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Microbial Production of Silver Nanoparticles (Agnps) By Some Bacterial Isolates

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Abstract
The five bacterial strains viz. Brevibacillus centrosporus DSM8445T, Brevibacillus invocatus NCIMB13772T, Brevibacillus choshinensis DSM8552T, Brevibacillus panacihumi DCY35 and Brevibacillus levickii LKG22481were screened for production of AgNPs using their extracellular (cell free supernatant) of bacterial cultures. The primary sight for AgNPs formation was change of colour of reaction mixture from pale yellow to dark brown.

In the present investigation it is evident that isolated bacterial strain Brevibacillus invocatus NCIMB13772T was the most efficient bacterial strain in production of AgNPs (0.996) followed by isolated bacterial strain Brevibacillus centrosporus DSM8445T (0.960), Brevibacillus panacihumi DCY35 (0.920), Brevibacillus levickii LMG22481 (0.382) and Brevibacillus chosinensis DSM8552T (Table- 2). The primary conformation of synthesis of nanoparticles in the medium was characterized by the changes in color from yellowish white to brown , the knowledge about the reduction of silver ions and formation of silver nanoparticles were still not clear, but it is believed that protein molecules and enzyme, the nitrate reductase act as good regulating agent in silver nanoparticles synthesis. The biosynthesis of AgNPs by these five bacterial strains has also been confirmed by FTIR, XRD and SEM techniques.

Key Words: *Biogenic nanoparticles; Biological nanoparticles, Cell free supernatant, Brevibacillus strains, Silver nitrate, UV- Vis Spectroscopy, XRD, SEM, FTIR*

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I. Introduction

The term "*nano*" is derived from a Greek word "*nanos*" which means dwarf and thus denotes a measurement on the scale of one-billionth (10^9) of a meter in size[1, 2] (Narayanan and Sakthivel, 2010; Thakkar et al. 2010). Nanoparticles are defined as particulate dispersions of solid particles with at least one dimension at a size range of 10-1000 nm[2, 3] (Thakkar et al. 2010; Mohanray and Chen, 2006). The most important feature of nanoparticles is their surface area to volume aspect ratio, allowing them to interact with other particles easier[1, 2] (Narayanan and Sakthivel, 2010; Thakkar et al. 2010).

In order to survive in environments containing high levels of metals, living organisms have adapted by evolving mechanisms to cope with them. These mechanisms may involve altering the chemical nature of the toxic metal so that it no longer causes toxicity, resulting in the formation of nanoparticles of the metal concerned. Thus nanoparticle formation is the "by-product" of a resistance mechanism against a specific metal, and this can be used as an alternative way of producing them. Nanoparticles have unique thermal, optical, physical, chemical, magnetic and electrical properties compared to their bulk material counterparts[4, 5] (Husseiny et al. 2007; Duran et al. 2007). These features can be exploited for next generation biosensors, electronics, catalysts and antimicrobials[1,5] (Narayanan and Sakthivel, 2010; Duran et al. 2007). Metallic nanoparticles are most important in present day science and widely studied group of materials, showing great diversity and many different uses.

There are important links between the way nanoparticles are synthesised and their potential uses. Silver nanoparticles (AgNPs) have been shown in numerous studies to display antibacterial properties[5, 6, 7, 8] (Duran et al. 2007; Guzman et al. 2012; Guzman et al. et al. 2009; Krishnaraj et al. 2010). For instance nanoparticles such as silver and gold have been shown to be effective in inhibiting growth of both Gram-positive and Gram-negative bacteria[7, 9] (Guzman et al. 2009; Lima et al. 2013). With the rise in antibiotic resistance in recent years and the development of fewer new antibiotics, research has begun to focus on these antibacterial nanoparticles

as potential new medical tools. Silver nanoparticles have also been used as optical sensors for the formation of small molecule adsorbates[10] (Mc Farland and Van Duyne, 2003). Whereas catalysts based on Pt nanoparticles have been shown to exhibit high activity for the electrooxidation of formic acid[11] (Waszczuk et al. 2002). The most common methods for preparing all of these nanoparticles are wet-chemical techniques, which are generally low-cost and high-volume. However, the need for toxic solvents and the contamination from chemicals used in nanoparticle production limit their potential use in biomedical applications[12] (Li et al. 2011). Therefore a "green", non-toxic way of synthesizing metallic nanoparticles is needed in order to allow them to be used in a wider range of industries. This could potentially be achieved by using biological methods.

Many bacteria, fungi and plants have the ability to synthesise metallic nanoparticles and all have their own advantages and disadvantages[13, 14, 15] (Suresh et al. 2004; Bhainsa and D' Souza, 2006; Song and Kim, 2009). Intracellular or extracellular synthesis, growth temperature, synthesis time, ease of extraction and percentage synthesised versus percentage removed from sample ratio, all play an important role in biological nanoparticle production. The right biological method for production of nanoparticles can depend upon a number of variables. Most importantly, the type of metal nanoparticle under investigation is of vital consideration, as in general organisms have developed resistance against a small number of metals, potentially limiting the choice of organism. However, synthetic biology; a nascent field of science, is starting to address these issues in order to create more generalized chassis, able to synthesise more than one type of metallic nanoparticle using the same organism[16] (Edmundson et al. 2014).

"Natural" biogenic metallic nanoparticle synthesis can be split into two categories. The first is bioreduction, in which metal ions are chemically reduced into more stable forms biologically. Many organisms have the ability to utilize dissimilatory metal reduction, in which the reduction of a metal ion is coupled with the oxidation of an enzyme[17] (Deplanche et al. 2010). This results in stable and inert metallic nanoparticles that can then be safely removed from a contaminated sample. The second category is biosorption. This involves the binding of metal ions from an aqueous or soil sample onto the organism itself, such as on the cell wall, and does not require the input of energy. Certain bacteria, fungi and plants express peptides or have a modified cell wall which binds to metal ions, and these are able to form stable complexes in the form of nanoparticles[18] (Yong et al. 2002).

Metallic nanoparticles are becoming increasingly important due to their potential application in many fields. The development of an environmentally friendly and inexpensive way of synthesizing them is therefore crucial. There are numerous organisms possessing the ability to synthesise nanoparticles and which therefore have the potential to be exploited and modified to optimise them to fulfil this purpose.

Several bacteria are known to produce metal nanoparticles by either reduction process or by both biosorption and reduction process both. *Thermomonospora sp, Rhodococcus* sp, *rhodopseudomonas capsulate, Pseudomonas aeruginosa,* and *Delfia acidovorans are known to produce gold nanoparticles (AuNPs)*[19, 20, 21, 1, 22] (Kasthuri et al. 2008; Park et al. 2011; He et al. 2007; Narayanan and Sakthivel, 2010; Johnston et al. 2013); *Eschericia coli to lead and platinum nanoparticles, (PbNP and PtNP)*[17] (Deplanche et al. 2010); *Shewanella* sp to selenium SeNP[23] (Raveendran et al. 2003) and Arsenic AsNPs[24] (Laudenslager et al. 2008) nanoparticles; *Desulfovibrio desulfuricansto* to lead (PbNP) nanoparticles[25] (Cai et al. 2009); *Bacillus sphaericus* JG- A12 to uranium (UNsP), copper (CuNPs) , lead (PbNPs), aluminium (AlNPs) and cadmium (CdNPs)[26] (Das et al. 2014). Similarly, the silver nanoparticles (AgNPs) are produced by a large number of bacteria viz. *Bacillus licheniformis*[23] (Raveendran et al. 2003), *Bacillus* sp[27] (Lloyd, 1998), *Klebsiella pneumonia, Escherichia coli, Enterobacter cloacae,*[28] (Kalimuthu et al. 2008), *Lactobacillus sp, Enterococcus faecium and Lactobacillus gravieae*[29] (Shahverdi et al. 2007).

Microbial source to produce the silver nanoparticles shows the great interest towards the precipitation of nanoparticles due to its metabolic activity. Extracellular synthesis of nanoparticles using cell filtrate could be beneficial over intracellular synthesis[4] (Husseiny et al. 2007).

In the present investigation the cell-free culture supernatants (extract) of five bacterial isolates viz. *Brevibacillus centrosporus* DSM8445T, *Brevibacillus invocatus* NCIMB13772T, *Brevibacillus choshinensis* DSM8552T, *Brevibacillus panacihumi* DCY35 and *Brevibacillus levickii* LKG22481 have been used to synthesize silver nanoparticles (AgNPs).

II. Materials And Methods

Bacteria were isolated from Gangetic alluvial soil of Patna by serial dilution and pour plate techniques using Nutrient Agar medium and the strains were identified by IMTECH, Chandigarh (India) following 16S-rRNA (ribotyping). The bacterial strains identified were *Brevibacillus agri, B. choshinensis DSM8552T, B. brevis, B. formosus, B. centrosporus DSM8445T, B. panacihumi DCY35, B. invocatus NCIMB13772T, B. borstelensisMTCC10644, B. bortelensis, B. thermoruber, B. levickii LMG22481, Bacillus acidicola 105- 2, Paenibacillus sp strain 324, P. popilliae ATCC14706T, P. dendritiformis MTCC and Fontibacillus aquaticus.* Of these strains five bacterial isolates viz. *Brevibacillus centrosporus* DSM8445T, *Brevibacillus invocatus*

NCIMB13772T, *Brevibacillus choshinensis* DSM8552T, *Brevibacillus panacihumi* DCY35 and *Brevibacillus levickii* LKG22481 were used for the production of Silver nanoparticles (AgNPs).

In order to screen the most efficient bacterial isolated strain (s) for the synthesis of AgNPs, all the five bacterial strains were inoculated separately in 50 ml Nutrient broth medium (peptone 5gm, Beef extract 3gm, NaCl 5gm per litre of distilled water) in 250 ml Erlenmeyer flask . The flasks were incubated at 37^0C for 48 hours in shaking incubator (150 rpm).

After incubation, cultures supernatants were obtained by centrifugation at 8000 rpm for 10 min. The final concentration of 1mM $AgNO_3$ in deionized water was added separately into 2ml of cell free supernatant in clean, sterile test tube. Three replicates were used for each strain. The cell free supernatant without addition of $AgNO_3$ used as control. The bio-reduction of silver ions was monitored by visual colour change and spectrophotometrically by UV/Vis Spectrometer with wavelength ranging from 200 to 800 nm for reaction mixture. The culture was then centrifuged at 10000 rpm for 10 minutes to recover the synthesized nanoparticles in the aliquot.

Characterization of Silver Nanoparticles

The silver nanoparticles produced by bacterial strains were characterized on the basis of following parameters:

1. Observed value:

The synthesis of silver nanoparticles was observed by colour change. The colour of the mixed solution changed to deep orange after 24 hours of agitated incubation with silver nitrate which further blackened in 72 hours. The black colour the culture culture confirmed the reduction of silver salt to silver nanoparticles.

2. UV- Vis Spectral analysis:

The microbial production of silver nanoparticles was confirmed by sampling the culture filtrate at different time intervals and the absorption was scanned by UV- Vis Spectrophotometer at wavelength of 300 – 800nm on Cole- Parmer Spectrophotometer.

3. FTIR analysis:

The dried samples were grinded with Potassium Bromide and the made pellets which were used for Fourier Transform Infra Red measurements. The spectrum was recorded in the range of 4000- 400/cm using Spectrum Two- FTIR Spectrophotometer.

4. XRD analysis:

The X- Ray Diffraction pattern was measured by powder diffractometer and the Co- K alpha radiation in the range of 20- 80 degree at a scan rate of 0.02 degree per minute with the time constant of 2 second.

5. SEM analysis:

The morphology and size of the silver nanoparticles were investigated by Scanning Electron Microscopic images using Phillips instrument.

III. Results And Discussion

The five bacterial strains viz. *Brevibacillus centrosporus* DSM8445T, *Brevibacillus invocatus* NCIMB13772T, *Brevibacillus choshinensis* DSM8552T, *Brevibacillus panacihumi* DCY35 and *Brevibacillus levickii* LKG22481were screened for production of AgNPs using their extracellular (cell free supernatant) of bacterial cultures. The primary sight for AgNPs formation was change of colour of reaction mixture from pale yellow to dark brown. This change in colour could be noticed by nacked eye. As the colour intensity increased, the accumulation of AgNPs increased (deep brown colour). The five extracellular culture supernatants and 1.0 mM AgNO3 mixture (formed colour) were scanned by spectrophotometer (200-800nm) by measuring the maximum absorbances, as indicated in Tables (1 and 3). The most efficient bacterial isolate *Brevibacillus invocatus* NCIMB13772T scane has been presented in Figure-1.

Table-1: Scanning for the maximum absorbance of dark brown colour formed by extracellular supernatants of five different bacterial strains

Bacterial stains	Wavelenght (nm)
Brevibacillus centrosporus SM8445T	421
Brevibacillus invocatus NCIMB13772T	436
Brevibacillus choshinensis DSM8552T	396
Brevibacillus panacihumi DCY35	415
Brevibacillus levickii LMG22481	365

Table- 2: The Absorbance of colour formed by five different bacterial strains at 420 nm

Bacterial stains	Absorbance at 420 nm
Brevibacillus centrosporus SM8445T	0.960
Brevibacillus invocatus NCIMB13772T	0.996
Brevibacillus choshinensis DSM8552T	0.365
Brevibacillus panacihumi DCY35	0.920
Brevibacillus levickii LMG22481	0.382

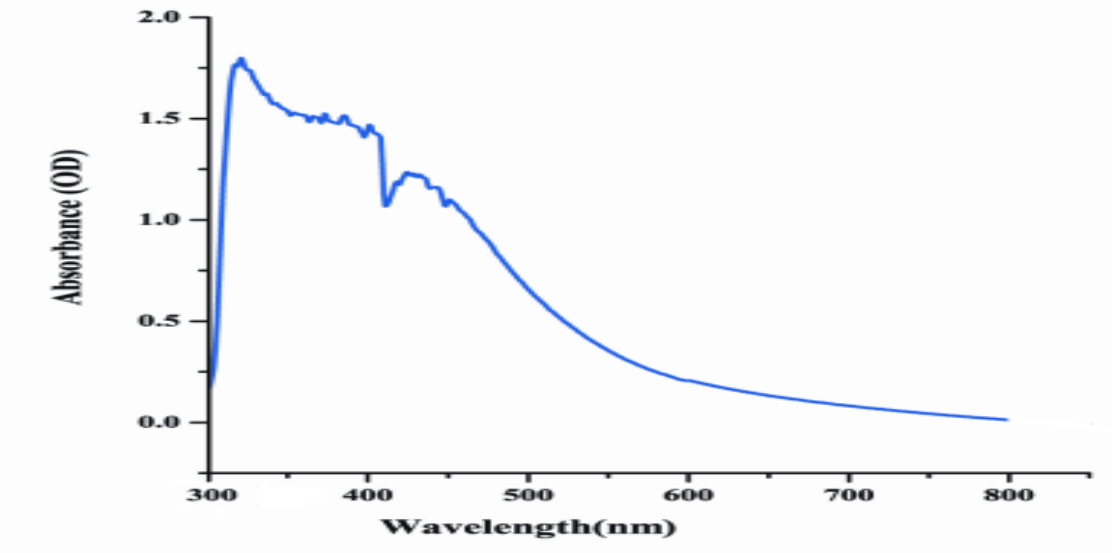


Figure- 1: Scan for the most efficient bacterial isolate *Brevibacillus invocatus* NCIMB13772T

From the results it is evident that isolated bacterial strain *Brevibacillus invocatus* NCIMB13772T was the most efficient bacterial strain in production of AgNPs (0.996) followed by isolated bacterial strain *Brevibacillus centrosporus* DSM8445T (0.960), *Brevibacillus panacihumi* DCY35 (0.920), *Brevibacillus levickii* LMG22481 (0.382) and *Brevibacillus chosinensis* DSM8552T (Table- 2). The primary conformation of synthesis of nanoparticles in the medium was characterized by the changes in color from yellowish white to brown**,** the knowledge about the reduction of silver ions and formation of silver nanoparticles were still not clear, but it is believed that protein molecules and enzyme, the nitrate reductase act as good regulating agent in silver nanoparticles synthesis[30] (Natarajan et al. 2010). As cleared by[31] (Kuber et al. 2006) biological method of synthesis of silver nanoparticles exhibit strong absorption of electromagnetic waves in the visible range due to their optical resonant property, called Surface Plasmon Resonance (SPR).

Mechanism of formation of AgNPs by FTIR

The formation of AgNPs by five different bacterial strains has been depicted by FTIR spectra (Figure- 2 to 6). The FTIR measurements were performed to identify the potential biomolecules in the in the bacterial cell free extracts responsible for the reduction Silver ions (Ag^+) to AgNPs. The peak was centred at 1345/cm which indicated the presence of Nitrate in the residual solution. The band at 3626/cm corresponds to O – H stretch Carboxylic acids. The stretch at 1651/cm corresponds to N – H bond primary amines. The peak at 1410/cm corresponds to C – N stretch of Aromatic amine group and the bands observed at 631/cm corresponds to C – N stretching esters.

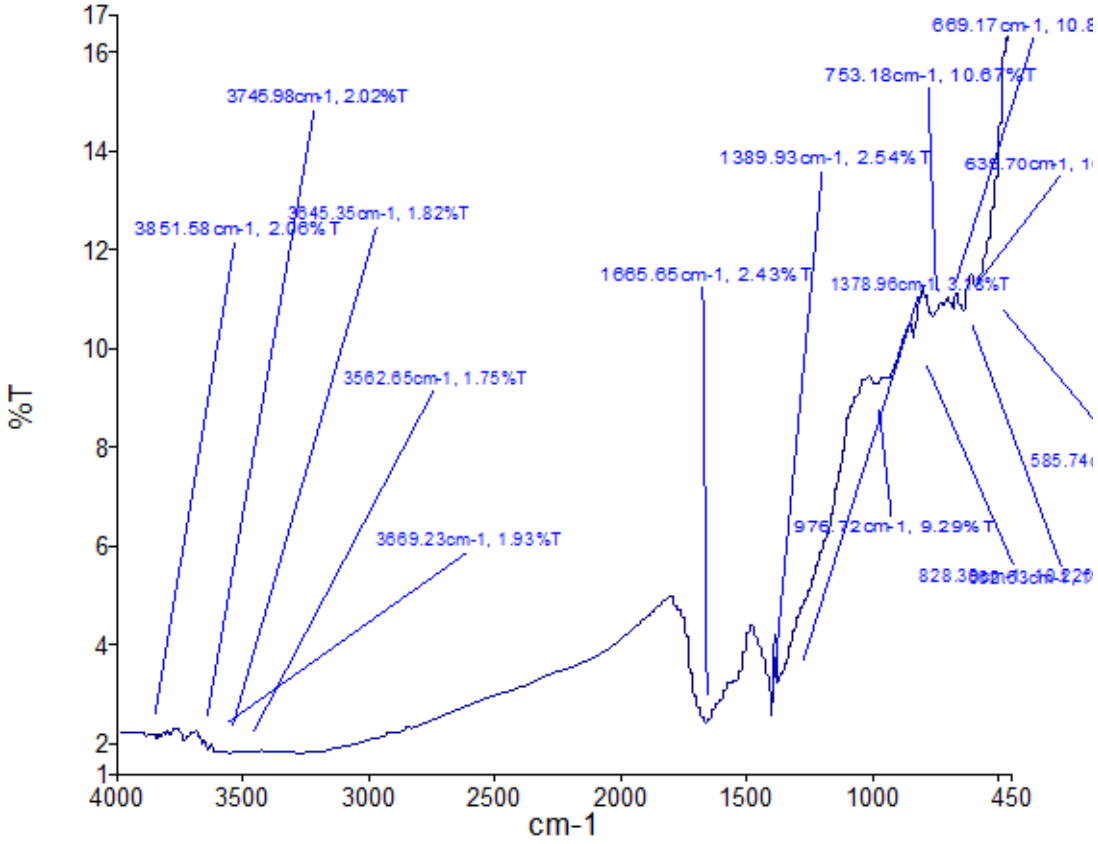


Figure- 2: FTIR Spectra of AgNPs produced by *Brevibacillus centrosporus* DSM8445T

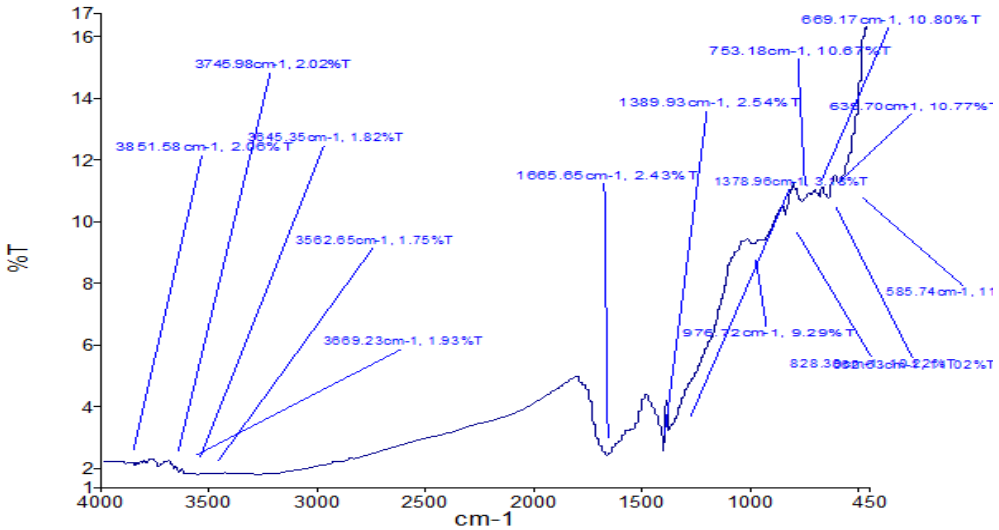


Figure- 3: FTIR Spectra of AgNPs produced by *Brevibacillus invocatus* NCIMB13772T

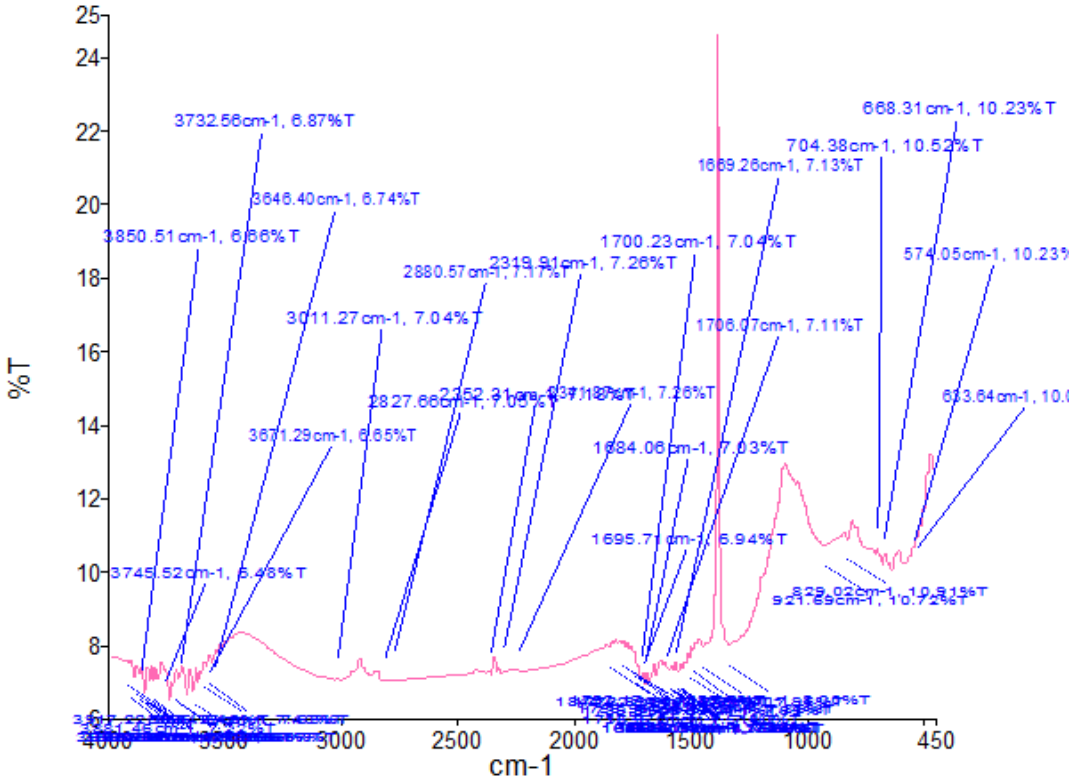
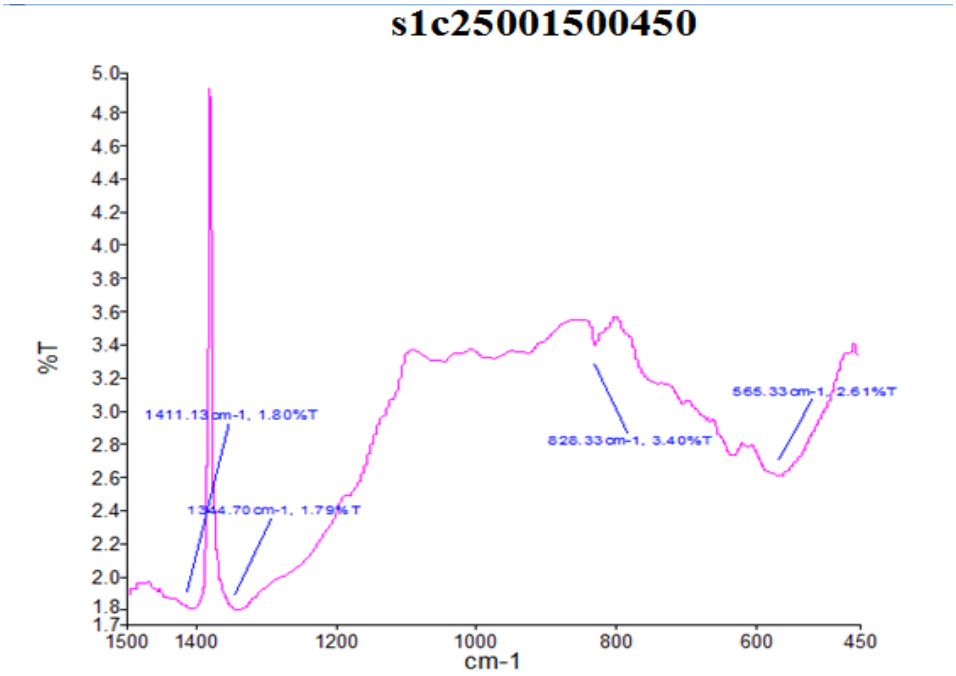
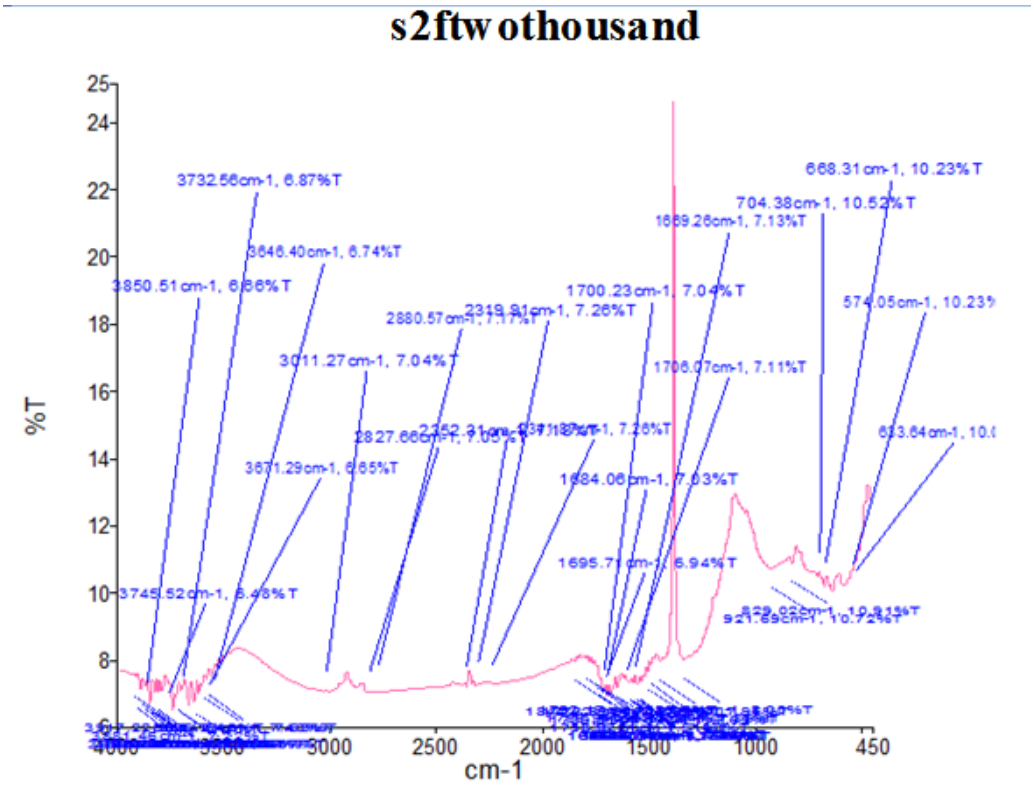


Figure- 4: FTIR spectra of AgNPs produced by *Brevibacillus Choshinensis* DSM8552T



Sample Name	Description	Saved or unsaved State	Spectrum quality check summary
s1c25001500450	Sample 032 By physics Date Thursday, October 16 2014	Saved	The Quality Checks give rise to multiple warnings for the sample.

Figure- 5: FTIR spectra of AgNPs produced by *Brevinnbacillus panacihumi* DCY35



Sample Name	Description	Saved or unsaved State	Spectrum quality check summary
s2ftwothousand	Sample 039 By physics Date Thursday, October 16 2014	Saved	The Quality Checks give rise to multiple warnings for the sample.

Figure- 6: FTIR Spectra of AgNPs produced by *Brevibacillus levickii* LMG22481

Confirmation of AgNPs production by XRD

In the present investigation the production of AgNPs by five bacterial isolates has been confirmed by XRD technique (Figure- 7 to 11; Table-3). The XRD patterns were obtained by measuring the angles at which X- ray beam is diffracted by the crystalline phases in the specimen. The XRD pattern of AgNPs produced by five bacterial strains showed four prominent peaks at 320 (2 theta), 460 (2 theta), 570 (2 theta), 760 (2 theta) which indicated the presence of (111), (200), (220), and (311) sets of lattice planes and accordingly could be indexed as Fee structures of AgNPs. Hence, from the XRD results it is evident that AgNPs formed by all the five bacterial strains using their extracts were essentially crystalline in nature (Table- 3; Figure- 7 to 11).

Peak list

2-theta (deg)	d (ang.)	Height (cps)	Int.I (cps*deg)	FWHM (deg)	Size	Phase name
28.51(3)	3.129(3)	51(13)	20.2(9)	0.29(3)	298(27)	Unknown,
32.928(13)	2.7179(10)	139(22)	58.0(18)	0.350(13)	247(9)	Unknown,
46.77(3)	1.9408(13)	129(21)	53.3(18)	0.30(3)	302(30)	Unknown,
55.32(3)	1.6592(9)	31(10)	11.8(9)	0.30(3)	311(33)	Unknown,
57.96(4)	1.5899(10)	43(12)	15.5(9)	0.28(5)	342(56)	Unknown,
77.05(6)	1.2366(8)	40(12)	19.2(14)	0.42(5)	254(29)	Unknown,

Fig : 3 Six Data parameters of Prominent Peak

Measurement profile

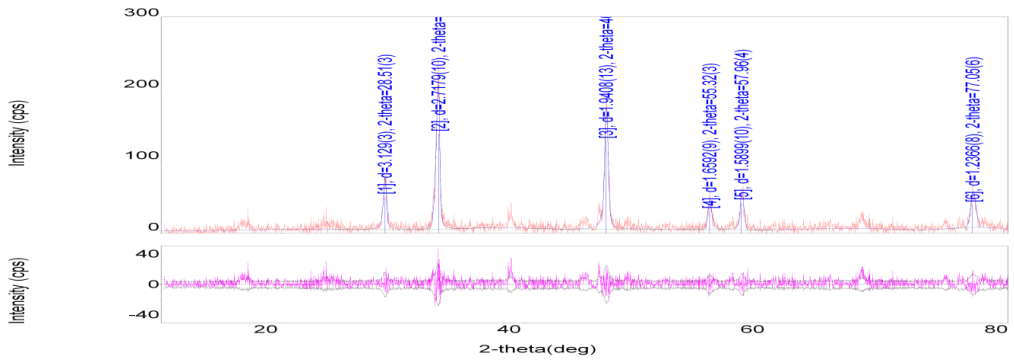


Figure- 7: XRD pattern of AgNPs of *Brevibacillus centrosporous* DSM8445T

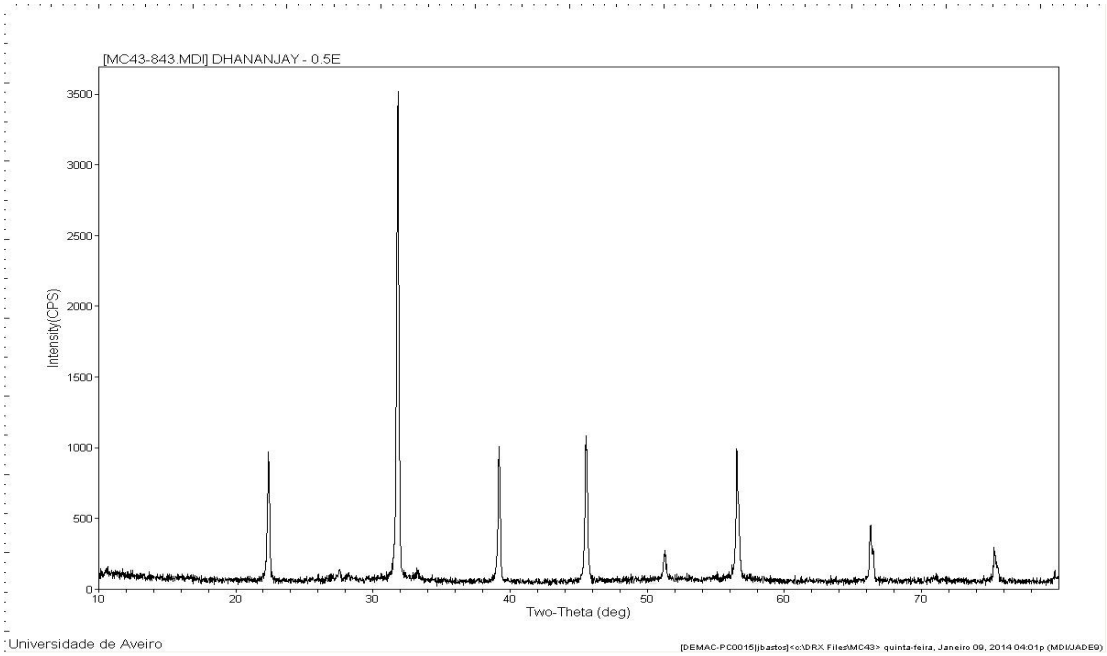


Figure- 8: XRD pattern of AgNPs of *Brevibacillus invocatus* NCIMB13772T

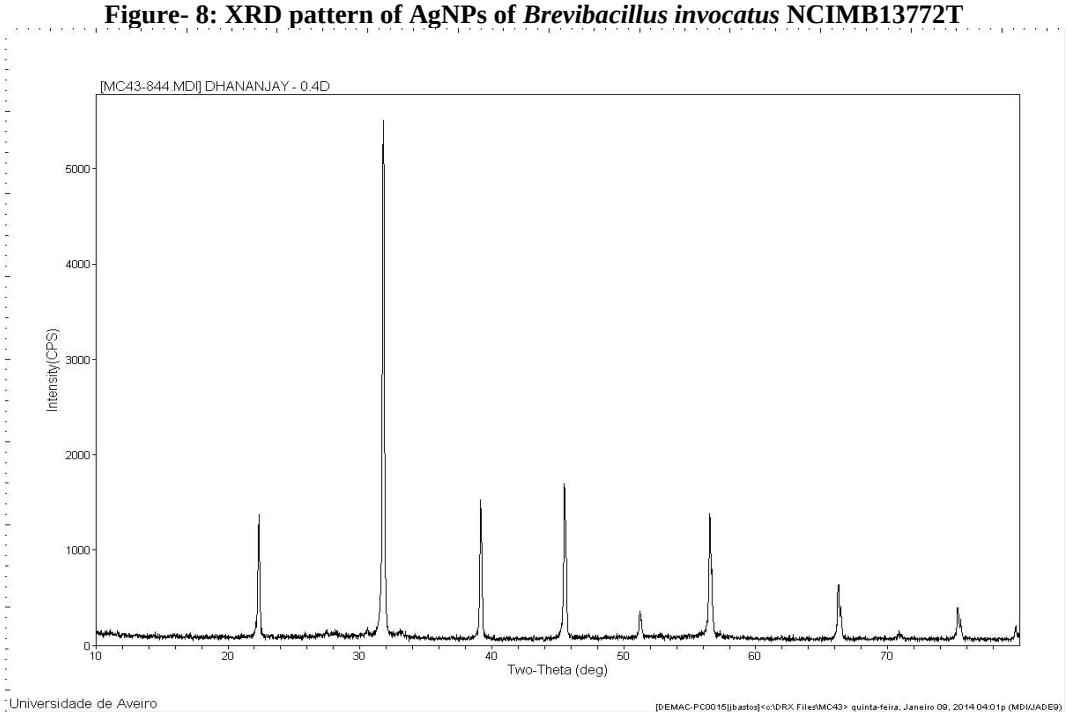


Figure- 9: XRD pattern of AgNPs of *Brevibacillus choshinensis* DSM8552T

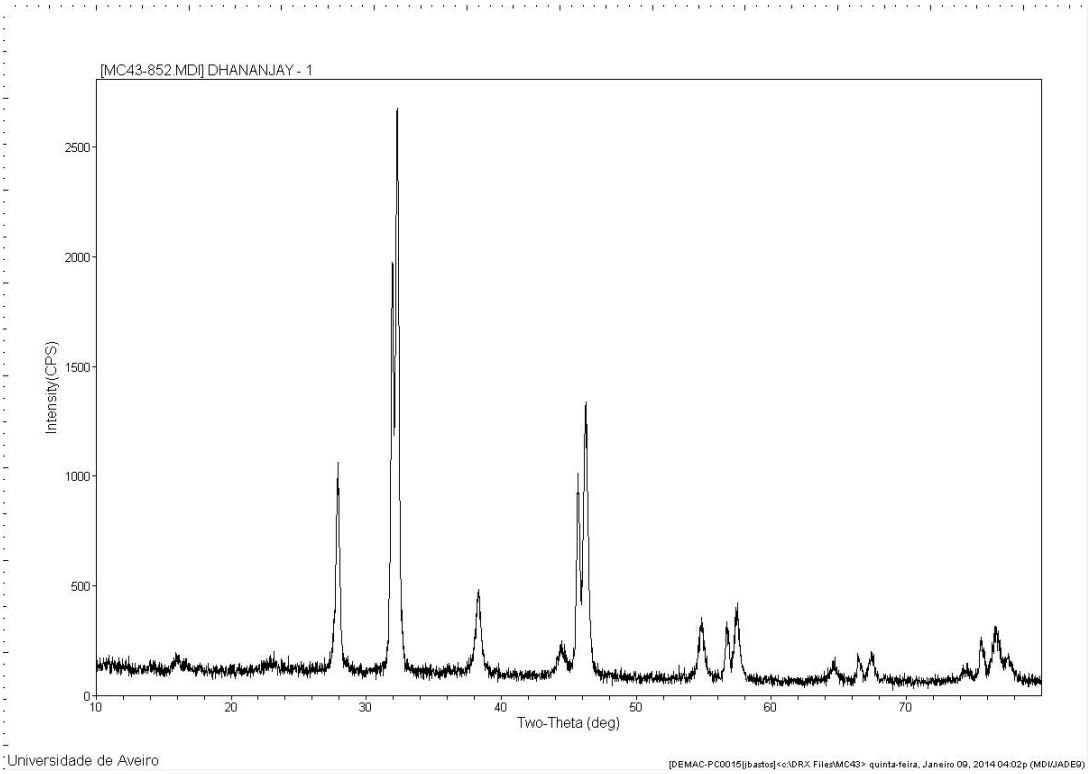


Figure- 10: XRD pattern of AgNPs of *Brevibacillus panacihumi* DCY35

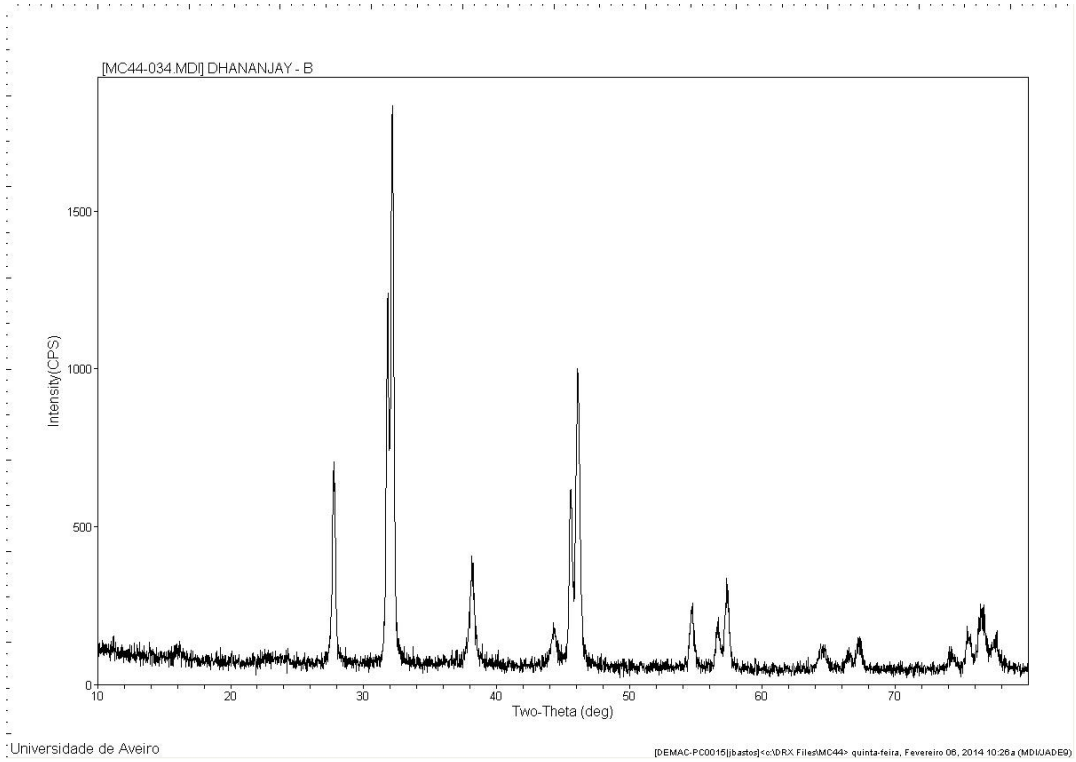


Figure- 11: XRD pattern of AgNPs of *Brevibacillus levickii* LMG22481

In the present investigation the size and shape of AgNPs synthesized by present five bacterial strains were also analyzed using Scanning Electron Microscopic (SEM) images (Figure- 12 to 16). The SEM images confirmed that the AgNPs produced by all the five bacterial strains were cubical in shape with diameter ranging from 5 nm to 15 nm.

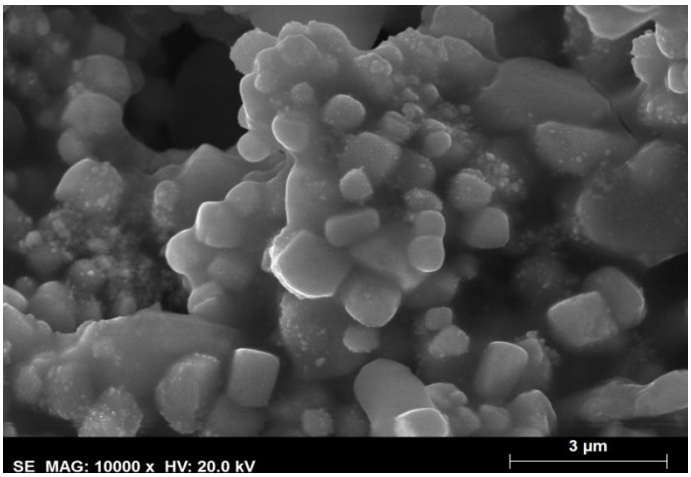


Figure- 12: SEM of AgNPs produced by *Brevibacillus centrosporus* DSM8445T

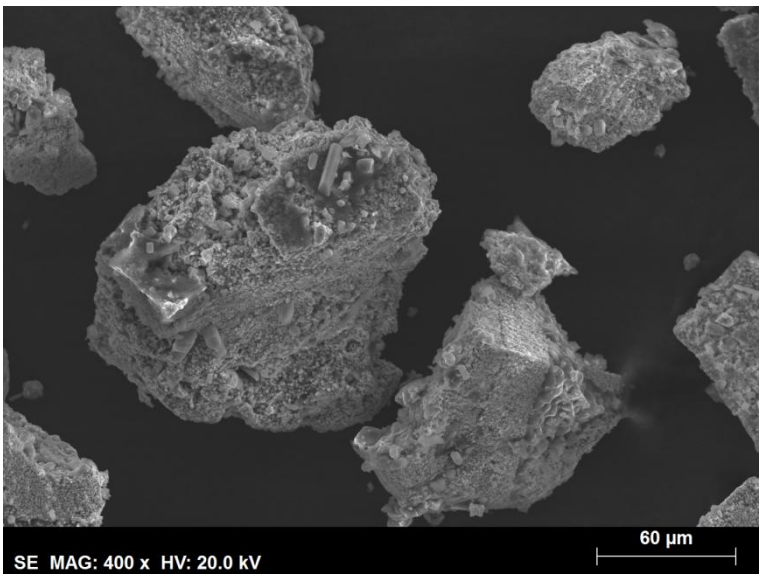


Figure- 13: SEM of AgNPs produced by *Brevibacillus invocatus* NCIMB13772T

Figure- 14: SEM of AgNPs produced by *Brevibacillus choshinensis* DSM8552T

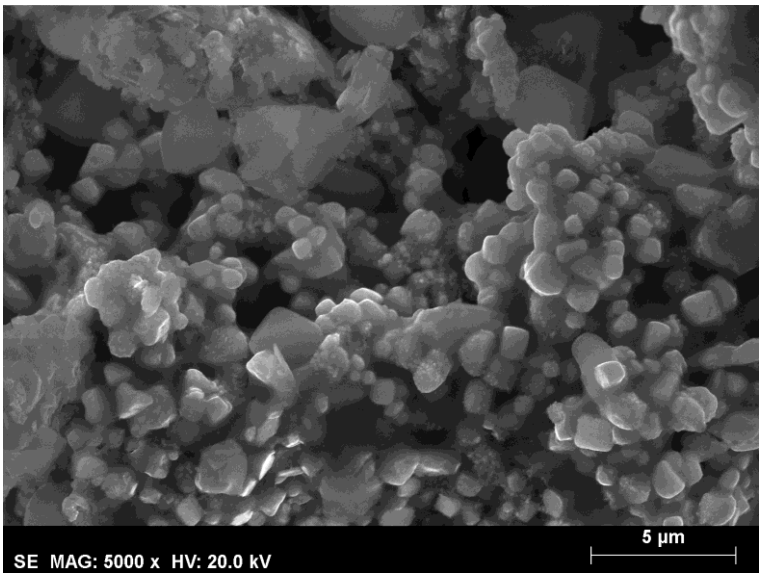


Figure- 15: SEM of AgNPs produced by *Brevibacillus panacihumi* DCY35

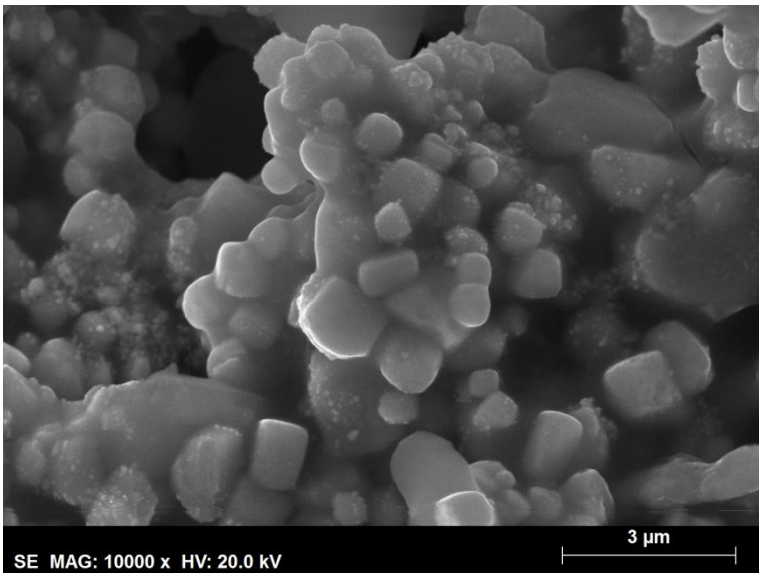


Figure- 16: SEM of AgNPs produced by *Brevibacillus levickii* LMG22481

SPR is highly influenced by shape and size of the nanoparticles. Likewise, the microorganisms has the metal-microbe interaction to produce inorganic metal ions, and have several applications in biotechnological fields, includes bioremediation, biomineralization, bioleaching and microbial corrosion[32] (Link et al. 2003).

The IR spectrum results showed the amide linkage of the protein has the stronger ability to bind silver so that the protein could most possibly to form a coat covering around AgNPs and it stabilize the aqueous medium. This evidence suggests that the biomolecules present in the cell free supernatant of B. flexus could possibly perform the function for the formation of stable AgNPs[33] (Priyadarshinia et al. 2013).

It was well known that the protein can bind to AgNPs through free amide groups. High negative f-potential of AgNPs due to capping with negatively charged proteins may be the reason for stabilization of AgNPs[34] (Sarkar et al. 2006). In a study of silver nanoparticle synthesis, it was observed that most of particles clearly attached on the surface of the cytoplasmic membrane[35] (Priyabatra et al. 2001).

Many bacterial species were tested to synthesise AgNPs Sintubin et al. (2009)[36] viz. *Lactobacillus spp., Pediococcus pentosaceus, Enterococcus faecium* and *Lactococcus garvieae* and a two-step process of AgNP formation was proposed. First, the Ag ions were accumulated at the cell wall via biosorption and then subsequent reduction of those ions formed the metallic nanoparticles (Sintubin et al. 2009)[36]. Sintubin et al. (2009)[36] also suggest that the cell wall may act as a capping agent for the nanoparticles, which keeps them stable by preventing aggregation and showed that by increasing the pH of the medium, the reduction rate of the

nanoparticles increased. The effect of pH on nanoparticle synthesis was also observed by He et al. (2007)[21]. By varying the pH levels, nanoparticles of differing size and shape were formed (He et al. 2009)[37]. They illustrated that by increasing the pH, AgNPs of around 10-20 nm were formed and by lowering the pH to 4, nanoplate formation was observed.

The present findings are in agreement with the work of Abo- State and Partila (2015)[38] who studied the production of Silver nanoparticles by *Pseudomonas aeruginosa* cell free extract. The present findings gain support from the work of Amar Kumar and Ashok Ghosh (2016)[39] who observed a more or less similar production of AgNPs by cell free extract of *Brevibacillus borstelensis* MTCC10642.

IV. Conclusions

It is clear that metallic nanoparticles have great potential in many different industries. The need for a process to synthesize such nanoparticles in a reliable and green way is becoming more pressing. Current chemical and physical methods involve toxic chemicals and high temperatures that are not only dangerous to the environment but costly too. Numerous groups have focused on alternative ways of synthesizing nanoparticles. Biological systems have been investigated in an effort to provide a sustainable, resource efficient and cheap method of synthesis. Many different biological chassis have been studied for their ability to resist the toxic effects of metal ions whilst producing metallic nanoparticles. Bacteria are relatively cheap and easy to cultivate and have a high growth rate compared to other biological systems such as fungi or plants. Their ease of manipulation gives them the advantage over plants and fungi as the chassis of choice for the near-term bio-production of nanoparticles that require optimized synthesis through genetic engineering.

Whatever the choice of biological chassis may be, whether it is a bacterium, fungus or plant, they all need to be studied comprehensively in order to gain a clearer understanding of mechanism and to close the knowledge gap in biological nanoparticle synthesis methods by different organisms. The risks of such nanoparticles must also be assessed, but here biological synthesis may offer yet another advantage. The rapidly developing field of synthetic biology aims to create predictable, standardized systems and with such new technologies directed towards the production of metallic nanoparticles, biogenic nanoparticle samples are likely to become more homogenous and more reproducible, therefore the environmental and health risks posed will be more easily and more reliably assessed.

The field of biological production of metallic nanoparticles is relatively new and underexplored. However, it shows great potential in the biotechnology sector. There are many aspects of these biological methods to be discovered, and later manipulated, as the technology emerges.

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