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Antimicrobial Activity & Anti Oxidant Activity of Extracellularly Synthesized Silver Nanoparticles from Marine Derived *Aeromonas salmonicida*

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Abstract: ***The process of development of reliable and eco-friendly metallic nanoparticles is an important step in the field of nanotechnology. Novel applications of nanoparticles and nanomaterials are rapidly emerging day by day. To achieve this, use of natural sources like biological systems become essential. In the present investigation, Silver Nanoparticles are biosynthesized extracellularly using Aeromonas salmonicida, isolated from marine sediment samples of Tuticorin coast, Tamil Nadu, India. The biosynthesized silver nanoparticles were initially confirmed by visual observation with the appearance of yellowish brown solution in the reaction mixture. In UV-visible spectroscopy, the spectra showed a strong surface Plasmon resonance centered at 420 nm. The silver nanoparticles were found to be face centered cubical in shape with variable size ranging from 20 to 80 nm as obtained by SEM analysis. X ray diffraction (XRD) data illustrated the crystalline nature of silver nanoparticles. The possible biomolecules such as amines and polyphenols may responsible for reduction of silver ions was identified through FTIR Spectroscopy. The antimicrobial activity of synthesized nanoparticles was affirmed against bacterial pathogens Bacillus subtilis, Enterobacter aerogenes, Escherichia coli, Klebsiella oxytoca, Staphylococcus aureus and Streptococcus pyogenes. Further the anti oxidant activity of silver nanoparticles was analyzed by DPPH assay.***

Keywords: Silver nanoparticles, *Bacillus subtilis,* antimicrobial activity, *Staphylococcus aureus*, *Escherichia coli*

1. Introduction

Nanotechnology can be defined as a research for the design, synthesis, and manipulation of structure of particles with dimension smaller than 100nm. Nanotechnology has dynamically developed as an important field of modern research with potential effects in electronic and medicine. Nanobiotechnology combines biological principles with physical and chemical procedures to generate nano-sized particles with specific functions (Ahmad *et al.,* 2005). This integration of nanoparticles with biological molecules has led to the development of diagnostic devices, contrast agents, and important tools in cancel therapy.

Noble metals such as palladium, silver, platinum and gold, exhibits wide range of material behavior along the atomic bulk transition (Shanmugam *et al.*, 2012). Among these noble metals silver have wide applications in jewellary, dental alloy and health additive in traditional Chinese and Indian Ayurvedic medicine (Singh *et al*, 2008). Silver is a naturally occurring precious metal, most often as a mineral ore in association with other elements. It has been positioned as the 47th element in the periodic table, having an atomic weight of 107.8 and two natural isotopes 106.90 Ag and 108.90 Ag with abundance of 52 and 48% respectively. It has been used in a wide variety of applications as it has some special properties like high electrical and thermal conductivity (Silambarasan *et al.,* 2013). Silver was known only as a metal until the recent advent of the nano-technology era, when it became recognized that silver could be produced at the nanoscale and metallic silver has been subjected to recent engineering technologies, resulting in ultra fine particles, the sizes of which are measured in nanometers (nm) and possess distinctive morphologies and characteristics. Nanoscale particles and molecules are a potential alternative for treatment of disease because they have unique biological effects based on their structure and size, which differ from traditional small-molecule drugs. (Saranyadevi *et al.,* 2012).

Marine microbial biotechnology has opened up unexpected new ways for finding new organism for trapping their potential resources (Shivakrishna *et al.,* 2013). The use of microorganisms for the synthesis of nano sized materials has recently emerged as a novel approach and designed for the production of heavy metals and metal nanoparticles (Bae *et al*., 2000). The biosynthesis of silver nanoparticles was carried out by using bio-reducing agent like bacteria, fungi and plant extracts (Ahmad *et al.,* 2003). The advantages of biological generation of nanoparticles are three fold; the bio-species can act as template, reducing and even as capping agent for nanoparticles. It is well explained for the formation of biogenic silver nanoparticles based on the intra and extra cellular activities of microbes, such as *Pseudomonas sp, Klebsiella sp, Escherichia coli, Vibrio cholera, Bacillus sp, Salmonella typhus and Staphylococcus aureus* (Basavaraja *et al.,* 2008). The metal reducing bacterial species were isolated from various soil environments like pond water sediment, ore, mine, inorganic pollutant areas and rare earth element environments. Marine habited microorganisms effortlessly adapted with heavy metals and it can produce unusual size and shape of inorganic nanoparticles via intra or extra cellular mechanisms (Dhandapani and Supraja, 2012). Hence the present study is aimed to synthesis and characterizes silver nanoparticles obtained by use of a marine bacterial strain and its various biological activities.

2. Materials and Methods

2.1 Materials

Silver nitrate Merck (Germany), Zobell Media was procured from Hi media (India). Analytical grade reagents were purchased from Sigma–Aldrich (Bangalore). All the samples were prepared in Milli-Q water.

2.2 Sample collection

The marine sediment sample was collected from Tuticorin Coast, Tamil Nadu, India at 10 m length and 5 m depth in sterilized glass bottle. The collected marine water sample was stored in ice box (4°C) and then transported to the laboratory.

2.3 Isolation of marine bacteria

10µl of sea water sample was spreaded over the surface of the marine agar (Zobell Marine Agar) with composition of Peptone 5. 0g, Yeast Extract 1.0g, Ferric Citrate 0.1g, Sodium chloride 19.45g, Magnesium chloride 8.8g, Sodium sulfate 3,24g, Calcium chloride 1.8g, Potassium Chloride 0.55g, Sodium bicarbonate 0.16g, Potassium Bromide 0.08g, Strontium Chloride 34.0mg, Boric acid 2.0mg, Sodium silicate 4.0mg, Sodium Fluoride 2.4mg, Ammonium Nitrate 1.6mg, Disodium Phosphate 8.0mg, Agar 15.0g. The plates were incubated at 30°C for 24 hours and the colonies obtained were purified by re-streaking on the isolation medium.

2.4 Amplification of 16S r DNA

The bacterial cells harvested from 10 ml broth were resuspended in 50µl sterile distilled water and boiled at 100°C for 10 min and centrifuged at 12,000 rpm for 5 min. Bacterial DNA was extracted by heat extraction method. Five µl lysate was used in polymerase chain reaction (PCR) to amplify 16s rDNA. The 16S rDNA PCR amplification was carried out in a total volume of 50 µl containing 10 X PCR buffer, 2.5mM $MgCl_2$,0.16mM of dNTPs,0.75 U of Taq DNA polymerase and 0.2 p mol forward primer 27F: AGA GTT TGA TCC TGG CTC CAG and reverse primer 1492R: TAC GGT TAC CTT GTT ACG ACT T. The PCR conditions consisted of an initial hot start at 95°C for 3 min, 30 amplification cycles of (extension), and finally one cycle of 72°C for 5 min.

The amplified PCR products were purified using the 'Wizard ®SV Gel and PCR Clean up system' by following guidelines provided by the manufacturer (Promega corporation). Both strands of amplified 16 S rDNA were sequenced using 27F and 1492R primers. Forward and reverse DNA strand sequence was aligned using BLAST (bl2seq) program available at NCBI. The finalized sequence of amplified 16SrDNA fragment from each isolate was blasted against the collection of non-redundant nucleotide sequence database of NCBI. The isolates were identified based on hits analysis from mega blast (highly similar sequences) output. The hits of 16S r DNA sequences were used in phylogenetic analysis of 16 S r DNA sequences of isolates to determine 16 S rDNA sequence based evolutionary relationship among the isolates and hits. The MSA of 16 S rDNA sequences were carried out using ClustalW (version 1.6) program. The MSA output from Clustal W was used the construction of a rooted dendogram using NJ (Neighbour Joining) method of Saitou and Nei provided at http://align.genome.jp/.

2.5 Biosynthesis of Silver Nanoparticles

Bacterial strain was grown in Zobell marine broth. The final pH was adjusted to 7.0. The flask were incubated at 200 rpm at 28 °C. After 24 hours of incubation, the biomass was separated by centrifugation. The supernatant and pellet was challenged with equal amount of with various concentrations (0.5, 1.0, 1.5, 2.0, 2.5 mM) of silver nitrate solution (prepared in deionized water) and incubated in dark condition at 28 °C. Simultaneously, a positive control of silver nitrate solution and deionized water and a negative control containing only silver nitrate solution were maintained under same conditions.

2.6 Characterization of Silver Nanoparticles

2.6.1 Ultra violet Visible Spectroscopy

The bio regeneration of the silver nanoparticles in the reaction mixture was measured by withdrawing 2 ml of the sample at pre determined time intervals and the absorbance was measured in the range of 360 to 600 nm at a resolution of 1 nm using a UV-Vis spectrophotometer (Hitachi, Modelno.3210) against sterile medium as the blank.

2.6.2 Scanning Electron Microscopy

Scanning Electron Microscopy (SEM) (Hitachi, Model: S-3400N) was used to observe synthesized Silver Nanoparticles. Thin films of the sample were prepared on a carbon coated copper grid by just dropping a very small amount of the sample on the grid, extra solution was removed using a blotting paper and then the film on the SEM grid were allowed to dry by plotting it under a mercury lamp for 5 minutes.

2.7 X-ray diffraction analysis

X-ray diffraction analysis was carried out by using an X- ray powder diffractometer (Philips X'pert Pro, Panalytical) having CuKa (k = 1.54 A °) radiation and a programmable divergence slit. The voltage and current of the X-ray source were 40 kV and 20 mA, respectively. The sample was drop-coated onto silica plate by applying many layer of small amount of samples on the plate with intermittent drying. This leads to a thick coat of sample.

2.8 FTIR spectroscopy analysis

For Fourier transform infrared spectroscopy (FTIR), freeze dried biomass was used. A small amount of dried biomass was grinded with potassium bromide (KBr). FTIR spectrum of sample was recorded on a PerkinElmer FT-IR system Spectrum GX model. All measurements were carried out in the range of 400– 4000 cm-1 at a resolution of 4 cm-1.

2.9 Antibacterial activity

A modified protocol of Kirby-Bauer as described by Sham (2010) was adapted for the *in vitro* antimicrobial activity of

the synthesized silver nanoparticles against *Bacillus subtilis, Enterobacter aerogenes, Escherichia coli, Klebsiella oxytoca, Staphylococcus aureus* and *Streptococcus pyogenes* by disc diffusion using Nutrient Agar (NA). The plates were prepared by pouring 15ml of molten media in to sterile Petri plates, allowed to solidify for 5 minutes. 0.1ml of inoculums suspension was swabbed uniformly on the surface and left to dry for 5 minutes. 0.01ml of (1mM) concentrations of Ag Nps solution was loaded on 5mm sterile individual disc formed. The loaded discs were then placed on the surface of the medium and the compound was allowed to diffuse for 5 minutes before incubating at 37°C for 24 hours. Streptomycin disc was used as positive control whereas for negative control, aqueous solution of $AgNO_3$ was used. At the end of incubation, inhibition zones formed around the disc were measured with a transparent ruler in millimeter. All assays were performed in triplicates and the mean value recorded for each of the organisms under study.

2.10 Determining the growth curves of bacterial cells exposed to different concentrations of Ag-NPs

To examine the growth curves of bacterial cells exposed to Ag-NPs, Muller –Hinton broth with different concentrations of Ag-NPs powder (0. 50, 100, 150, 200, 250 µg/ml) was used, and the bacterial cell concentration was adjusted to 10^5 CFU /ml. Each culture was incubated in a shaking incubator at 37°C for 24 hours. Growth curves of bacterial cell cultures were attained through repeated measures of the optical density (O.D.) at 600 nm (Kim *et al*., 2011).

2.11 Antioxidant activity

DPPH radical scavenging activity

In DPPH radical scavenging method, 0.1 mM solution of DPPH in ethanol was prepared and 1 ml of this solution was mixed with 5 ml of 0.01% sample solutions in water at different concentrations. After 30 minutes, the absorbance was measured at 517nm. A decrease in absorbance of DPPH solution indicates an increase in the DPPH radical scavenging activity (Muniyappan and Nagarajan 2014).

$$\%\text{DPPH radical scavenging} = \frac{\text{Control OD} - \text{Sample OD}}{\text{Control OD}} \times 100$$

3. Results and Discussion

In the present study a total of five marine bacterial isolates (MB1, MB2, MB3, MB4, and MB5) were isolated from the sea water sample collected from Tuticorin coastal area. Of these five isolates MB1 showed a considerable growth on Zobell agar medium and was taken for further studies. The 36 h old bacterial culture of MB1 was grown in marine medium at 37^0C under aerobic condition at 120 rpm were collected and the respective genomic DNA was isolated. The PCR was performed in a thermocycler, producing multicopies of the specified DNA. The PCR reaction was allowed for 30 cycles for amplification of 16s rRNA gene forward primer 27F: AGA GTT TGA TCC TGG CTC CAG and reverse primer 1492R: TAC GGT TAC CTT GTT ACG ACT T. Then the PCR product was run on 2% Agarose gel electrophoresis along with 100 bp DNA mixed and visualized under UV light (Figure 1).

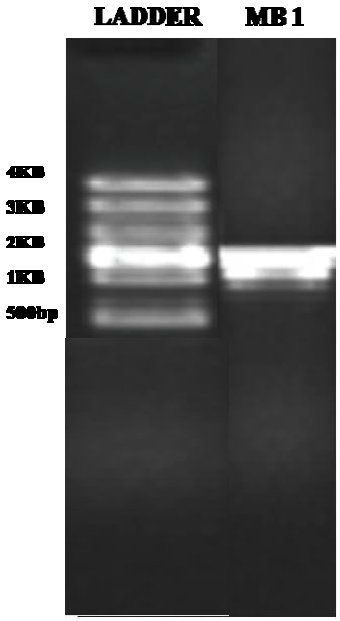

Figure 1: Polymerase chain reaction (PCR) amplified 16s rRNA fragments of MB 1.

The nucleotide sequence of PCR product of both forward and reverse primer confirmed as *Aeromonas salmonicida* 16s rRNA of 1244 bp. BLASTN homology analysis was carried out to compare with others 16s rRNA sequences available in the GenBank of NCBI. All the sequences reported by BLAST revealed that the bacterial species showed a very high percentage of similarity (99%) with the sequences of *Aeromonas salmonicida* subsp. *salmonicida* strain A449 with a reasonably high score and E-value being zero.

In a phylogenic tree (Figure 2) each node with descendants represents the most ancestors of the descendants and the edge length in some trees corresponds to time estimates.

Figure 2: Rooted dendogram showing clustering of *Aeromonas salmonicida*

Each node is called a taxonomic unit. Internal nodes are generally called hypothetical units as they cannot directly observe. The sequences showing the maximum similarity were used for alignment using CLUSTAL W2 to derive the phylogenetic relationship. There exists a clear evolutionary relation between all the 16s rRNA sequences as this represents a highly conserved sequences.

The Silver nanoparticles production was carried out using cell free extract of 24 hours grown marine broth with the supplementation of Silver Nitrate. Visual observations showed a change in silver nitrate solution from yellow to brown (Figure 3), whereas no color change was observed in the culture supernatant without silver nitrate. The appearance of a yellowish brown colour in silver nitrate treated culture supernatant suggested the formation of silver nanoparticles (Sastry *et al.*, 2003). The brown colour of the medium could be due to the excitation of surface Plasmon vibration of silver nanoparticles (Ahmad *et al.*, 2005).

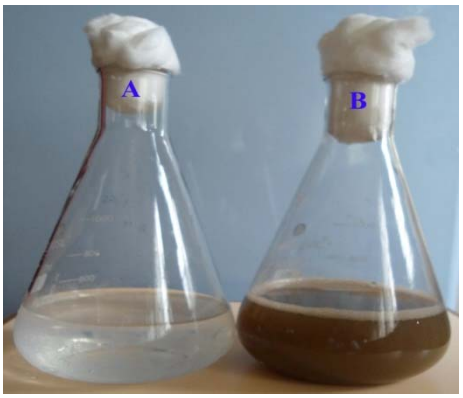

Figure 3: Conical flasks containing *Aeromonas salmonicida* culture supernatant in aqueous $AgNO_3$ solution: (A) at the beginning of reaction showing no colour change; (B) after 24 h of reaction showing brown colour.

The exact mechanism of biosynthesis of silver nanoparticles is not known. However, it has been hypothesized that silver ions required the NADPH-dependent nitrate reductase enzyme for their reduction, which was secreted by the bacteria in its extracellular environment (Kalishwaralal *et al.*, 2008). The use of this enzyme has previously been demonstrated in the *in vitro* synthesis of silver nanoparticles under anaerobic conditions. Nitrate reductase is known to shuttle electron from nitrate to metal group. Thus, these results substantiate the role of nitrate reductase enzyme in the biosynthesis of silver nanoparticles (Gajbhiye *et al.*, 2009).

The synthesized silver nanoparticles were characterized by UV-Vis spectroscopy. In the UV-Vis absorption spectrum, a strong, broad peak located between 420 and 430nm was observed (Figure 4). Observation of this peak, assigned to a surface Plasmon, is well documented for various metal nanoparticles with sizes ranging from 2-100nm (Tillmann 2004).

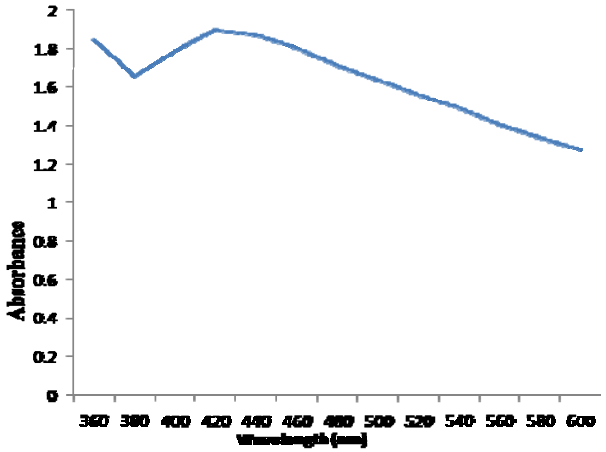

Figure 4: Absorption spectrum of silver nanoparticles synthesized by the culture supernatant of *Aeromonas salmonicida* (420 nm)

X-ray diffraction (XRD) was carried out to confirm the crystalline nature of the particles and the XRD pattern obtained is shown in Figure 5. The XRD pattern shows four intense peaks in the whole spectrum of 2θ values ranging from 20-80nm.

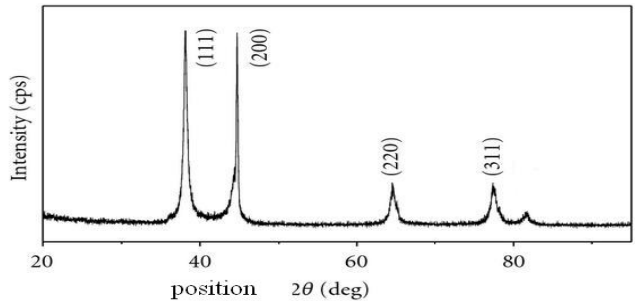

Figure 5: Representative XRD pattern of silver nanoparticles formed after reaction of culture supernatant with $AgNO_3$(1×10^{-3}M) for 72 h. JCPDS (Joint Committee on Powder Diffraction Standards)- File No: 04-0783

A comparison of the XRD spectrum with the standard confirmed that the silver nanoparticles formed in the present study were in the form of nanocrystals, as evident from the peaks at 2θ values of 36.97, 46.58, 67.85, 77.24 corresponding to (111), (200), (220) and (311), respectively for silver.The formation of silver nanoparticles as well as their morphological dimensions were studied by Scanning Electron Microscopy (Figure 6) which demonstrated that, the average size of silver nanoparticles was 20-80nm, with inter particle distance and the shape of the nanoparticles was proved to be cubical.

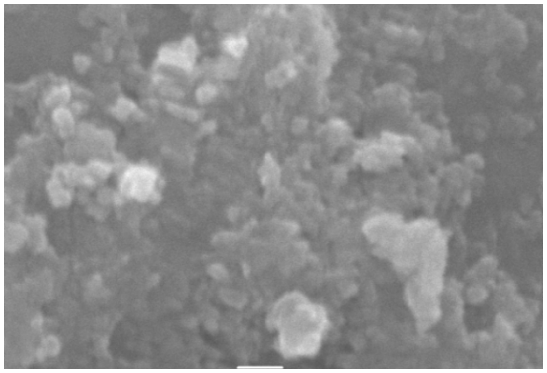

Figure 6: SEM micrograph of silver nanoparticles formed after reaction of culture supernatant with $AgNO_3$ (1×10^{-3} *M*) for 72 h.

FTIR measurements were carried out (Figure 7) to identify possible interaction between silver salts and protein molecules, which could account for the reduction of silver ions and stabilization of silver nanoparticles.

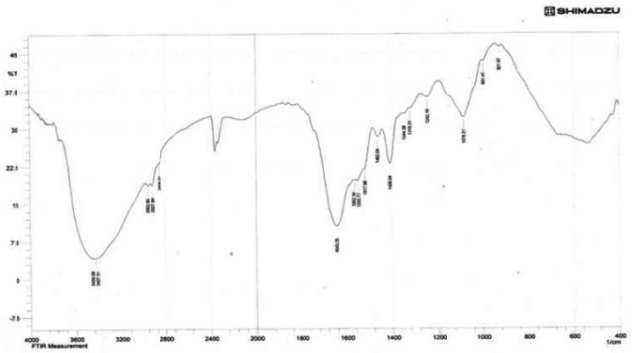

Figure 7: FTIR spectra recorded from powder of silver nanoparticles synthesized using *Aeromonas salmonicida*

The amide linkages between amino acid residues in proteins give rise to well known signatures in the infrared region of the electromagnetic spectrum. The bands seen at 3427.51 cm^{-1} and 2927.94 cm^{-1} were assigned to the stretching vibrations of primary and secondary amines, respectively. The bands seen at 1344.38 cm^{-1} and 1078.21 cm^{-1} corresponds to –C-N stretching vibrations, while the band at 1406.04 cm^{-1} is characteristic of amine and amino-methyl stretching groups. The band seen at 1517.98 cm^{-1} is characteristic of –C=O carbonyl groups and –C=C- stretching. The overall observation confirms the presence of protein in samples of silver nanoparticles. It has also been reported earlier that protein can bind to nanoparticles either through their free amino groups or cysteine residues (Gole *et al.*, 2001). Therefore, stabilization of silver nanoparticles by proteins is a clear possibility.

The anti-microbial activities of biosynthesized silver nanoparticles against *Bacillus subtilis, Enterobacter aerogenes, Escherichia coli, Klebsiella oxytoca, Staphylococcus aureus* and Streptococcus *pyogenes* were also investigated. Both silver ions and silver nanoparticles were known to have excellent anti-microbial activities (Furno *et al.* 2004). The result obtained showed the silver nanoparticles to be effective against the bacterial isolates (Table 1). The highest toxicity was observed against *Escherichia coli* (15mm) this could be attributed to its cell wall nature which allow easy passage of the particles. The second highest activity was observed against *Enterobacter*

aerogenes (13mm) followed by *Streptococcus pyogenes* (12mm), *Staphylococcus aureus* (11mm) and *Klebsiella oxytoca* (10mm). The least antimicrobial activity was observed against *Bacillus subtilis* (9.5 mm). The lower activity was found in silver nitrate alone.

Table 1: Antibacterial activity of silver nanoparticles synthesized using marine bacteria

S.No	*Microorganisms*	*Zone of Inhibition (Diameter in mm) (Concentration – 100µg/ml)*		
		Silver nanoparticles	*Streptomycin*	*Silver nitrate*
1	*Bacillus subtilis*	9.5±0.57	22±0.45	6.5±0.43
2	*Enterobacter aerogenes*	13±0.67	20±0.56	7.5±0.44
3	*Escherichia coli*	15±0.23	28±0.45	5.5±0.32
4	*Klebsiella oxytoca*	10±0.45	25±0.24	4.5±0.34
5	*Staphylococcus aureus*	11±0.25	23±0.13	2.5±0.54
6	*Streptococcus pyogenes.*	12±0.56	21±0.64	2.6±0.55

The growth curves of bacterial cells treated with AgNPs indicated that AgNPs could inhibit the growth and reproduction of bacterial cells. The growth curves of AgNPs treated *Staphylococcus aureus* & *Enterobacter aerogenes* are shown in figure 8 (a) & 8 (b) respectively. The bacterial growth of cells treated with 100 and 150 µg/ml AgNPs were inhibited. After 4 hours, almost all treated bacterial cells were dead. The bacterial growth of the cells treated with 50 µg/ml AgNPs was also slightly lower than that of cells in the control group. These findings indicate that the antibacterial activity of AgNPs (50 µg/ml) could slightly inhibit bacterial growth but not enough to outpace the speed of reproduction of bacterial cells.

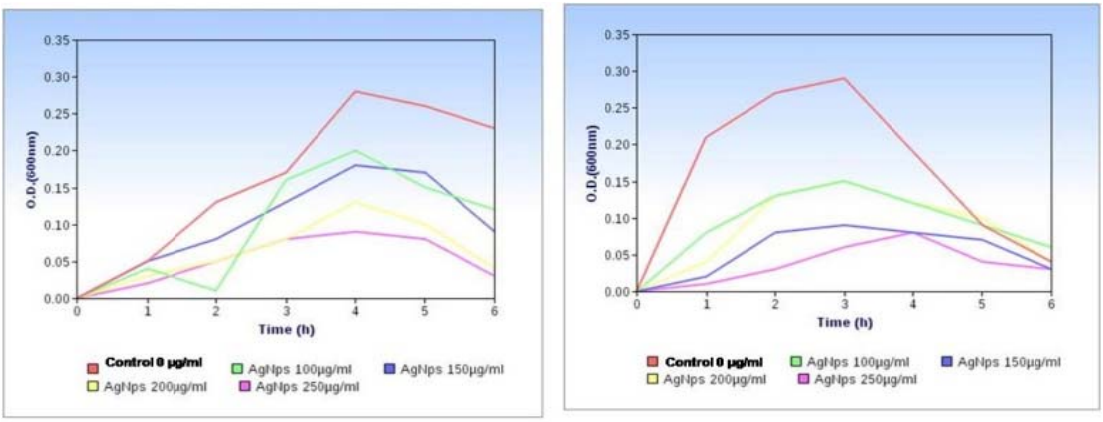

Figure 8: Growth curves of *Staphylococcus aureus* (a) & *Enterobacter aerogenes* (b) cells exposed to different concentrations of Ag-NPs

The antioxidant activity of AgNPs and Albumin solution were studied using DPPH assay. In this method, the antioxidant reacts with the stable DPPH (deep violet color) and converts it into 1,1-diphenyl-2-picrylhydrazine with discoloration. Figure 9 demonstrates that the percentage of inhibition of free radicals at different concentrations (40, 80,100,140 & 160 50 µg/ml) for AgNPs and Albumin solution. Due to the presence of sulfhydryl group in albumin, it has free radicals scavenging activity. The result revealed that when compared to albumin the synthesized AgNPs also has antioxidant activity.

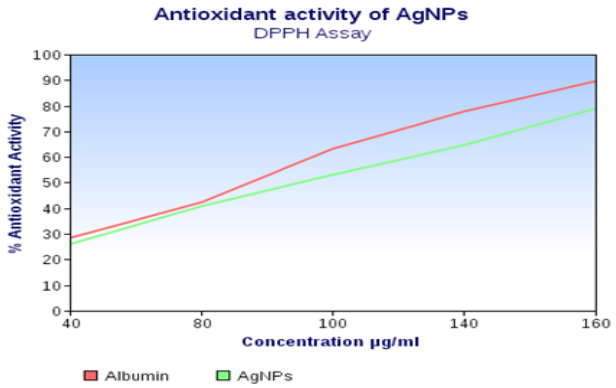

Figure 9: Antioxidant activity of AgNPs with albumin as standard

4. Conclusion

Silver nanoparticles with an average size of 20-80nm were synthesized by the marine bacteria *Aeromonas salmonicida*. The synthesized silver nanoparticles were characterized by UV-Vis, SEM, XRD and FTIR measurements. This green synthesis method is alternative to chemical method, since it is cheap, pollutant free and eco-friendly. Further, these synthesized silver nanoparticles shows the antimicrobial activity against various human pathogenic bacteria. It also shows the maximum rate of growth inhibition and also high free radical scavenging activity. It is concluded that the application of such eco-friendly nanoparticles makes this method potentially exciting for the large scale synthesis of nanoparticles. In future, our aim is to synthesize the silver nanoparticles by using different marine microbes and also to study the biochemical and molecular mechanism of nanoparticles formation by the cell filtrate in order to achieve better control over size and polydispersity of the nanoparticles.

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