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Extracellular synthesis of silver nanoparticle by *Pseudomonas hibiscicola* – Mechanistic approach

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Abstract. Biosynthesis of nanoparticles has acquired particular attention due to its economic feasibility, low toxicity and simplicity of the process. Extracellular synthesis of nanoparticles by bacteria and fungi has been stated to be brought about by enzymes and other reducing agents that may be secreted in the culture medium. The present study was carried out to determine the underlying mechanisms of extracellular silver nanoparticle synthesis by *Pseudomonas hibiscicola* isolated from the effluent of an electroplating industry in Mumbai. Synthesized nanoparticles were characterized by spectroscopy and electron microscopic techniques. Protein profiling studies were done using Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (1D-SDS PAGE) and subjected to identification by Mass Spectrometry. Characterization studies revealed synthesis of 50 nm nanoparticles of well-defined morphology. Total protein content and SDS PAGE analysis revealed a reduction of total protein content in test (nanoparticles solution) samples when compared to controls (broth supernatant). 45.45% of the proteins involved in the process of nanoparticle synthesis were identified to be oxidoreductases and are thought to be involved in either reduction of metal ions or capping of synthesized nanoparticles.

Keywords: bio-inspired nanomaterials; silver nanoparticles; biosynthesis; nanobiotechnology; nano-biotechnology

1. Introduction

Nanoscience is rapidly making an impact in all domains of human life due to its wide applications. (Filipponi and Sutherland 2013) The physical methods are energy consuming while chemical methods involve a lot of toxic chemicals and both are equally expensive, hence, a cost effective and environmentally benign method is the need of the hour. Biological methods are the key solution to this problem. The potential ability of microorganisms to synthesize nanoparticles has led microbiologist around the world to screen array of microorganisms for this capability and develop a simple and cost effective method to synthesize nanoparticles of constant size, shape and monodispersity. (Babu *et al*. 2011, Horikoshi and Serpone 2013)

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Certain microbes have developed genetic and proteomic responses to toxic environments leading to synthesis of metallic nanoparticles. They resist the inhibitory effects of such sites by various mechanisms like complexation, efflux, or reductive precipitation, making them capable of surviving in environments containing high concentrations of heavy metal. As reported in case of *Klebsiella pneumoniae* reduction of silver ions was observed mainly due to conjugation shuttles with the participation of the reductases. Thereby, indicating that the cell-associated nitro-reductase enzymes may be involved in the photo reduction of silver ions. Singh *et al* highlights multiple electron transport pathways that are available in a bacterial cell for reduction of silver ions, along with involvement of other enzymes and reducing cofactors in order to completely understand the mechanism of nanoparticle synthesis by bacteria. (Li *et al*. 2011, Iravani 2014, Singh *et al*. 2015)

This study is an attempt to understand the mechanistic approach of extracellular nanoparticle synthesis from *Pseudomonas hibiscicola*, an isolate obtained from the effluent of an electroplating industry. Some microbes have appetite for gas, oil, metals and toxic chemicals. Microorganisms often produce inorganic materials of nano-size either extracellularly or intracellularly. Microbial systems are able to detoxify heavy metals by virtue of their ability to reduce the metal ions or convert the soluble toxic ions into insoluble non-toxic metal nanoparticles. A great deal of study has been carried out on synthesis of nanoparticles by prokaryotic bacteria since they are the easiest organisms to handle and can be manipulated most easily. Bacteria are able to form nanoparticles both intracellularly via bioaccumulation and extracellularly using its enzymes. Nanoparticle synthesis was optimized and characterized to determine its size shape and conformity. Preliminary proteomic studies were undertaken to understand and identify the differential proteins that may be involved in extracellular synthesis of nanoparticles.

2. Materials and methods

2.1 Extracellular synthesis of silver nanoparticles

The bacterial isolate of *Pseudomonas hibiscicola* (previously reported in one of our study) was used for synthesis of silver nanoparticles. (Punjabi *et al*. 2017) Briefly, the culture was inoculated in nutrient medium. After incubation the broth (containing extracellular proteins) was centrifuged and supernatant collected for further use. The supernatant was then challenged with metal solution i.e., 1 mM $AgNO_3$ in this case and further incubated for 72 hrs on shaker. Synthesis of silver nanoparticles (AgNPs) was considered complete upon observation of dark colour of the reaction mixture after the incubation. Synthesis was further confirmed by analyzing the mixture using UV-Vis spectrophotometer in which typical resonance for silver nanoparticles was expected. (Kumar *et al*. 2011)

2.2 Optimization and stability of silver nanoparticle synthesis

To optimize the synthesis of nanoparticles critical parameters like pH, concentration of $AgNO_3$ (Sigma Aldrich, USA) and time of incubation of reaction mixture were analyzed. To determine the favorable pH for complete reaction of supernatant with $AgNO_3$ the supernatant was adjusted to acidic, neutral and alkaline pH such as 4, 7 and 9 respectively keeping all the other factors constant. Similarly, increasing concentration of $AgNO_3$ from 0.1 mM, 1.0 mM, 2.0 mM, 5.0 mM and finally up to 10.0 mM were challenged in 1:1 v/v ratio with supernatant to determine the optimal concentration for AgNP synthesis. (Banerjee 2013) In order to determine the optimum temperature

required for the stability of synthesized silver nanoparticles, they were stored at two different conditions- room temperature (RT) and refrigeration at 4°C. Samples were aliquoted at regular time intervals and analyzed using UV–Vis spectrophotometer for a period of six months. The spectra were evaluated to understand changes that may be occurring as an estimate to its stability. (Basavaraj 2012)

2.3 Characterization of AgNPs

Synthesized nanoparticles were characterized by various analytical methods involving spectral studies and electron microscopy to identify the size, shape and conformity. The nanoparticles were subjected to Transmission electron microscope TEM (Philips CM 200), Nanoparticle tracking and Analysis (NTA, Model LM 20 from Nanosight UK), Direct Light Scattering-Particle Size Analyzer (DLS – PSA) and Zeta potential analysis to detect the size of nanoparticles and surface charges acquired by nanoparticles respectively, which can be used to gain further insights into the stability of the obtained colloidal nanoparticles. Particles with zeta potential values more positive than +30 mV or more negative than −30 mV are considered to be stable (Meléndrez *et al*. 2010). Particle size analysis and Zeta potential analysis was done using NanoPlus DLS Particle Size and Zeta Potential (Micromeritics Instrument Corporation).

2.4 Protein profiling studies

To understand the mechanism of synthesis of nanoparticles from extracellular proteins secreted by the *P. hibiscola*, protein analysis carried out using one dimensional SDS PAGE. Untreated broth supernatant containing extracellular proteins was used as control while the supernatant challenged with $AgNO_3$ (hereafter referred to as nanoparticle solution) was used as test.

2.4.1 Determination of total protein content

The proteins in both control and test solutions were precipitated and concentrated using trichloroacetic acid (TCA) method. (Nandakumar *et al*. 2003) Briefly, the solutions were treated with 20% v/v TCA in ice for 30 min to precipitate protein. The precipitate was collected by centrifugation at 5000 rpm for 30 min at 4°C. (Mohammadian *et al*. 2007) The pellet obtained was dissolved in 1% SDS and stored at -80°C until further use. The total protein in test and control protein lysates was estimated by Bradford method using Bovine serum albumin as a standard.

2.4.2 SDS PAGE

20-30 μg proteins were resolved on SDS PAGE with 5% stacking and 12% resolving gel. The samples (control and test) were run along with a standard 12 KDa to 225 KDa marker at constant voltage of 120V. The bands were visualized using a mass spectrometry compatible silver staining protocol. (Mohammadian *et al*. 2007) The gels were scanned using the ImageScanner III scanner and LabScan 6.0 software and stored in 5% glacial acetic acid at 4°C until further use.

2.5 Protein extraction and Mass spectrometry

The bands of interest were excised manually from the gel and washed with acetonitrile. The bands were subjected to reduction and alkylation prior to in-gel trypsinisation and extraction of peptides. The fractions obtained were completely dried and stored at -20°C till MS analysis. The samples were reconstituted with 0.1% TFA in 50% ACN and loaded for MS analysis carried out

using Matrix associated laser desorption ionization—time of flight (MALDI-TOF) instrument (Bruker Daltonik GmbH) at ACTREC, Kharghar, Navi Mumbai. The data obtained was analyzed using database searching software such as MASCOT. (Oosthuizen *et al*. 2002, Shevchenko *et al*. 2006, Ngo *et al*. 2014, Khusro and Sankari 2015)

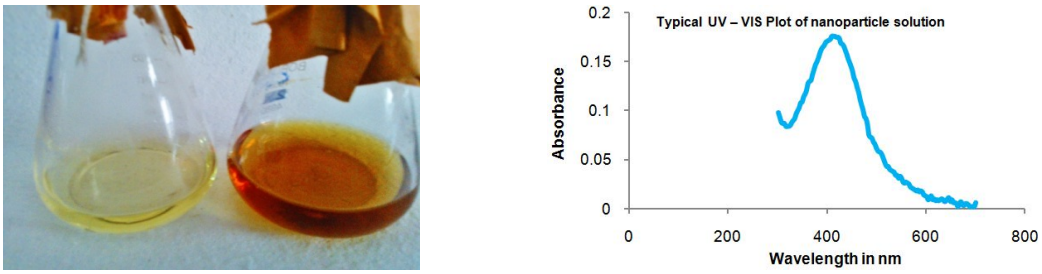
3. Results

3.1 Extracellular synthesis of silver nanoparticles

The extracellular synthesis of silver nanoparticles was achieved by the reaction of culture supernatant of *Pseudomonas hibiscicola* and $AgNO_3$. The characteristic change in colour of the reaction mixture from pale yellow to dark brown Fig. 1 indicated nanoparticle synthesis. The typical spectra, showing a peak in the range of 390–450 nm confirmed the presence of nanoparticles in the reaction mixture.

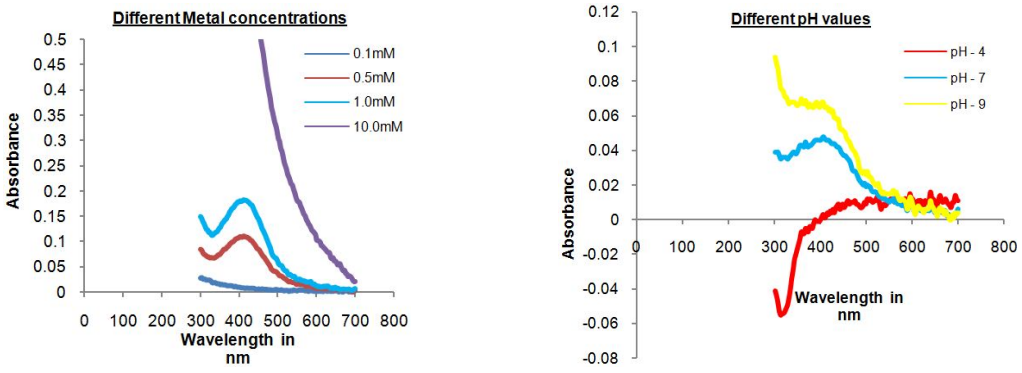
3.2 Optimization and stability of silver nanoparticle synthesis

The process of nanoparticle synthesis using the isolate was optimized based on $AgNO_3$



(a) (b)

Fig. 1 Change in colour of reaction mixture and characteristic peak in a UV – Vis spectra

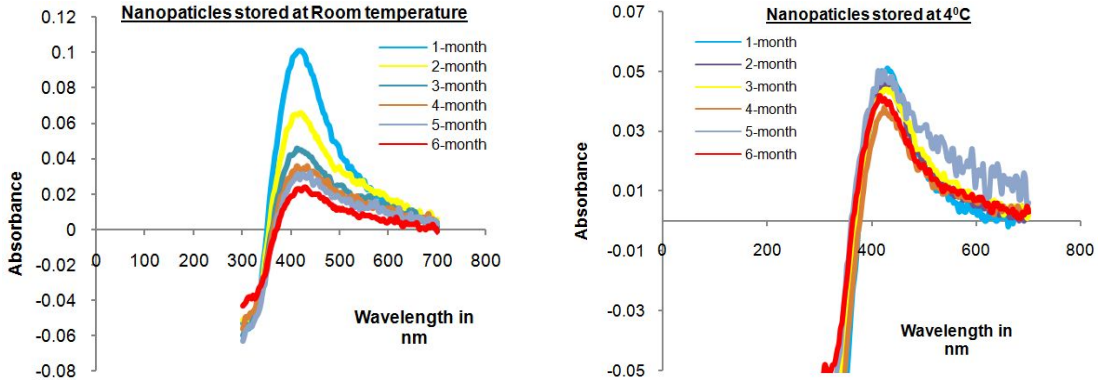


(a) (b)

Fig. 2 Changes observed in spectral studies upon deviation from optimal condition of 1 mM metal concentration and pH 7 of supernatant

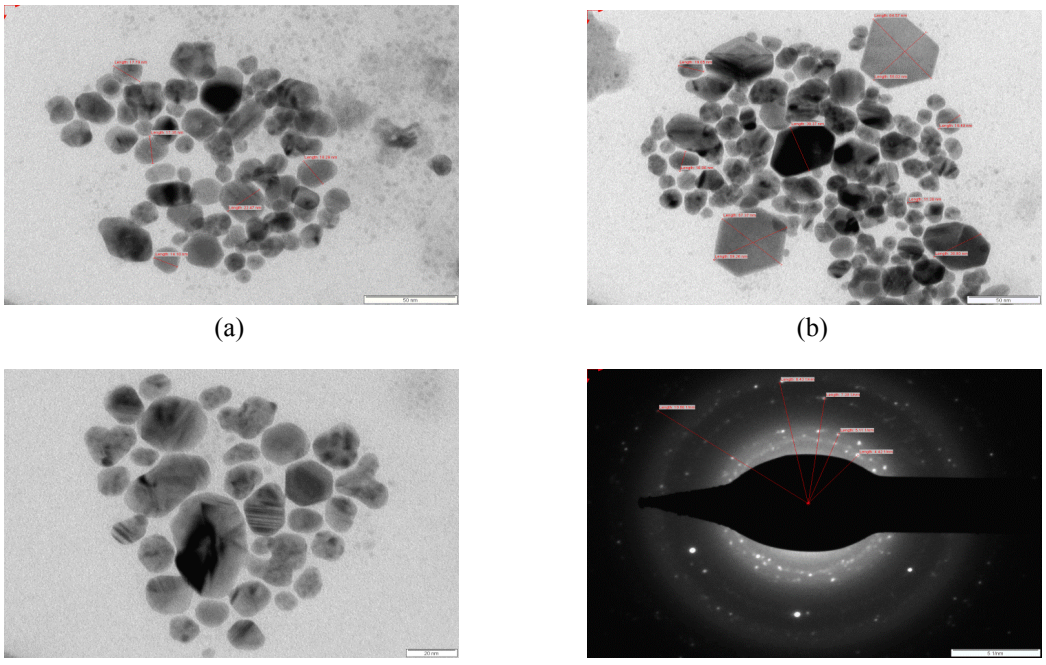
concentration and pH. It was found that reaction of supernatant with 1 mM $AgNO_3$in 1:1 v/v ratio, pH adjusted to 7 and incubation of reaction mixture for 72 hrs resulted in optimal synthesis of nanoparticles. Fig. 2 depicts the shift in peak observed in case of variable pH and metal concentration mixtures.

In addition nanoparticle stability was also analyzed using UV–Vis spectral analysis. When stored at two different temperatures i.e., 4°C and room temperature (RT) changes in spectra such as reduction in peak height over a time period were observed indicative of alteration in



(a) (b)

Fig. 3 Stability of silver nanoparticles at room temperature and refrigeration temperature



(a) (b)

(c) (d)

Fig. 4 TEM Micrographs and SAED pattern of synthesized silver nanoparticles

nanoparticle structure. Spectral changes were observed at both temperatures however, the pattern of deviation was found to be different. In case of refrigeration (4°C) the reduction in peak height was observed as early as 1 month but further reduction was considerably controlled while in case of room temperature (RT) the reduction of peak was far less for the first few months but significantly reduced after 90 days. The Fig. 3 shows the difference in stability of nanoparticles at two different temperatures.

3.3 Characterization of AgNPs

DLS– particle size analyzer revealed that the average particle diameter was 41.0 nm and Polydispersity index was 0.515. The TEM micrographs showed presence of nanoparticles of various shapes of about 50 nm in size Fig 4. NTA results indicated the mean size of AgNPs synthesized was 39 nm and concentration of nanoparticles was 3.79×10^8 particles/ml Fig. 5. The zeta potential of the silver nanoparticles was found to be −21.41 mV Fig. 6.

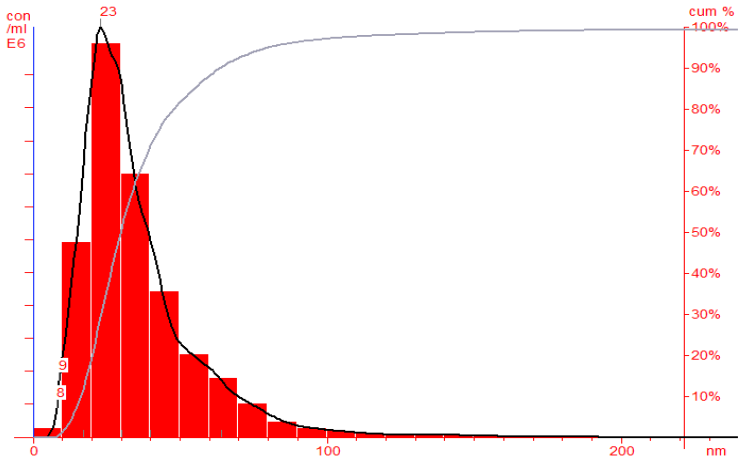


Fig. 5 NTA analysis of synthesized silver nanoparticles

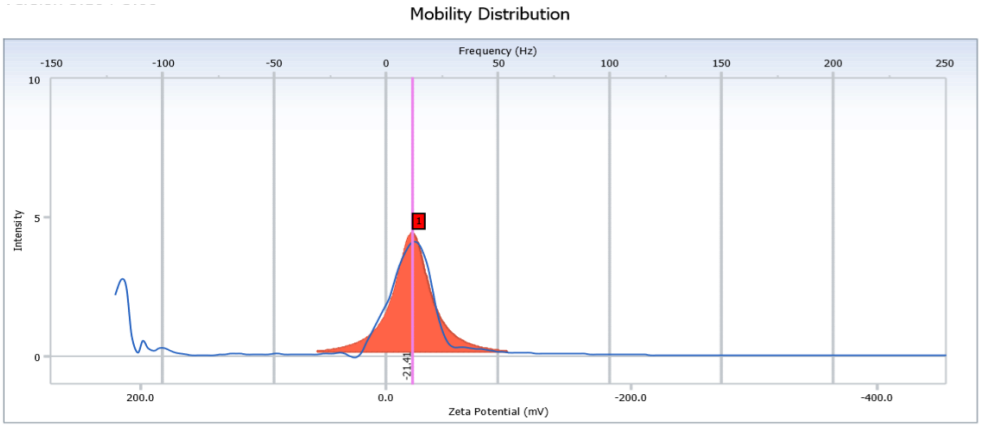


Fig. 6 Zeta potential of synthesized silver nanoparticles

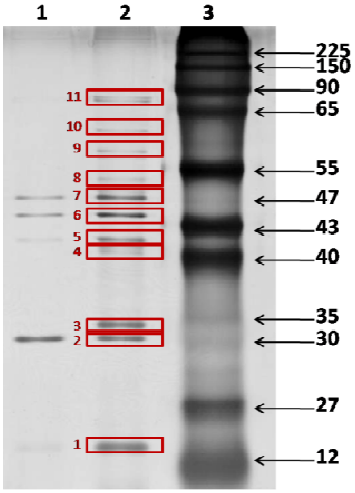


Fig. 7 Representative picture of Silver stained SDS PAGE gel. Left to right: Lane 1- Test (nanoparticle solution), Lane 2- Control (Untreated supernatant), Lane 3- Ladder. around the band indicates sequentially numbered and excised bands 1 to 11 for easy reference

Table 1 Probable list of proteins fractions identified using mass spectrometry

Sample /Band	Sample/Band	Molecular function	Intensity in test solution*
1	Molybdenum cofactor guanylyltransferase	GTP Binding, **Metal ion binding**, Molybdenum cofactor guanylyltransferase activity	↓
2	Crossover junction endodeoxyribonuclease	Crossover junction endodeoxyribonuclease activity, **magnesium ion binding**, nucleic acid binding	NC
3	3-phosphoshikimate 1-carboxyvinyltransferase	Aromatic amino acid family biosynthetic process, chorismate biosynthetic process	ND
4	4 hydroxy 3 methylbut 2 enyl" diphosphate reductase	3 iron, 4 sulfur cluster binding, hydroxy 3 methylbut 2 enyl" diphosphate reductase activity, **metal ion binding**	ND
5	Adenylate kinase	adenylate kinase activity, ATP binding, **Zinc ion binding**	↓
6	Adenylate kinase	adenylate kinase activity, ATP binding	↓
7	Shikimate kinase	ATP binding, **magnesium ion binding**, shikimate kinase activity	↓
8	NADH-quinoneoxidoreductase subunit 1	4 iron, 4 sulfur cluster binding, FMN binding, **metal ion binding**, NAD binding, NADH dehydrogenase, quinone binding	ND
9	NADH-quinoneoxidoreductase subunit B	4 iron, 4 sulfur cluster binding, **iron ion binding**, NADH dehydrogenase (ubiquinone) activity, quinone binding	ND

Table 1 Continued

Sample /Band	Sample/Band	Molecular function	Intensity in test solution*
10	Enoyl-[acyl-carrier-protein] reductase [NADH]	Enoyl-[acyl-carrier-protein] reductase [NADH] activity	ND
11	D-amino acid dehydrogenase	D-amino acid dehydrogenase activity	ND

* in comparison to control solution;
↓- Downregulation, NC – Visually no changes observed, ND – Visually not detectable/absent

3.4 Protein profiling studies

3.4.1 Total Protein content and SDS PAGE

The total protein content of culture supernatant was found to be in the range of 3-5 μg/μl. 20-30 μg of proteins were resolved on SDS page and proteins were visualized using silver staining. Based on the differences in the number of proteins and their intensity in control and test, the proteins were fractionated sequentially from 1-11 (in lane 2) for the purpose of excision and MS analysis Fig. 7. Only 45% of the total number of proteins in control was observed in test solution, even those with decreased intensity. Visual absence of proteins in test solution i.e., protein fractions 3, 4, 8 9, 10 and 11 in indicates participation of these proteins in metal reduction process and capping of nanoparticles.

3.5 Protein extraction and mass spectrometry

Protein excision from 1D SDS PAGE was carried out for all the 11 protein fractions mentioned previously. Table 1 is a list of probable proteins identified using MS analysis. The differences observed in test solution when compared to controls may be attributed to either partial or complete utilization of proteins for synthesis or catalyzing the synthesis of nanoparticles.

4. Discussion

Owing to their abundance in the environment and ability to acclimatize to extreme conditions, there has been an increased focus on prokaryotic cells for bio-synthesis of nanoparticles. These include especially those bacteria which are fast growing, inexpensive to cultivate and easy to manipulate by modifying growth conditions such as temperature, oxygenation, incubation time to synthesize desired nanoparticles etc. (Pantidos and Horsfall 2014)

Screening of unexplored microorganisms for synthesis of nanoparticles can lead to attainment of well-defined nanostructures using comparatively easier and environmental friendly mode of synthesis. Microbial synthesis of metal nanoparticles can take place either intracellularly or extracellularly, however additional steps such as sonication or reactions with suitable detergents to release the synthesized nanoparticles are required to extract proteins which are a result of intracellular synthesis. In contrast, extracellular synthesis is cheap and has simple downstream processing that can easily be scaled up to support large-scale production of nanoparticles. Thus, several studies are focusing on extracellular methods for the synthesis of metal nanoparticles. (Das *et al*. 2014)

In this study bacteria isolated from an industrial effluent, *Pseudomonas hibiscicola* was used for extracellular synthesis of silver nanoparticles. Synthesis of silver nanoparticles using the culture supernatant was confirmed using UV Vis spectra measured at 420 nm. (Durán *et al*. 2005)

Neutral pH (7.0) and 72 h incubation was found to be optimum for 1 mM $AgNO_3$ in 1:1 v/v ratio. Further, the synthesized nanoparticles were stable upto 3 months at RT. Both these results are in accordance with studies reported by Patil (2015). The stability of nanoparticles is primarily dependent on its capping agents which in these cases are predominantly biomolecules. The binding of these biomolecules to nanoparticles is influenced by parameters like time and temperature. (Patil 2015, Wabale *et al*. 2015)

The DLS PSA of the synthesized nanoparticles revealed the average diameter of particles to be 41.0 nm. The nanoparticles were found to be monodispersed based on its size and PDI which was found to be 0.515. Polydispersity is a measure of the heterogeneity of size of particles and the maximum PDI value is arbitrarily limited to 1. The zeta potential determines whether the particles within a liquid tend to flocculate or not. Nanoparticles with zeta potential values greater than +25 mV or less than -25 mV typically have high degrees of stability. (Thakkar *et al*. 2016) The zeta potential of synthesized nanoparticles in the current study was found to be −21.41 mV, indicating stable nanoparticles. The TEM analysis revealed an average size of 50.0 nm which is in accordance with results obtained by DLS PSA and closely matches the mean size obtained in NTA analysis. The surface morphology and topography of synthesized AgNPs revealed nanoparticles of variable shapes.

Total protein content of the supernatant was found to be in the range of 3-5 μg/μL. The protein lysates of supernatant (control) and nanoparticle (test) solution and were resolved on 1-D SDS PAGE and distinct differential patterns were observed between the two. The number of visually detectable protein fractions in nanoparticle solution was only 45% of that of control supernatant suggesting that a large number of proteins from the latter are being utilized for the synthesis of nanoparticles. All the 11 protein fractions were identified by MS analysis and a probable list of proteins participating in nanoparticle synthesis was obtained (Table 1). The proteins were classified into different groups depending on their functions Fig. 8. 45.45% of the proteins identified in the present study were the oxidoreductases, followed by kinases (27.27%), transferases (18.18%) and ribonuclease (9.09%).

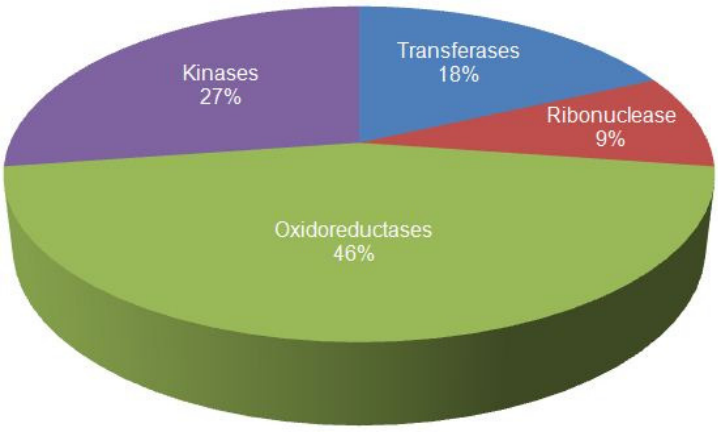


Fig. 8 Percentage distribution of functional classification of MS identified proteins

One of the suggested mechanisms of biosynthesis of nanoparticles is that the metal ions are reduced by the use of specific microbial reducing enzymes such as NADH-dependent reductases. In addition, quinine derivatives also act as redox centres in the reduction of AgNPs. (Ramezani 2010)

Reduction of metals by enzymatic action of microbial synthesized NADH and NADH dependent enzymes, primarily nitrate reductase has been reported for synthesis of silver nanoparticles by *B*. *licheniformis*. The counter effect of metallophillic microbes to their toxic environments is anticipated to the genomic and proteomic response to regulate the metal homeostasis. (Li *et al*. 2011)

In this study, five such reductases were identified. This may also be the plausible explanation for complete absence of the protein bands 4, 8, 9, 10 and 11 from the test solution. Further, Chowdhury *et al*. have reported that SDS - PAGE, showed an intense band of 85 kDa which was not seen in the nanoparticle solution as compared to its cell filtrate, and, it is likely that this 85 - kDa protein acts as a capping material and confers stability to the silver nanoparticles. Detection of extracellular proteins responsible for synthesis and stability of silver nanoparticles has also been reported in few other literatures. The presence of natural capping proteins eliminates the postproduction steps of capping which is necessary for most applications of nanoparticles in the field of medicine. (Jain *et al*. 2011, Chowdhury *et al*. 2014) Since, in our study, majority of the proteins that were not observed in nanoparticle solution but were distinctly present in the supernatant or cell filtrate included the oxidoreductases as well as a carbo-vinyl transferase, hence it is difficult to identify the exact role of these proteins. However we hypothesize that all six protein fractions that are completely absent from the test solution are involved in either of the two important processes i.e., reduction or capping that has resulted in successful synthesis of stable nanoparticles.

Further higher resolution techniques such as 2-Dimension gel electrophoresis must be done to further resolve these protein fractions that will help us identify the exact number and functions of the proteins involved in nanoparticle synthesis. In addition, the data can be validated using other complementary techniques such as western blotting.

5. Conclusions

Microbial synthesis of metal nanoparticles has been central to the green nanotechnology. Understanding the underlying mechanism of microbial synthesis of nanoparticles can help us explore methods to enhance and encourage the large-scale production of nanoparticles using such safe, easy to handle and cost-effective techniques. The present study is only a preliminary assessment of the probable list of proteins from the extracellular microbial secretions that may be actively involved or utilized in the synthesis of nanoparticles, further studies are warranted to elucidate the exact mechanism. These studies authenticate the extracellular release and involvement of proteins in manifesting nanoparticles in the system. The study also leaves a trailing sequence directing critically six out of the set of eleven proteins are the ones playing role in synthesis and capping of nanoparticles by *Pseudomonas hibiscicola*. This is a valuable piece of data to further the role of *Pseudomonas hibiscicola* in either scale up or purification of specific peptide to simplify the upstream and downstream of bulk nanoparticle synthesis. A comparable data of proteins identified with other reports also implies relevance of the information and ready base model for any further extension of this work to a higher level.

Ethical statement

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest

The authors declare no conflict of interest.

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