BIOSYNTHESIS AND CHARACTERIZATION OF SILVER NANOPARTICLES USING BIOFILM FORMING BACTERIA *ESCHERICHIA COLI.*

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Abstract:

Synthesis of nanoparticle is a novel and effective approach. Microbiological synthesis of silver nanoparticles using marine biofilm forming bacteria will explore many advantages. Biological synthesis of nanoparticles is thus an effective and economic approaches which even control the size and shape of the nanoparticles/. In the present study marine biofilm forming bacteria isolated at Visakhapatnam port i.e. *Escherichia coli* strain is used for the synthesis of silver nanoparticles. The selected marine biofilm forming bacterial strain *E. coli* has showed the ability to synthesize silver nanoparticles by extracellular synthesis mechanisms. This was observed by the changes in the colour from pale yellow to brown. UV-visible spectra of silver nanoparticles synthesized by the selected isolates of *E.coli* strain showed an absorption peak at 422 nm by treating the cell free supernatant with $AgNO_3$. The FTIR spectrum of silver nanoparticles between the wave number 400-4000 cm^{-1} showed the active biomolecules of cell free supernatant responsible for the reduction of Ag^+ ions into metallic silver nanoparticles. The XRD pattern of the silver nitrate treated with cell free extract by extracellularly corresponds to that of silver nanoparticles. Diffractions patterns at 2Θ values 38.37º, 44.02º and 63.87º indicated the 111, 200 and 220 reflections of metallic silver. SEM micrographs indicated that `the presence of cubic in nature of individual nanoparticles that are approximately in the range of 30-50 nm in size and the EDS analysis also showed a peak in the silver region, confirming the formation of silver nanoparticles. The TEM micrograph also confirmed the size of nanoparticles, which were in the range of 10 to 15 nm. From the results obtained in this effort, one can conformed that marine biofilm forming bacterial strain *E. coli* can play an important role in the bioreduction and stabilization of silver ions to silver nanoparticles.

IndexTerms - Silver nanoparticles, UV-Visible spectroscopy, FTIR, XRD, SEM, EDS, TEM.

1.INTRODUCTION

Microbiological synthesis of nanoparticles is an effective and novel approach for the synthesis of nanoparticles using biological specimen. In that the plant, animal or microbial biomass involved as a reducing agents [1] which are environmentally safe, non toxic and has significant advantages over other processes since it takes place at relatively at optimum temperature and pressure [2]. Biological synthesis of nanoparticles is thus an effective and economic approaches which even control the size and shape of the nanoparticles [3-5].

Biosynthesis of silver nanoparticles was first carried out by exploiting the bacteria *Pseudomonas stutzeri* reported [6]. Microbial synthesis of nanoparticles can take place either intracellularly or extracellularly [7-9]. Intracellular cellular microbial synthesis of nanoparticles requires additional steps such as ultrasonication or reactions with suitable detergents to release the synthesized nanoparticles [10, 11]. At the same time the extracellular biosynthesis of nanoparticles is cheap and it requires simpler downstream processing. This favours the large scale production of silver nanoparticles its potential applications.

Because of this reasons like cheap and potential application with silver nanoparticles, many studies were carried out with extracellular methods for the synthesis of metal nanoparticles [12]. Few journals have reported the extracellular synthesis of nanoparticles formation by the culture supernatant of *Bacillus megaterium* with aqueous silver nitrate solutions [13]. Studies using culture supernatants of bacteria like *Pseudomonas proteolytica, Pseudomonas meridiana, Arthrobacter kerguelensis, Bacillus indicus,* etc.[14], *Bacillus lichniformis*[15], *Corynebacterium* sps [16] *E. coli* [17,18] were proven to its property of

extracellular nanoparticles effectively. Of which, the extracellular synthesis of nanoparticles is still continually emerging in order to understand the mechanisms of synthesis, easy downstream processing and rapid scale – up processing. For these reasons, a microbial system could prove to be a potential source for the extracellular synthesis of interesting nanoparticles without using toxic chemicals. However, the screening of unexplored marine microbial organisms for silver nanoparticles synthesizing property is very much important. Hence the present study was planned to synthesis of silver nanoparticle by using biofilm forming bacteria identified *Escherichia coli* strain isolated from the boats at fishing harbour, Visakhapatnam port.

2. MATERIALS AND METHODS

2.1. Screening of bacterium for silver nanoparticle production

In the present study screening of an efficient bacterium for the production of silver nanoparticles samples were collected from the Visakhapatnam port harbour (Lat 17.6831° N, Log 83.2399° E) during the period of August - October 2018. The bottom of the boat was gently swabbed with a sterile cotton swab, placed in tubes containing 10 mL sterile water. Then they were inoculated in specific media for the isolation of microbes. The bioflilm bacterial strains used in the antibacterial assay were isolated by the pour plate technique. The screened *E. coli* strain culture was routinely maintained on Luria-Bertani (LB) agar slant at 37°C.

2.2. Synthesis of silver nanoparticles (SNPs)

Synthesis of SNPs was carried out according to the method described by Kalimuthu *et al.*[11] and Kalishwaralal *et al.*[28]. The sterile LB medium was prepared and then inoculated with stock culture of the bacterial isolate and incubated for 24 h in an orbital shaker at a speed of 120 rpm and 37º C temperature. After incubation, the bacterial culture solution was centrifuged at 10,000 rpm for 15 minutes and then the supernatant separated was used for the synthesis of silver nanoparticles. Two Erlenmeyer flasks, one containing 100ml of 1mM silver nitrate ($AgNO_3$) solution without the supernatant taken as a control and the second flask containing 10 ml of supernatant and $AgNO_3$ solution at the same concentration were incubated for 24h is taken as test. The absorption spectrum of the sample was recorded on a UV-visible spectrophotometer (Applied Biosystems) in the range of 200-1100 nm. The bioreduction of SNPs was monitored by visual inspection for a change in the color of the culture medium from a clear, pale yellow to brown and by the measurement of the peak exhibited by SNPs in the UV-visible spectra measurement. The supernatant thus obtained was a brown homogenous mixture and clear suspension of silver nanoparticles.

2.3. Lyophilization for the reluctant sample mixture

After the desired reaction period, the broth containing silver nanoparticles were lyophilized for XRD and SEM analysis. The reluctant samples were centrifuged for 10,000 rpm for 15 minutes and the reaction was carried out for five times. After the desired reaction period remove the supernatant and collected the fractions and freeze dried. The lyophilized samples were kept in the deep freezer at -20ºC for further analysis.

2.4. Characterization of Nanoparticles

An important phage in the biosynthesis of nanoparticles is physico-chemical characterization of produced nanoparticles. Knowing about size, shape, surface area, homogeneity and other features will provide valuable information of nanoscale systems and insight into synthesis control of nanoparticles for commercial applications. Some common techniques of characterization are UV-visible spectroscopy, Fourier transform infrared spectroscopy (FTIR), powder X-ray diffraction, scanning electron microscopy (SEM) with Energy dispersive spectroscopy (EDS), transmission electron microscopy (TEM).

2.4.1. UV-vis analysis

UV-visible spectroscopy has proven to be very useful for analyzing silver nanoparticles [29]**.** To determine the time-point of maximum production of silver nanoparticles, the absorption spectra of the supernatants were taken using a UV-visible spectrometer (Applied Biosystems). Based on the rapid reduction of $AgNO_3$ into SNPs the efficient bacterial strain was selected and further characterized.

2.4.2. Fourier transform infrared spectroscopy (FTIR)

A FTIR can be useful for preliminary investigation of surface chemistry of biogenic nanoparticles (i.e. those chemicals that contain carbon). This technique is widely used for identification of chemical residues such as amine, carbonyl and hydroxyl functional groups in a molecule [20, 21]. The FTIR analysis

was performed with cell free culture reduced silver nanoparticles. The synthesized SNPs sample was mixed with KBr to make a pellet in the ratio of 1:100. The FTIR instrument (SYMADZO, Japan) is with diffuse reflectance mode attachment. All measurements were carried out in the range of 400 - 4,000 cm^{-1} at a resolution of 4 cm^{-1} [22].

2.4.3. X-ray diffraction (XRD)

XRD is one of the primary analytical techniques used for the phase identification and determination of crystalline structure in the solid-state materials. The biologically synthesized silver nanoparticles were freeze dried on lyophilzer and the powdered sample was used for X-ray diffraction (XRD) analysis. The XRD analysis was performed by X'PERT PRO diffractometer with Cu-Kα radiation (k = 1.54056 Å) in the range of 30-70º at 40 keV.

2.4.4 Scanning Electron Microscope (SEM) with Energy Dispersive Spectrum (EDS)

In addition, the presence of silver metals in the sample was cast on the glass slide and dried at an ambient temperature and then the glass slides were fixed on copper supports. The samples were covered with in a thin layer of gold by sputtering. The surface was examined using the scanning electron microscope (SEM) with resolution 3.5 nm to identify the morphology of the produced SNPs and determine their mean grain sizes. The SEM is equipped with an energy dispersive spectroscopy (EDS) unit for qualitative and quantitative analyses and elemental mapping for the produced nanoclusters.

2.4.5. Transmission Electron Microscope (TEM)

The morphology and size of SNPs was studied by TEM. For this purpose, an aliquot of an aqueous suspension of SNPs was transferred onto a carbon coated copper grid and then allowed to be dried [30]. The grid was then scanned using high resolution transmission electron microscope.

3. RESULTS

3.1. Screening of potential bacterium for silver nanoparticle production

The potential bacterial strain *Escherichia coli* isolated from the bottom of the boat at fishing harbour, Visakhapatnam port was gently swabbed with a sterile cotton swab, placed in tubes containing 10 mL sterile water was shown good growth and maximum activity of silver nanoparticles production. Since the isolated strain *E. coli* was utilized for the synthesis and characterization of silver nanoparticles.

3.2. Synthesis of silver nanoparticles (Ag NPs)

The biosynthesis of silver nanoparticles using cell free filtrate of *E. coli* bacterium was investigated primarily through the observation of colour change of the experimental sample in the presence of aqueous 1 mM $AgNO_3$. A rapid change in colour from pale yellow to brown occurred in the reaction flask within 24 h of incubation as shown in fig 1. The positive result as observed by the formation of brown colour was maintained throughout the 48 h period of observation. At the same time, experimental control containing silver nitrate showed no colour change. This suggests the colour change observed in the cell free supernatant sample was due to the formation of colloidal silver nanoparticles.

Fig 1: Biosynthesis of silver nanoparticles by cell free supernatant of *E. coli* treated with 1mM $AgNO_3$ solution.

3.2.1. UV-vis analysis

UV-visible spectrophotometer was used for the structural characterization of silver nanoparticles. Incubation was carried out after the reduction of silver ions in to flask containing cell free supernatant solution resulting in an increasing absorption in the spectral range (350 - 600 nm) was observed. In the UV-visible absorption spectrum, a strong, broad peak, located at about 422 nm, was observed for nanoparticles synthesized using the cell free supernatant of *E.coli* strain and is an indication of formation of nanoparticles as shown in fig. 2. The colour change and UV absorption data analysis thus confirms the reduction of $AgNO_3$ to silver nanoparticles by the cell free supernatant of *E.coli* strain.

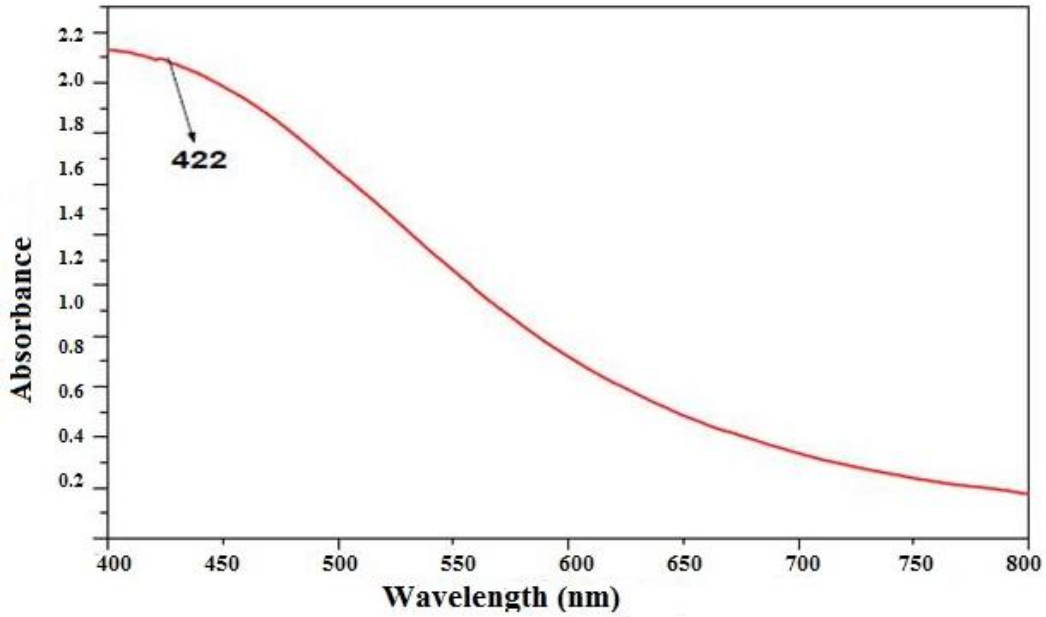

Fig 2: UV-Visible absorption spectrum of silver nanoparticles synthesized by cell free supernatant of *E. coli*.

3.2.2. Fourier transform infrared spectroscopy (FTIR)

To determine the interaction between protein and metallic particles, FTIR spectroscopy used. This could account for the reduction of silver ions and stabilization of silver nanoparticles showed intense absorption bands at 3371, 2947, 2835, 2353, 1666, 1408, 1022 and 705 cm^{-1} as shown in fig. 3. An intense broad absorbance at 3371 cm^{-1} results due to the stretch, H-bonded O-H of alcohol group. The band at 2947 cm^{-1} assigned to C-H stretching of alkane group. The band at 2835 cm^{-1} were assigned to = C-H stretching vibrations of aldehyde group.

The band at 2353 cm^{-1} assigned to the C ≡ C stretching modes of vibration in the alkynes groups. The band at 1666 cm^{-1} designates the C = C stretching of alkenes. The band at 1408 cm^{-1} can be assigned to C - C stretching (in-ring) of aromatics. The intense band at 1022 cm^{-1} can be assigned to the C-N stretch vibrations of aliphatic amines. The weak band at 705 cm^{-1} indicates that C - H bend of monosaccharides. Thus, the results of present study clearly evidenced that proteins were responsible for the stabilizing the silver nanoparticles synthesized by using the cell free filtrate of *E. coli*.

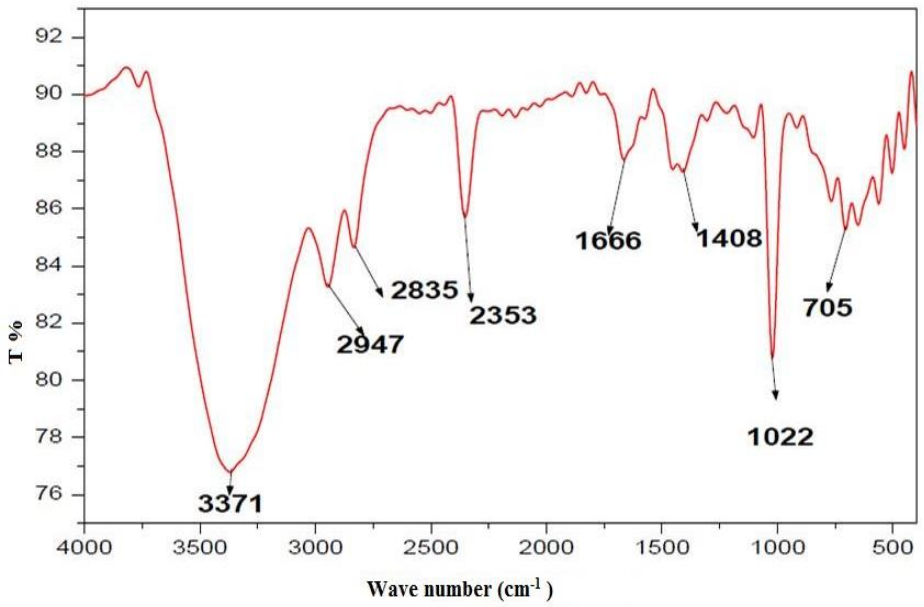

Fig 3: FTIR spectrum of synthesized SNPs using cell free extract of *E. coli*

3.2.3. X-ray diffraction (XRD)

The X-ray diffraction to confirm the crystalline nature of the particles, and the XRD pattern obtained is shown fig.4. The XRD pattern shows three intense peaks in the whole spectrum of 2Θ values ranging from 30 to 70º. It is important to know the exact nature of silver particles formed and this can be deduced from the XRD spectrum of the sample. XRD spectra of pure crystalline silver structures have been published. A comparison of our XRD spectrum with the standard confirmed that the silver particles formed in our experiments were in the form of nanocrystals, as evidenced by the peaks at 2Θ values of 38.37º, 44.02º and 63.87º, corresponding to 111, 200 and 220 planes for silver, respectively. No impurity peak is observed which confirm the high phase purity of the samples.

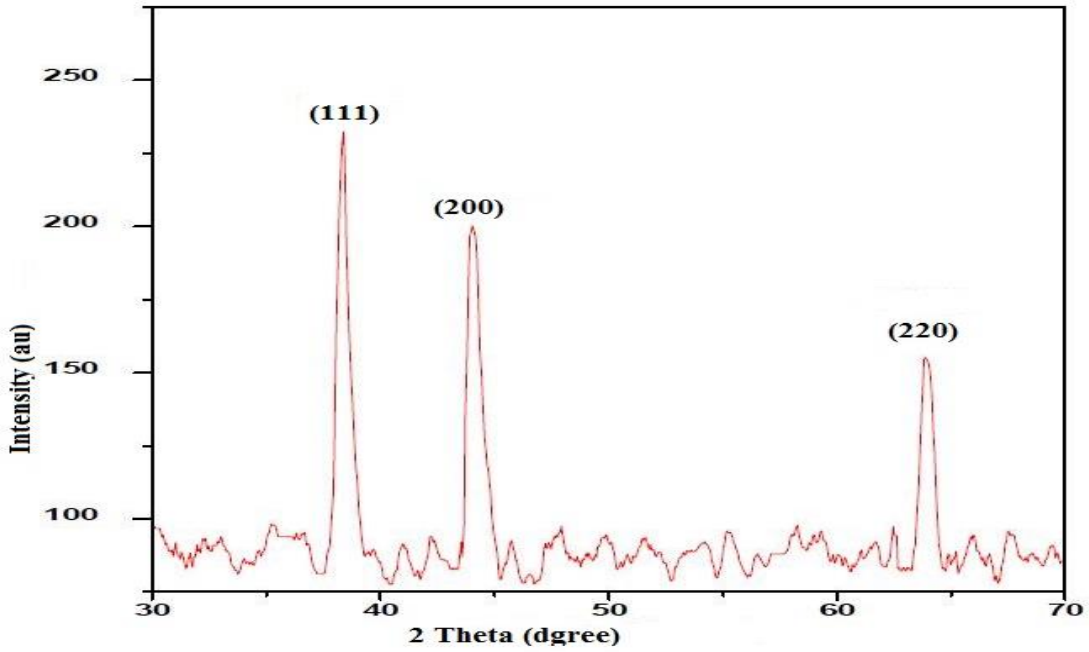

Fig 4: XRD patterns for the SNPs produced by cell free filtrate of *E. coli.*

3.2.4. Scanning electron microscope (SEM) with energy dispersive spectrum (EDS)

The SEM determinations of the above mentioned sample showed the formation of nanoparticles, which was confirmed to be of silver by EDS. It can be observed that the surface morphology nanoparticles biosynthesized from *E. coli* strain cell free supernatant treated with silver nitrate. As shown in fig 5 clearly indicates that they are cubic in shapes with sizes in the range of 30-50 nm. It is known fact that the FESEM cannot provide exact size of the particles since it is a two dimensional approach. To specify the size exactly we should go for TEM measurement.

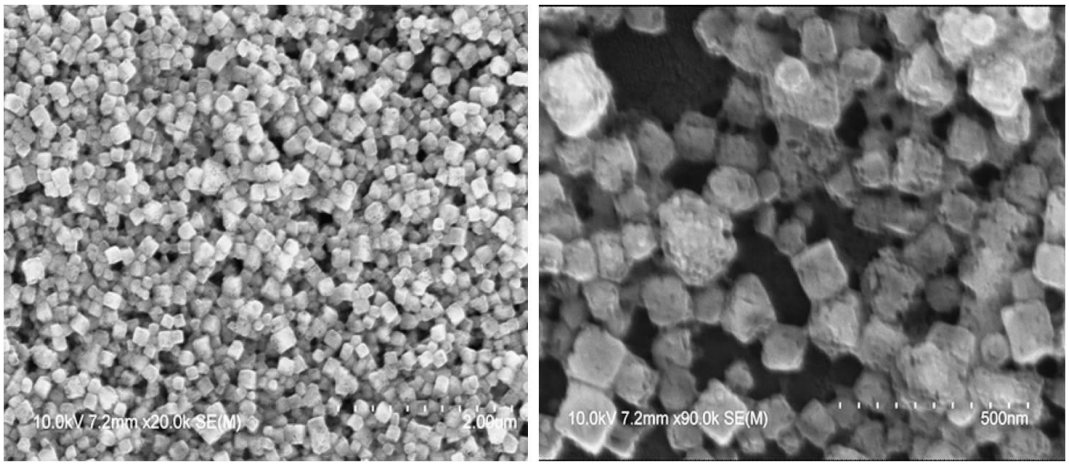

Fig 5: FESEM micrograph of SNPs

The EDS analysis also showed a peak in the silver region, confirming the formation of silver nanoparticles with the bacterial strain as shown in fig 6. Appearance of strong signal of silver indicates that the silver nanoparticles were successfully formed in the cell free supernatant of *E. coli* strain. The optical absorption peak is observed approximately at 3 keV.

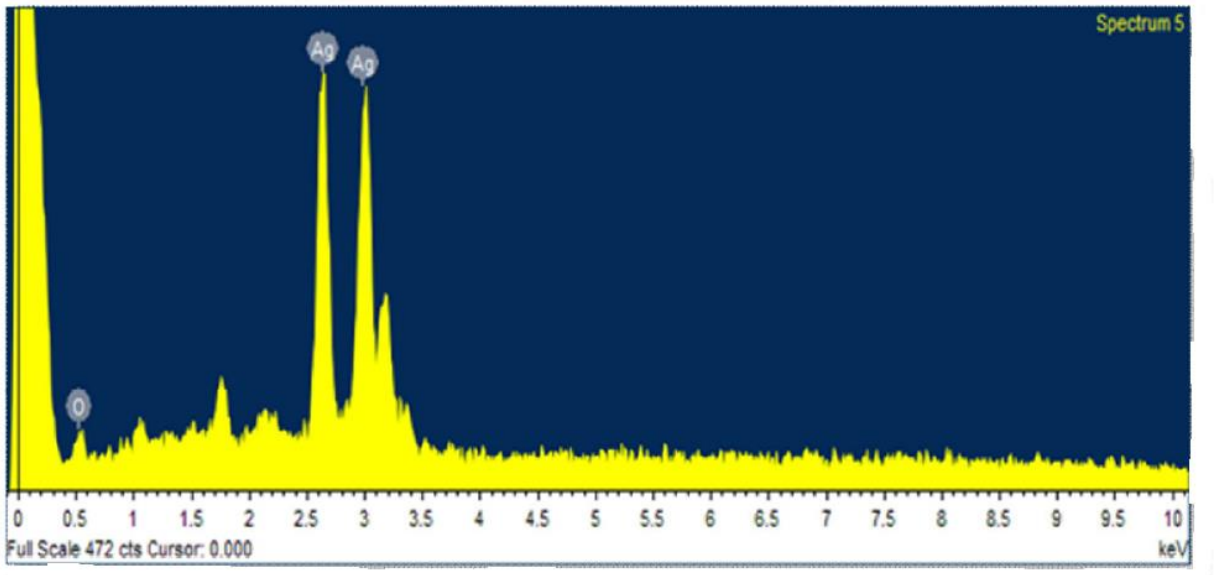

Fig 6: EDS spectrum of Silver nanoparticles

3.2.5. Transmission electron microscope (TEM)

TEM has provided further insight into the morphology and size of the silver nanoparticles formed by the cell free supernatant of *E.coli* strain. Fig 7 showed the selected area electron diffraction (SAED) pattern of silver nanoparticles. All the diffraction patterns were indexed to cubic phase of SNPs in agreement with the XRD patterns. The clear lattice fringes display indicates that the nanoparticles are good crystals. These also represents the TEM image of Ag nanoparticles of almost monodispersed with average diameter of 2 nm and the TEM images of silver nanoparticles also showed the formation of well-defined cubic phase. However few of the particles are spherical in morphology and this may be due to difference in film preparation and detection technique. The TEM images of the Ag nanoparticles show that the sizes of the individual particles are in the range of 10 -15 nm.

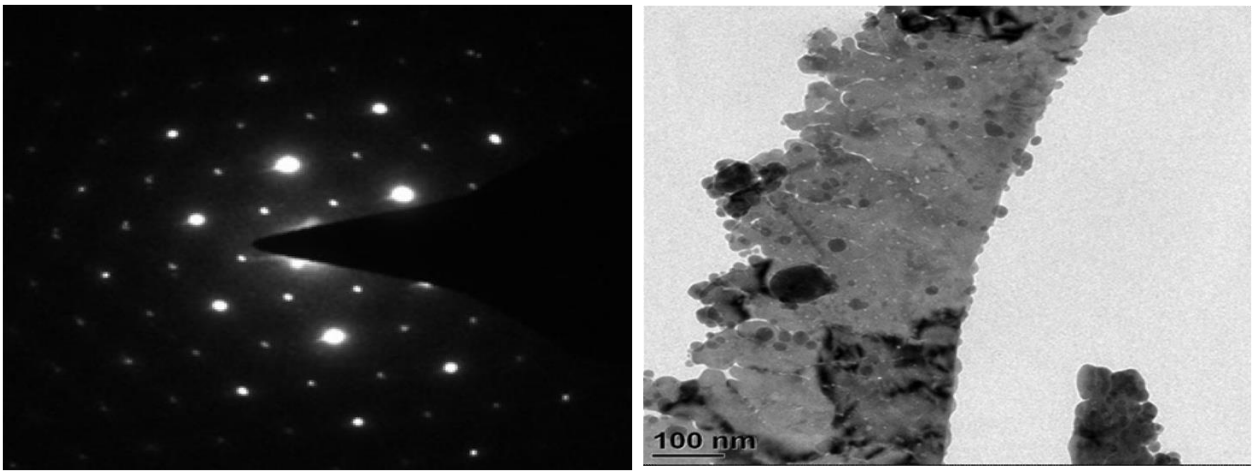

Fig 7: TEM images of synthesized SNPs using cell free supernatant of *E. coli*.

4. DISCUSSION

The biological agents such as in the form of microbes are efficient strains for the synthesis of nanoparticles. Silver nanoparticle had gain more attention because of their huge advantage in the field of chemistry, electronics, medicine and biotechnology. In the current study, the selected marine biofilm forming bacterial strain *E. coli* has showed the ability to synthesize silver nanoparticles by extracellular synthesis mechanisms. This was observed by the changes in the colour from pale yellow to brown. Observation on colour change is a method generally used for primary screening of microbial isolates for silver nanoparticle synthesis reported by Kalimuthu *et al. [11]*. The excitation of surface Plasmon vibration in the silver nanoparticles was considered as the basis for formation of brown colour. Similar observation was previously reported for the supernatant of *E. coli*, where pale yellow to brown colour was formed due to the reduction of silver ions to silver nanoparticles [17]. This maintains the fact that changes in colour as observed in the experiment can be considered as an indication of silver nanoparticles formation.

This was further confirmed by UV-visible spectroscopy which measures the absorption spectra of silver nanoparticles formed due to the collective excitation of conduction electrons in the metal. Thus, methods based on UV-visible spectroscopy have been shown to be an effective technique for the analysis of nanoparticles formation [23] UV-Vis spectra of silver nanoparticles synthesized by the selected isolates of *E.coli* strain showed an absorption peak at 422 nm by treating the cell free supernatant with $AgNO_3$. Many reports have discussed the biosynthesis of silver nanoparticles [17, 19, 24]. The mechanism behind the extracellular synthesis of nanoparticles using microbes is not fully understood. But it is considered that the enzymes like nitrate reductase secreted by microbes help in the bioreduction of metal ions to metal nanoparticles [12]. This was reported in *Bacillus licheniformis* where nitrate reductase secreted by the bacteria was found to be responsible for the reduction of Ag^+ to nanoparticles [11].

The FTIR spectrum of silver nanoparticles between the wave number 400-4000 cm^{-1} showed that the active biomolecules of cell free supernatant responsible for the reduction of Ag^+ ions into metallic silver nanoparticles, which reveals that the bond stretching were identified as O-H, C-H, =C-H, C≡C, C=C, C-C, C-N and C-H. The IR spectrum results showed the amide linkage of the protein has the stronger ability to bind silver so that the biomolecules present in the cell free supernatant of *E. coli* strain could possibly perform the function for the formation of stable silver nanoparticles. The results obtained from the present study were comparatively similar to reports produced [13,25].

The XRD pattern of the silver nitrate treated with *E. coli* strain by extracellularly corresponds to that of silver nanoparticles. Diffractions patterns at 2Θ values 38.37º, 44.02º and 63.87º indicated the 111, 200 and 220 reflections of metallic silver. However, the peak positions indicated the presence of residual cell free filtrate strain in the crystal structure. The results illustrated that strain has the largest ability for nanosilver production. Similar appearance of diffraction patterns were observed with silver nanoparticles synthesized using the cell filtrates of *Bacillus licheniformis, Bacillus cereus*, *Bacillus casei* and *Klebsiella pneu*moniae.

The morphology and crystal structure of silver nanoparticles powder have been evaluated by SEM with EDS. These SEM micrographs indicated that `the presence of cubic in nature consist of individual nanoparticles that are approximately in the range of 30-50 nm in size, which are composed of cubic shape particles. Priyadarshini *et al. [26]* employed a similar method for biosynthesis of silver nanoparticle using *Bacillus flexus* which produced spherical and triangular shape of silver nanoparticles with a size range of 12

to 61 nm. The EDS analysis also showed a peak in the silver region, confirming the formation of silver nanoparticles. The optical absorption peak is observed approximately at 3 keV, which is typical for the absorption of metallic silver nanoparticles due to surface plasmon resonance [27].

The biosynthesized silver nanoparticles were further confirmed by the analysis of TEM. The nanoparticles show variable shape but most of them are in cubic in nature. The TEM micrograph also confirmed the size of nanoparticles, which were in the range of 10 to 15 nm. Similar method of production of silver nanoparticles by treating silver nitrate solution with the culture cell free supernatant of *Klebsiella pneumoniae* belonging to the family of Enterobacteriaceae has also been reported in which the particles range in size from 28.2 to 122 nm and possess an average size of 52.5 nm was reported by Shahverdi *et al.[24]* and Kalishwaralal *et al.*, [28] also reported that a study on the synthesis of spherical silver nanoparticles with 20 nm size by using *Morganella* sp. belonging to the family of *Enterobacteriaceae*. Moreover, Gurunathan *et al. [17]*, also reported that the ambient conditions for maximum synthesis of silver nanoparticles through the reduction of Ag+ ions by the culture supernatant of *Escherichia coli* and distribution of nanoparticles, with an average size of 50 nm.

5. CONCLUSION

In conclusion, the present research work demonstrates the synthesis of silver nanoparticles from the marine biofilm forming bacterium *E. coli* strain isolated from the coastal region of Visakhapatnam port was found to be a potential strain to form the silver nanoparticles by extracellularly at room temperature within 24h. The synthesized silver nanoparticles were characterized by UV-visible spectroscopy, FTIR, XRD, SEM with EDS and TEM. From the results obtained in this effort, one can conformed that marine biofilm forming bacterial strain *E. coli* can play an important role in the bioreduction and stabilization of silver ions to silver nanoparticles.

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