealed a sharp peak.

Figure 6. GC-MS chromatogram of the ethyl acetate extract from culture supernatant of *B. cereus* A30.

Table 1. Bioactive components present in the ethyl acetate extract of *Bacillus cereus* A30.

S. no	R_T	Compound name	Molecular weight	Formula	Area (%)
1	18.420	Distearyl thiodipropionate	682	$C_{42}H_{82}O_4S$	11.70
2	22.70	Hentriacontane	436	$C_{31}H_{64}$	1.459
3	23.077	1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) ester	278	$C_{16}H_{22}O_4$	0.868
4	23.467	Hentriacontane	436	$C_{31}H_{64}$	3.971
5	24.202	Hentriacontane	436	$C_{31}H_{64}$	6.709
6	24.618	Hentriacontane	436	$C_{31}H_{64}$	9.659
7	25.618	Hentriacontane	436	$C_{31}H_{64}$	12.010
8	26.298	Hentriacontane	436	$C_{31}H_{64}$	12.308
9	27.048	Hentriacontane	436	$C_{31}H_{64}$	12.889
10	27.903	Hentriacontane	436	$C_{31}H_{64}$	10.409
11	28.904	Hentriacontane	436	$C_{31}H_{64}$	9.602
12	30.079	Hentriacontane	436	$C_{31}H_{64}$	5.190
13	31.480	Hentriacontane	436	$C_{31}H_{64}$	3.227

significant activity in *S. aureus*. The gram-positive microorganism *S. aureus* showed a less significant MIC than the gram-negative microorganisms.

3.7. Cytotoxicity on brine shrimp

Cell free supernatant, synthesized AgNPs and ethyl acetate extract of *Bacillus cereus* treated against brine shrimp nauplii caused variable toxicity during the bioassay (Figure 11(a)). The supernatant (14.66%) as well as the AgNPs (21.33%) showed only a minor percentage of mortality as compared to the ethyl acetate extract (66.66%) at maximum concentration. The lethality rate was found to be directly proportional to the concentration of the extract. The LC_{50} value of the Cell free supernatant, AgNPs and ethyl acetate extract were; $0.74 \pm 0.02 \mu\text{l}$,

$0.56 \pm 0.02 \mu\text{l}$ and $0.22 \pm 0.12 \mu\text{l}$, respectively, after 24 h. The morphological images of the control and test samples treated *Artemia* are shown in Figure 11(b).

4. Discussion

The marine environment was found to provide a rich source of bioactive compounds with unlimited compounds of functional diversity. More than 30,000 products have been obtained from marine organisms and most of them are under clinical trials [28]. Several researchers have reported that marine water bodies and seabottoms are important species-rich habitats of the planet [29,30]. Marine bacteria might be indigenous to aquatic environments, or exogenous, transiently and occasionally present in the water as a result of shedding from animal, vegetal, or soil surfaces [31]. Also, more than 90% of marine bacterial strains are able to produce bioactive compounds. The present investigation was focusing on the isolation of halophilic bacteria from seawater and microbial extracts and AgNPs obtained from the isolated microbes were tested against multidrug resistant bacteria. Halophiles, inhabiting the saline environments are considered to be a suitable source of useful salt stable enzymes [4]. Their enzymes possess unique structural features that are necessary to catalyse the reactions under high salt conditions. Generally, halophiles require more than 0.5 M NaCl for their optimal growth that have developed earlier with two different basic mechanisms of osmoregulatory solute accumulation to endure with ionic strength substantial water stress [32]. These mechanisms allow halophiles to proliferate in saturated salt solutions and to survive entrapment in salt rocks. Velankar [33] in an earlier contribution has isolated

Table 2. Antibacterial sensitivity patterns of selected multidrug pathogens.

Antibiotic	Zone of inhibition				Moderate sensitive				Result			
	A	B	C	D	A	B	C	D	A	B	C	D
Amikacin (AK)	19	18	17	12	15–16	15–16	15–16	15–16	S	S	S	R
Amoxicillin (AMC)	6	23	16	0	14–17	14–17	14–17	14–17	R	S	MS	R
Ampicillin/Sulbactam(A/S)	9	20	24	0	12–14	12–14	12–14	12–14	R	S	S	R
Aztreonam (AT)	12	24	UT	UT	16–21	16–21	UT	UT	R	S	UT	UT
Carbenicillin (CAC)	12	28	UT	19	20–22	20–22	UT	20–22	R	S	UT	R
Cofactor (CF)	9	25	UT	UT	15–17	15–17	UT	UT	R	S	UT	UT
Cefepime (CPM)	16	UT	UT	UT	18–20	18–20	UT	UT	MS	UT	UT	UT
Cefotaxime (CXM)	13	22	21	UT	15–22	15–22	15–22	UT	R	MS	MS	UT
Cefpodoxime (CPM)	23	26	22	UT	18–20	18–20	18–20	UT	S	S	S	UT
Ceftriaxone (CTX)	13	26	22	0	14–20	15–22	14–20	14–20	R	S	S	R
Cefuroxime (CFS)	20	25	UT	9	15–17	15–17	UT	15–17	S	S	UT	R
Ciprofloxacin (CIP)	6	30	22	20	16–20	16–20	15–20	16–20	R	S	S	MS
Co-Trimoxazole (CTR)	14	25	14	0	11–15	11–15	11–15	11–15	MS	S	MS	R
Gentamycin (GEN)	12	24	12	0	13–14	13–14	13–14	13–14	R	S	R	R
Imipenem (IPM)	29	29	UT	20	14–15	14–15	UT	14–15	S	S	UT	S
Levofloxacin (LE)	12	24	23	19	16–18	16–18	16–18	16–18	R	S	S	S
Nalidixic acid (NA)	0	24	UT	UT	14–18	14–18	UT	UT	R	S	UT	UT
Netilmicin (NET)	16	17	20	0	13–14	13–14	15–16	11–15	S	S	S	R
Nitrofurantoin (NIT)	17	14	UT	0	15–16	15–16	UT	15–16	S	R	UT	R
Cefoperazone (C)	UT	UT	UT	16	UT	UT	UT	16–20	UT	UT	UT	S
Norfloxacin (NX)	0	23	UT	UT	13–16	13–16	UT	UT	R	S	UT	UT
Ofloxacin (OF)	8	24	16	10	13–15	13–15	18–20	13–15	R	S	S	R
Piperacillin/tazobactam(PIF)	22	19	UT	13	18–20	18–20	UT	18–20	S	MS	UT	R
Tetracycline (TE)	6	22	19	10	16–18	13–14	11–12	16–18	R	S	S	R
Azithromycin (AZM)	UT	UT	19	UT	UT	UT	14–17	UT	UT	UT	S	UT
Cefazolin (CZ)	UT	UT	24	UT	UT	UT	15–17	UT	UT	UT	S	UT
Chloramphenicol	UT	UT	26	UT	UT	UT	13–17	UT	UT	UT	S	UT
Cefoxitin (CX)	UT	UT	17	UT	UT	UT	15–17	UT	UT	UT	MS	UT
Linezolid (LZ)	UT	UT	28	UT	UT	UT	20	UT	UT	UT	S	UT
Methicillin (MET)	UT	UT	8	UT	UT	UT	10–13	UT	UT	UT	R	UT
Tobramycin (TOB)	UT	UT	19	UT	UT	UT	13–14	UT	UT	UT	S	UT

Notes: A = *Escherichia coli* B = *Klebsiella pneumoniae* C = MRSA D = *Pseudomonas aeruginosa*.
 UT = untreated; S = Sensitive; R = Resistant; MS = Moderate Sensitive.

Figure 7. Antibacterial assay of synthesized AgNPs by *B. cereus* A30 using well diffusion test against (A) *E. coli*, (B) *K. pneumoniae*, (C) *P. aeruginosa*, (D) MRSA. AgNO_3 was used as control.

bacteria from the sea-water and mud of the Mandapam coast (India). Presently, the isolated halophilic bacteria from the sea-water of the Mandapam Coast (Southeast

India) were grown under optimal lab conditions as prescribed in an earlier report [34]. Fritze [35] suggested that the phenotypic representation of results should not be

Figure 8. Antibacterial assay of ethyl acetate extract from culture supernatant of *B. cereus* A30 - using well diffusion test against MRSA, *P. aeruginosa*, *K. pneumoniae* and *E. coli*.

compared solidly without relating to the full background information of the precise environmental/culture conditions. According to Stackebrandt and Swiderski [36], this is particularly true for the group of Gram-positive endospore-forming bacteria that were formerly allocated to the genus *Bacillus* but have now been reclassified as separate lineages based upon phylogenetic diversity. Presently, the 16S rDNA sequence analysis was used to ascertain the accurate taxonomic position of this halophilic strain. The sequence of strain A30 shared a close relationship with the sequences of *Bacillus* species and it showed closest similarity with *Bacillus cereus*. In earlier studies of Garabito et al. [37], true marine species, such as *Bacillus marinus*, *B. salexigens* and *B. dipsosauri* were isolated. Similarly, Ivanova et al. [38] reported that the strains of *B. subtilis* and *B. pumilus* were found to show a close relation with sponge-associated microbes, ascidians, soft corals, and they were present in seawater as well. Their study has also reported abundant *Bacillus* species, such as *B. badius*, *B. subtilis*, *B. cereus*, *B. licheniformis*, *B. firmus*, *B. pumilus*, *B. mycoides*, and *B. lentus* from marine environments [39]. Antibiotic-resistant pathogens from agricultural animals are generally released into the drainage from contaminated sites by urine, feces, and manure. The wastewater from hospitals and intensive farming facilities are also a major source of antibiotic-resistant pathogens that are spreading to the environment [40]. Therefore, it is indispensable to create public awareness on active barrier measures about the prevention of integration of resistant and pathogenic bacteria into the

environment. The preliminary antibacterial activity of cell free supernatants of isolated halophilic bacteria was evaluated against selected clinical pathogens. With the initial evaluation of the supernatants from all isolates, the strain A30 exhibited a maximum inhibition, and hence that species was screened and subjected to AgNPs synthesis and ethyl acetate extraction. The UV-visible spectrum of the cell free supernatant exposed to AgNO_3 solution was recorded at 420 nm which is the characteristic of AgNPs, as reported earlier by Gopinath et al. [41]. Also, no precipitation was found after a week of incubation [42]. The bioreduction of AgNPs from the bacterium A30 might reduce metal oxides through the secretion of small, diffusible redox compounds which could be employed as an electron shuttle [43]. The presence of amine groups as interpreted from the FT-IR spectrum showed a vital role in reducing and controlling the formation of AgNPs and otherwise the secondary structures of the proteins might be changed after the reaction of supernatants with silver ions [44,45]. In XRD patterns, the five peaks recorded in the diffractograms were in agreement with Bragg's reflections of AgNPs [46]. In line with an earlier report of El-Shanshoury et al. [47], the synthesized AgNPs were of nano-crystalline nature which could be attributed to their Bragg's reflections. The micrographic images of FESEM illustrate the spherical morphology of the AgNPs within the size range of 50 nm which supports the nature of silver as reported earlier by several researchers [48,49]. Through EDX patterns, the presence of metallic silver was confirmed and further weak signals of

Figure 9. Analysis of antibacterial activity of AgNPs (A) and ethyl acetate extract (B) of *Bacillus cereus* A30 against multidrug resistant pathogens *E. coli*, *K. pneumoniae*, *P. aeruginosa*, MRSA.

Table 3. One way ANOVA of the test samples using Tukey's HSD post hoc comparison test.

Samples	Tukey's HSD	<i>E. coli</i>	<i>S. aureus</i>	<i>K. pneumoniae</i>	<i>P. aeruginosa</i>
SNPs	F value	10.800	61.143	9.538	6.00
(df:3,8)	P value	0.003**	0.000***	0.005**	0.019*
Ethyl acetate	F value	3.769	13.600	10.756	27.759
(df:3,8)	P value	0.059 ^{ns}	0.002**	0.004**	0.000***

Notes: HSD – Honest significant difference; ns – no significance; *significant; **moderately significant; ***highly significant; df – degrees of freedom.

oxygen and other atoms were observed from the *B. cereus* mediated AgNPs. The presently obtained negative zeta potential value around -26.3 mV might possess an ideal surface charge as reported by Chanthini et al. [50]. The dispersion would be stable, when the zeta potential is lesser than -30 mV or higher than 30 mV. Ultimately, the zeta potential of the particles depends on the pH and the amount of electrolyte dispersed in physiological saline [51]. The presence of bioactive components in the ethyl acetate extract of strain A30 supernatant was identified from the GC-MS spectrum through the NIST and WILEY database library with their corresponding retention time. The major component (1), hentriacontane was

reported earlier from green tobacco leaves which was found to be transparent to the UV region [52]. The earlier study of Kunwar et al. [53] have documented the biological applications of hentriacontane such as antimicrobial, cancer treatment, acne control etc., from *Bauhinia variegata* and *Vitex negundo*. The bioactive component (2), distearyl thiodipropionate which is a diester of stearyl alcohol and thiodipropionic acid was used as an antioxidant [54]. The bioactive component (3), 1, 2-benzenedicarboxylic acid, mono (2-ethylhexyl) ester was reported earlier as a secondary metabolite, possessing antimicrobial, antioxidant and anti-proliferative activities [55–57]. Bacterial resistance was found to be the major reason for the treatment of infectious diseases resulting in increased morbidity, mortality, and costs [58]. Antibiotic resistance has been reported in a wide range of human pathogenic bacteria such as *K. pneumoniae*, *P. aeruginosa*, *E. coli* and MRSA [59]. Several researchers have earlier identified many genes that are responsible for intrinsic resistance to different classes of antibiotics, including β -lactams, fluoroquinolones, and aminoglycosides. This could be achieved using high-throughput screens of high-density genome mutant libraries which were developed by the targeted insertion or random transposon mutagenesis

Figure 10. Growth curves of different clinical strains exposed to Ag-NPs synthesized and Ethyl acetate extract from the cell free supernatant of *Bacillus cereus* during 24 h: (A) *P. aeruginosa* (3.12 µg/ml), (B) *K. pneumoniae* (>3.12 µg/ml) (C) *S. aureus* (12.5 µg/ml).

Table 4. Minimum inhibition concentration (MIC) value of test samples.

Clinical pathogen	Supernatant	Ag NPs	Ethyl acetate
<i>Pseudomonas aeruginosa</i>	>25	3.12	<25
<i>Klebsiella pneumoniae</i>	>25	>3.12	25
<i>Staphylococcus aureus</i>	>25	12.5	12.5
<i>Escherichia coli</i>	>25	6.25	>25

in bacteria such as *Staphylococcus aureus* [23], *E. coli* [60], *K. pneumoniae* [63] and *Pseudomonas* sp. [61]. Our susceptibility test on selected pathogens against AgNPs revealed the resistance-deterioration of the multidrug resistance-gene of the parental/resistance species (that transfers the genetic information), like the antibiotic resistance property of various offsprings. Radha et al. [24] have also reported that *E. coli* and *P. aeruginosa* were resistant against most antibiotics. Frank [62] has demonstrated that these challenging resistant pathogens were characterized to exterminate their virulence as well as to inhibit the respective enzymes by superimposing AgNPs and ethyl acetate extract of strain A30. In this study, AgNPs synthesized with extracts of the halophilic bacterium, *Bacillus cereus* showed significant antibacterial activity at the maximum concentration against *P. aeruginosa* (25 mm) followed by *K. pneumoniae* (19 mm) and *E. coli* (17 mm). The inhibitory effects of AgNPs on bacteria could have been due to the three most common mechanisms as proposed earlier by Catalina and Hoek [63]. Which neither disrupted the ATP production and DNA replication by the endorsement of free Ag ions nor

reactive oxygen species (ROS) generation by Ag and AgNPs nor the direct damage of AgNPs to the cell membranes. Dutta et al. [64] reported that the AgNPs conjugate and probably generate ROS with clinical pathogens in culture medium supported by a growth inhibition study in the presence of standard ROS scavengers. Whereas, ethyl acetate extract exhibited significant activity on MRSA, *P. aeruginosa* and *E. coli* which could be due to the presence of hentriacontane, that possesses antimicrobial properties as reported earlier by several researchers [56–58]. The antibacterial activity on antibiotic resistant MRSA was tested earlier by Jeyanthi and Velusamy [65], from the halophilic bacterium, *Bacillus amyloliquefaciens* MHB1, which is also in agreement with the presently evaluated inhibitory effect. Overall, the evaluated antibacterial activities of AgNPs synthesized from the halophilic bacterium, *Bacillus cereus* A30 was found to be more effective than ethyl acetate extracts on multi-drug resistant bacteria. The results indicated that marine bacteria based synthesized AgNPs showed a good activity against human pathogens.

The presently recorded antimicrobial activity of AgNPs is in agreement with earlier reports, where the MIC of AgNPs was found to be significant effective against clinical pathogens [66]. Also, the ethyl acetate extract showed considerable antimicrobial activity as compared to AgNPs. Here, the effect of AgNPs could be arisen from the metabolites associated with the bacterium, *Bacillus cereus*, against the tested gram-positive

Figure 11. (A) Percentage mortality of *Artemia salina* treated with the test samples at different concentration; (B) Stereo microscopic images of *Artemia salina* nauplius after 24 h treatment.

and gram-negative microorganisms through the structure of their respective cell walls [67].

He et al. [68] reported that the extent of brine shrimp cytotoxicity could be co-related to the smaller size of the AgNPs, which had stronger effect on their uptake by cells. In contrast, in the present investigation, the AgNPs exhibited only lesser toxicity as compared to the ethyl acetate's effect. This could be in chance of toxins present in the ethyl acetate extract leading to brine shrimp death and causing internal membrane damage as evidenced by Figure 11(b).

5. Conclusion

A rapid method is presented here for the synthesis of AgNPs using the halophilic bacterium, *Bacillus cereus* A30 isolated from marine waters. AgNPs were found to be highly stable and crystalline in nature, as confirmed by XRD patterns as well as the Zeta potential. FESEM distinguished the spherical morphology with size ranges from 26 to 46 nm of AgNPs. The AgNPs showed significant activity on the tested pathogens. Also, the ethyl acetate extract revealed pronounced antibacterial activity by the three bioactive components, Hentriacontane, Distearyl thiodipropionate and 1,2-benzenedicarboxylic acid, mono-(2-ethylhexyl)-ester. The antimicrobial efficacy of AgNPs and ethyl acetate extract on both Gram-positive

and Gram-negative pathogens could be utilized as alternative sources for the development of new antibiotics against multi-drug resistant pathogens.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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