International Research Journal of Biotechnology (ISSN: 2141-5153) Vol. 1(5) pp.071-079, December, 2010
Available online http://www.interesjournals.org/IRJOB

Full Length Research Paper

Bacteria mediated extracellular synthesis of metallic nanoparticles

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Accepted 25 October, 2010

Bacillus megaterium **isolated from fluvial zone of North Bihar has been used for the extracellular synthesis of metal nanoparticles (NPs) viz silver, lead and cadmium. The synthesized nanoparticles got accumulated on the surface of the cell wall of bacteria.** ***Bacillus megaterium*** **was grown aerobically and the cultures were challenged with the solutions of silver nitrate, lead nitrate and cadmium nitrate in requisite ambience of laboratory. Cell lysate obtained, after the centrifugation of cultures were characterized through optical absorption by UV-Vis Spectrophotometer, X-ray Diffraction (XRD), Transmission Electron Micrography (TEM) and Energy Dispersive Spectroscopy (EDS). These nanoparticles of Ag, Pb and Cd showed absorption peaks at ~ 435 nm, ~ 330 nm and ~ 410 nm respectively corresponding to the plasmon resonance of silver nanoparticles (AgNPs), cadmium nanoparticles (CdNPs) and lead nanoparticles (PbNPs). XRD Spectrum of these nanoparticles confirmed the formation of Ag, PbS and CdS particles by showing their respective characteristic peaks. TEM showed the production of these metal nanoparticles with particle size in the range of 10-20 nm.**

Keywords: Extracellular; AgNPs; CdNPs; PbNPs; TEM; Selected Area Electron Diffraction

INTRODUCTION

Green approach for the synthesis of nanomaterials utilizes of biological components, primarily prokaryotes and eukaryotes. Microbes play direct or indirect roles in several biological activities. Metals and non-metals present on earth are in constant association with biological components. This interaction of metals and microbes are natural and continuous since the inception of life provides the exciting areas of research. Nanoparticles of noble metals have been synthesized by several methods viz. hard templating
(Zhou et al.,1999), bio-reduction (Canizal et al., 2001) and solution phase analyses (Yu et al., 1997 ; Jana et al., 2001; Lisiecki et al., 2000). The most abundant organisms in our biosphere are bacteria. Slight climate changes can potentially be disastrous to the life processes of bacteria; this can result in the prolific advantage for the production of nanoparticles. Synthesis of gold and silver (Mukherjee et al., 2001) nanoparticles by eukaryotic cells such as fungi is reported. Synthesis of gold nanoparticles by *Shewanella algae* (Konish et al., 2004) and silver nanoparticles by fungus *Verticillium* (Mukherjee et al., 2001) were also reported. Several strains of ***Fusarium*** **viz** ***Fusarium oxysporum*** (Duran et al., 2005), ***Aspergillus fumigatus and Aspergillus flavus*** (Vighneshwaran et al., 2007) were used for successful production of metal nanoparticles. Recently white rot fungus ***Coriolus versicolor*** has also been used for the synthesis of stable silver nanoparticles. Biologically synthesized nanoparticles have wide application viz., biosensors (Narn et al., 2003) biolabelling (Tkachenko, et al., 2003), in cancer therapeutics (Hirsch et al., 2003) and in coating of medical appliances (Matsumura et al., 2003). In the present study silver, cadmium and lead nanoparticles have been synthesized by Bacillus species, viz *Bacillus*

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megaterium and characterization of these nanoparticles have been carried out by UV-Vis Spectroscopy, XRD and TEM techniques.

MATERIALS AND METHODS

Culture and isolation

The soil samples were collected from different sites of the North Bihar of India along the river Ganges. Inoculums were prepared on nutrient agar plates by serial dilution and streaking. Pure culture was isolated after the requisite period of incubation (Figure 1). The identification and characterization of the culture was done on morphological and biochemical basis. The strain was characterized as aerobic or facultative, endospore forming bacteria, versatile chemoheterotrophic capable of respiration using a variety of simple organic compounds. They are mostly mesophilic. This strain showed positive result for Starch hydrolysis, Nitrate reduction, Catalase test, Oxidase test and Oxidation / Fermentation test for Dextrose, Lactose, Maltose, Sucrose and Mannitol. They showed negative result with H_2S production, Cytochrome Oxidase, Indole test , Voges Proskauer test and Growth on Mckonkey agar. One of the most resistant strain *Bacillus megaterium* was selected for this study. Selection of this strain was made on the basis of the exploitation of **the** local biodiversity. Most Bacillus species are versatile chemoheterotrophs capable of respiration using a variety of simple organic compounds. *B. Megaterium* requires no organic growth factors while other microbes require amino acids, B- vitamin or both. Many morphological and biochemical experiments were done for the identification of the test microbes (Table-1 and Table-2). The tests were done to identify the strain upto genus level. The observed characteristics were compared with Bergey's Manual of Determinative Bacteriology for proper identification of the organisms. The species of the strain was identified by Institute of Microbial Technology (IMTECH), Chandigarh, India. which has given the MTCC Number to these bacteria; *B. cereus* (MTCC No-8527), *B. megaterium* (MTCC No-8755) and *B. koreensis* (MTCC No-8529). It was cultured aerobically in 250ml MGYP (glucose 1%, malt extract 1%, yeast extract 0.3%, peptone 0.5%) medium in 500 ml Erlenmeyer flask for the experiment. The flasks were incubated at 37.5°C on a rotary shaker set at 100 rpm for 24hrs.

Synthesis of Nanoparticles

Metal salts i. e. Silver Nitrate ($AgNO_3$), Cadmium Nitrate $Cd(NO_3)_2$ and Lead Nitrate
$Pb(NO_3)_2$ were added to the freshly prepared (regularly sub-cultured) innoculum, which showed fair growth when put on shaker for 24h-72h.The experiment was done at different pH ranging from 5.6- 10.9, however, the nanomaterials were synthesized at pH 8.3.The media was made slightly alkaline because at this pH the maxima optical density (OD) showed prominent peak. One of the set of culture was treated as control for the experiment (without the silver, cadmium and lead salts). Silver Nitrate($AgNO_3$), Cadmium Nitrate ($Cd(NO_3)_2$) and Lead Nitrate ($PbNO_3)_2$ were added in different concentrations ranging from 50, 100, 500, 1000, 1500, 20000 ppm respectively.The concentration range was selected to check the viability of microbes with increasing concentration. Earlier work protocol has mentioned concentration upto 250mg/litre (Gericke and Pinches, 2006). After 72h the cultures were filtered through Whatman filter paper (0.22 mm) and the cell free supernatant was observed on UV-VIS spectrophotometer on wavelength range 200-600 nm. The absorbance maximum of supernatant was taken at different time intervals i.e. after 1h of addition of $AgNO_3$, $Cd(NO_3)_2$ and $(PbNO_3)_2$. Further absorbance maxima were taken after 24h, 48h, 72h, 7days and 15days.These time intervals were selected to observe the stability of the nanomaterials. AgNPs, PbNPs and CdNPs showed absorbance maxima at ~ 435nm, ~ 330nm and ~ 410nm respectively. The cultures were then centrifuged at 5000 rpm for 15 min, they were then washed with distilled water several times and allowed to dry at 200°C. The cultures were stored for one month and the particles were found to be stable.

Characterization of nanoparticles

UV-Vis Spectroscopy

The optical density of these nanoparticles suspended in distilled water was measured by UV-Visible spectrophotometer (Systronics 2202 double beam model) from wavelengths 200-700nm. **AgNPs, PbNPs and CdNPs showed absorbance maxima at ~ 435nm, ~ 330nm and ~ 410nm respectively.**

X- Ray Diffraction Analysis

The formation and quality of nanoparticles were checked by XRD technique. X-ray Diffraction (XRD) measurements of drop-coated films of synthesized nanoparticles on glass substrate were recorded in a wide range of Bragg angles 2θ at a scanning rate of $2^0 min^{-1,}$ carried out on a Philips PW 1830 instrument that was operated at a voltage of 40 kV and a current of 30 mA with Cu Kα radiation (λ=1.5405 Å).

TEM and Electron Diffraction Analysis

High Resolution Transmission Electron Microscopy (HRTEM) was performed by TECHNAI G20-STWIN (200KV) machine with a line resolution 2.32 (in °A). These images were taken by drop coating AgNPs, CdNPs and PbNPs on a carbon-coated copper grid. Energy Dispersive Absorption Spectroscopy photograph of these metal nanoparticles were carried out by the HRTEM equipment as mentioned above.

RESULTS

The results of this experiment i.e. extracellular synthesis of AgNPs, CdNPs and PbNPs were observed. The colour of the challenged solution changed to deep orange after 24h of agitation with $AgNO_3$ (Figure 2a) which further blackened in 72hrs. The black colour of the culture confirmed the reduction of silver salt to AgNPs, however the bacterial strain treated with ionized water retained its original colour. The bacteria were found to be reducing the silver extracellularly. There was perceptible reduction in time of biosynthesis when the bacteria in log phase with $AgNO_3$ were exposed to sunlight. Change in colour of aliquot treated with $Cd(NO_3)_2$ and $(PbNO_3)_2$ was also observed (Figure 2b and 2c) which confirmed the reduction of lead and cadmium salts to their nanoparticles. Colour of solution challenged with Cd $(NO_3)_2$ and Pb $(NO_3)_2$ were found to be turbid yellow and light brown respectively with respect to control after **72 h**

Table 1. Results of Morphological and Biochemical Tests of the Bacillus Species

Colony morphology	Margin	Elevation	Surface	Opacity	MR	O/F	Gas from glucose	V/P
Round	Entire	Low convex	Smooth and shiny	Opaque	+	+	–	–

MR- Methyl Red test, O/F- Oxidation-Fermentation, V/P- Voges Proskauer test

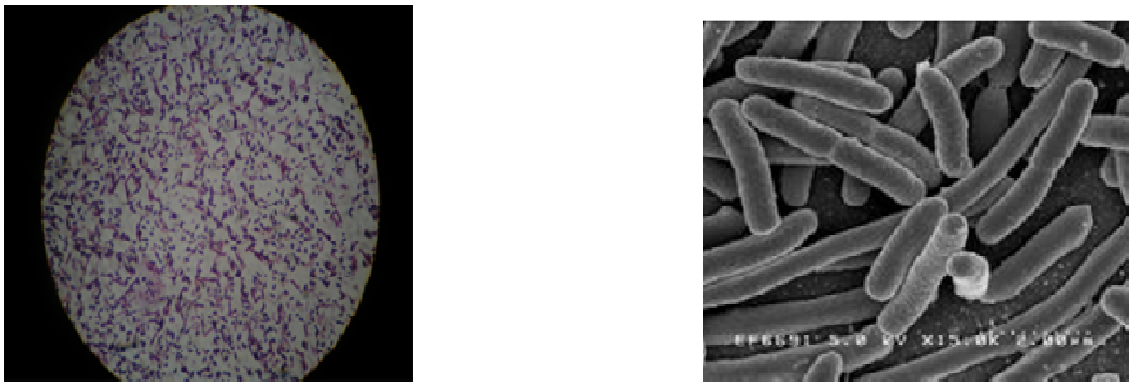


Figure 1. Photograph of *Bacillus megaterium***,** the bacterial strain

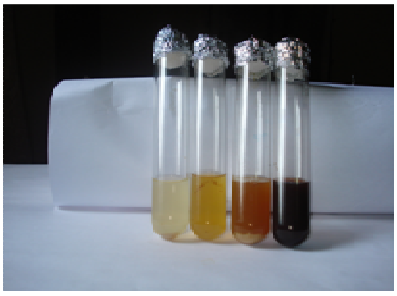


Figure 2a

Figure 2b

of agitation.

The absorbance scan taken by UV-VIS spectrophotometer showed a sharp plasmon peak at ~ 435 nm **(Figure** 3) confirming the presence of silver. The absorbance of the lead and cadmium nanoparticles was obtained at ~ 330nm and 410nm respectively. The plasmon peaks showed variance in values. The yield of nanomaterial increased between pH 5.6-8.1 and was

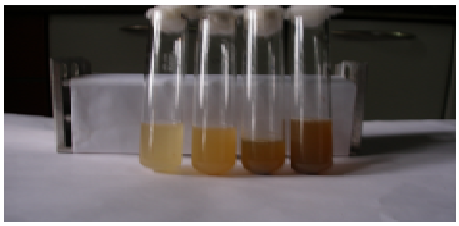


Figure 2c

Figure - 2 a, b and c: Photographs of change in colour of aliquot after addition of $AgNO_3$, $Cd(NO_3)_2$ and $Pb(NO_3)_2$ during different time period : 24hrs, 48hrs and 72hrs

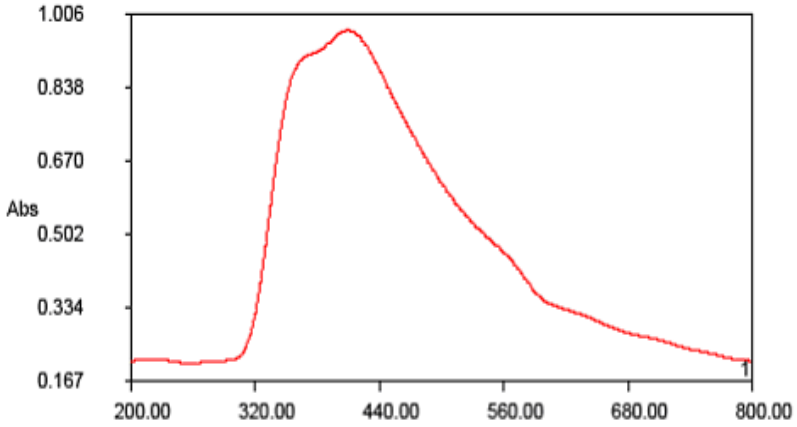


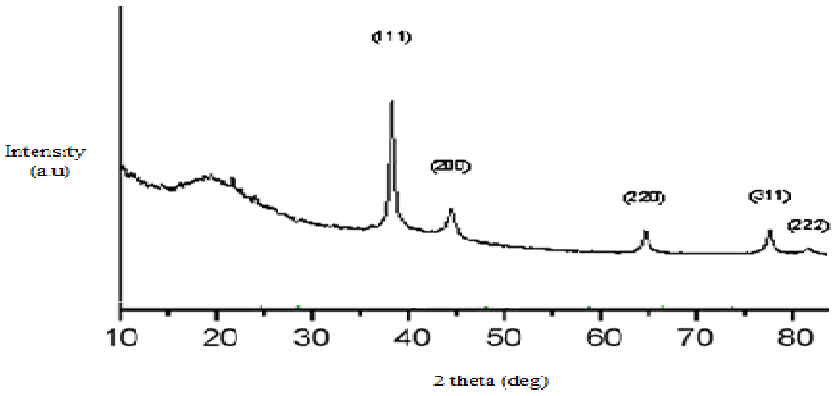
Figure 3. UV-Vis absorbance Maxima of AgNPs after 24h of Agitation

maximum at pH 8.6, after which the yield started decreasing. This clearly implies that at pH 5.6- 8.1 is optimal for this particular bacteria and the heavy metal resistance by the microbes is maximum at this **pH.** The colour of the culture changed in course of synthesis, its absorbance was recorded after regular intervals of time (24h, 48h, 72h, 7days, 15days and 30 days). The suspension was stored for about one month to observe the stability of the synthesized nanocrystallites. Thus it was observed that acidification of nanocrystallites led to attenuation of absorbance maxima whereas alkalinity resulted in enhanced absorbance and this was also observed by others (Lawrence et al., 1972).

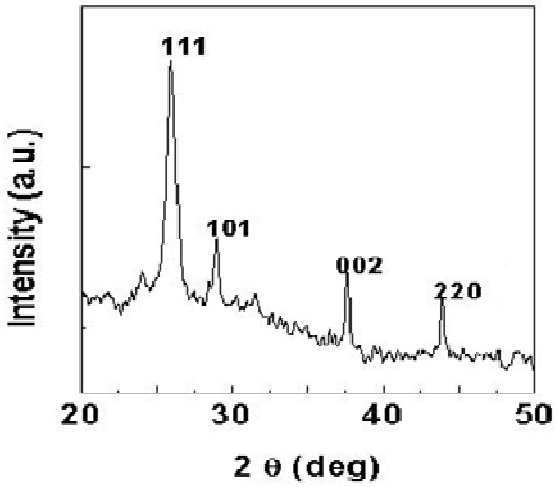
X-ray Diffraction (XRD) pattern (Figure **4**a) shows intense Bragg's reflections that can be indexed on the basis of the fcc structure of silver. The XRD pattern thus obtained clearly shows [111], [200], [220], and [311] planes, and exhibit that the synthesized AgNPs by the *Bacillus megaterium* were crystalline in nature. The nanocrystalline nature of CdS and PbS nanoparticles (**Figure 4b and 4c** respectively) was also exhibited by the respective characteristic peaks of the XRD pattern. The diffraction peaks were found to be broad around their bases indicating that the silver particles are in nanosizes. The peak broadening at half maximum intensity of the X-ray diffraction lines is due to a reduction in crystallite size, flattening and microstrains within the diffracting domains. Scherrer's equation for broadening resulting from a small crystalline size, the mean, effective or apparent dimension of the crystalline composing the powder is,

$$P_{hkl} = k\lambda / \beta_{1/2} \cos\theta$$

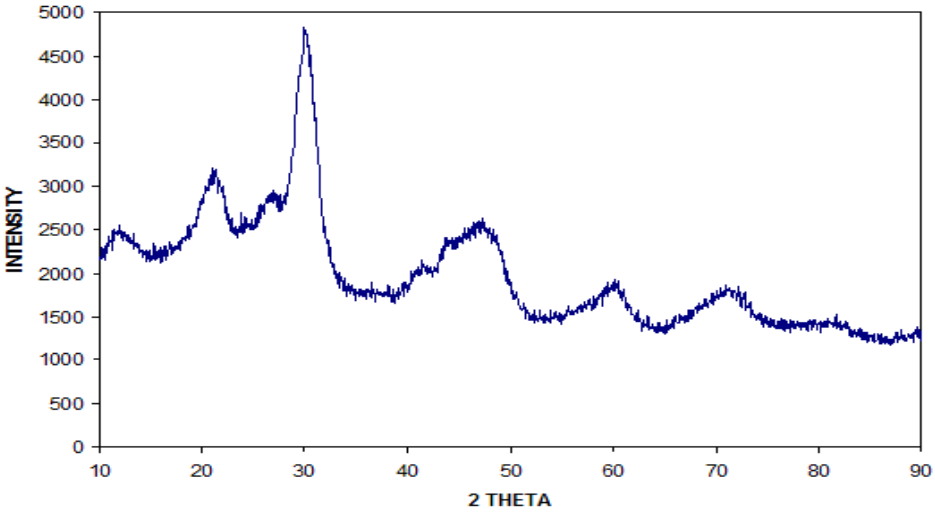
where θ is the Bragg angle and λ is the X-ray wavelength, β is the breadth of the pure diffraction profile in radians on 2θ scale and k is a constant approximately equal to unity and related both to the crystalline shape and to the way in which β is defined. The best possible value of k has been estimated as 0.89. The particle sizes of all the samples in our study have been estimated by using the above Scherer's equation and was found to be ~15nm (AgNPs, CdNPs and PbNPs) for the strongest peak. Transmission electron microscopy (TEM) images of nanoparticles that were synthesized by *Bacillus megaterium* indicated that the nanoparticles were in the size range of 10 to 20nm (**Figure 5a for AgNPs, 5b for** CdNPs and 5c for PbNPs) which is in close agreement with the particle size calculated from the XRD profile.



4a

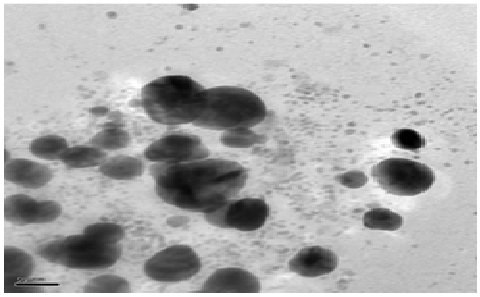


4b

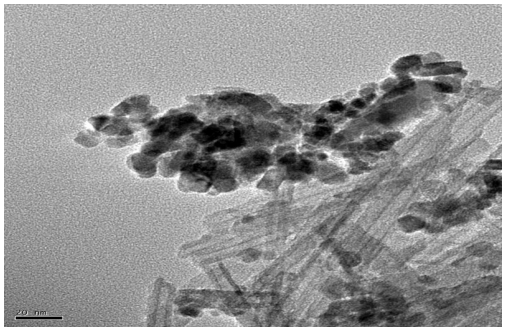


4c

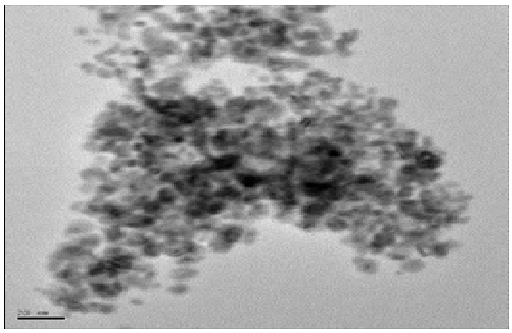
Figure 4 a, b and c: Room temperature XRD pattern of AgNPs, CdNPs and PbNPs respectively



5a



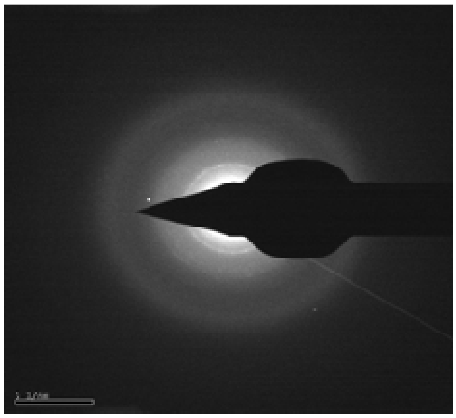
5b



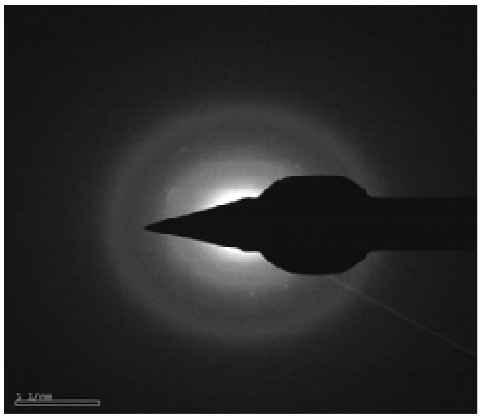
5c

Figure 5. a, b and c: TEM photographs of CdNPs, AgNPs and PbNPs at different magnifications

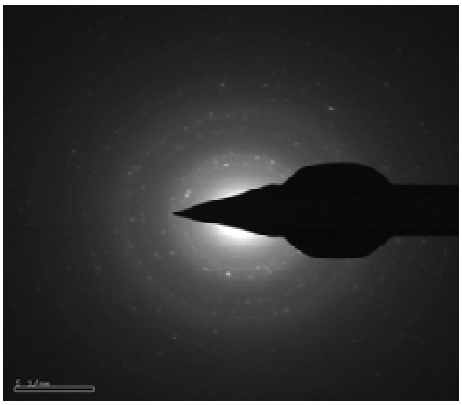
Selected area electron diffraction (SAED) spots for AgNPs (Figure 6) corresponded to the (from inside to outside of the central ring) [111], [200], [220], [311] and [222] planes of the face-centered cubic (fcc) structure of silver. HRTEM image shows the d spacing of 2.02Å, which matches with the [200] plane of nanoparticles. **Figure 7** shows the Energy Dispersive Absorption Spectroscopy photograph of AgNPs. All the peaks of Ag are observed and are assigned. Peaks for Cu and C are from the grid used and the peaks for S, P and N correspond to the protein capping over the AgNPs. It is reported earlier that proteins can bind to nanoparticles either through free amine groups or cysteine residues in the proteins (Mandal et al., 2005) and via the electrostatic attraction of negatively charged carboxylate groups in enzymes present in the cell wall of bacteria (Sastry et al.,2003) and therefore, stabilization of the AgNPs by protein is a possibility. The amide linkages between amino acid residues in proteins give rise to the well-known signatures in the infrared region of the electromagnetic spectrum and have been shown by the FTIR spectrum (Vigneshwaran et al., 2005). In future, it would be important to understand the biochemical and molecular mechanism of the synthesis of nanoparticles



6a



6b



6c

Figure 6. Selected area electron diffraction pattern of AgNPs

by the cell filtrate in order to achieve better control over size and polydispersity of the nanoparticles.

DISCUSSIONS

The mechanism for the synthesis of nanoparticles by bacteria is not exactly deciphered till date but several possible ways of synthesis is being explained. The cell surface of *Bacillus* is a laminated structure that consists of a capsule, a proteinaceous surface layer (S- layer), several layers of peptidoglycan sheeting, and the proteins on the outer surface of the plasma membrane. It has been demonstrated that the S-layer can physically mask the negatively charged peptidoglycan sheet and the layer may play some role in bacteria – metal interaction. Since heavy metal ions cannot be degraded or modified like toxic organic compounds, there are only three possible mechanisms for heavy metal resistance system. First, the accumulation of the respective ion can be diminished by efflux, or active extrusion of the heavy metal ion from the cell. Secondly cation specially "Sulphur lovers" can be seggregated into complex compounds by Thiol containing molecules. Third some metal ions may be reduced to less toxic oxidative state. And for many metals, resistance and homeostasis involve combinations of two or three of basic mechanisms mentioned. Thus if the cell chooses to detoxify such a compound by reduction, an efflux system should be present to export the reduced production which might result in the reduction of metal ions to metals.

Some bacteria reduce metal oxides by producing and secreting small, diffusible redox compounds that can serve as electron shuttle between the microbes and the iron substrate (Newman and Kolter, 2000). This extracellular enzyme shows an excellent redox property and it can act as an electron shuttle in the metal reduction. It was evident that electron shuttles or other reducing agents e.g. hydroquinone released by microorganisms are capable of reducing ions to nanoparticles (Baker and Tatum,1998) . The possible

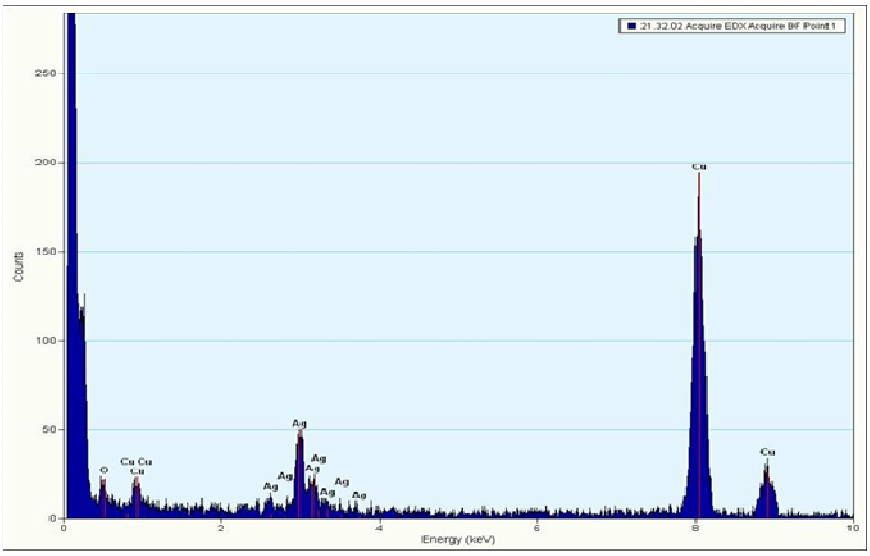


Figure 7. Energy Dispersive Absorption Spectroscopy photograph of AgNPs

chemical reactions in the culture medium may be as follows:

$$C_6H_{12}O_6 \rightarrow CH_3\text{-}CO\text{-}COOH$$
(Glucose) (Pyruvate)

$$NaHCO_3 \leftrightarrow Na^+ + HCO_3^-$$

$$HCO_3 \leftrightarrow OH^- + CO_2$$

$$MS + (OH)_2 \rightarrow MO + H_2O + N_2$$
(Metal salts of Ag,Pb, Cd) (metal oxides)

$$MS\ (OH)_2 \rightarrow MNPs \downarrow + H_2O$$
(Metal Ag, Pb, Cd nanoparticles)

Sodium symport or contransport, an important process in cells is used in sugar and amino acid uptake. ATP binding employ special substrate binding proteins, which are attached to membrane lipids on the external face of gram positive bacteria (*B megaterium*). A proton gradient can power active transport indirectly and most of the bacteria which have electrokinetic potential readily attract the cations and this probably acts as an initiator for the biosynthesis of nanoparticles. Thus silver resistance in gram positive organisms is by efflux through ATPase and additional complexation by intracellular compounds. Cadmium resistance in gram positive bacteria was also reported to be mediated by CadA- like protein. Lead resistance was also reported to be based on ion efflux. The possible mechanism for the reduction of metal ions comes probably through H^+ ions released in the process of Glycolysis which involves breakdown of glucose present in bacterial cell resulting in liberation of energy and molecular oxygen.

Under aerobic condition, cells obtain energy from the breakdown of glucose. The pyruvate formed during glycolysis undergoes oxidative decarboxylation to form Acetyl Co-A, resulting in liberation of electrons and ATP. Pyruvate molecules are decarboxylated (they loose a molecule of carbon dioxide) in the mitochondria. They are then oxidized and converted to acetylcoenzyme A, usually abbreviated to acetyl CoA and proceed to the mitochondria through the Krebs cycle and further release of energy.

$$2CH_3COCOO^- + 2NAD^+ + 2H_2O \rightarrow 2CH_3COO^- + 2NADH + 2H^+ + 2CO_2$$

Further the NAD and H^+ ions produced act as a reducing agent in presence of enzyme called Nitrate reductase which is present in both aerobes and anaerobes. This enzyme basically converts nitrate into nitrite and used for the nitrogen cycle. The same enzyme during the conversion may shuttle the electron to the silver ions formed in aqueous solution. This mechanism seems to be similar to the formation of magnetic Fe_3O_4 particles by magentotactic bacteria.

CONCLUSIONS

Biological synthesis of metal nanoparticles is a reliable and with ecofriendly protocol. The nanoparticles for the Ag, Pb and Cd have particle size in the range of 10-20nm. The mechanisms of extracellular exclusion and accumulation both resulted in the immobilization and the detoxification as some microorganisms have the ability to resist and detoxify metals The mechanism of synthesis of metal nanoparticles by microbes is not clearly explored .However , in this paper a step has been taken to

understand the possible mechanism of metal and microbes interaction which may be due to structural specificity of the cell of microbes and the breakdown of polysaccharides into simpler form and further their oxidation. How metal availability influences microbial resistance and, hence, the microbial detoxification of a metal has important implications for future technologies

A**CKNOWLEDGMENT**

The authors wish to acknowledge University Grants Commission, New Delhi, India for the financial support under the major research project scheme.

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