Delhi Technological University, Delhi, India

Green Synthesis and characterization of Silver Nanoparticles using Bacterial culture and its antimicrobial activity

to be submitted as Major Project in partial fulfilment of the requirement for the degree of

Master of Technology

In

Industrial Biotechnology

Submitted By

KRATIKA

(2K15/IBT/05)

Under the Supervision of

Dr. PRAKASH CHANDRA
(ASSISTANT PROFESSOR)

DEPARTMENT OF BIOTECHNOLOGY
DELHI TECHNOLOGICAL UNIVERSITY
(Formerly Delhi College of Engineering)
Shahbad Daulatpur, Bawana Road
Delhi-110042, INDIA

CERTIFICATE

This is to certify that the M. Tech thesis entitled "**Green Synthesis and characterization of Silver Nanoparticles using Bacterial culture its and antimicrobial activity**" submitted by **Kratika (2K15/IBT/05)** in the partial fulfilment of the requirements for the reward of the degree of Master of Technology, Delhi Technological University (Formerly Delhi College of Engineering, University of Delhi), is an authentic record of the candidate's own work carried out by her under my guidance. The information and data enclosed in this thesis is original and has not been submitted elsewhere for honouring of any other degree.

Date:

Dr. PRAKASH CHANDRA
Supervisor
ASSISTANT PROFESSOR
Department of Biotechnology
Delhi Technological University
(Formerly Delhi College of Engineering, University of Delhi)

Prof. D. Kumar
(Co-Supervisor)
Head of Department
Department of Biotechnology

DECLARATION

I declare that my major project entitled "**Green Synthesis and characterization of Silver Nanoparticles using Bacterial culture and its antimicrobial activity"**submitted to Department of Biotechnology, Delhi Technological University as a result of the work carried out by me at Molecular Biology and Genetic engineering Laboratory, Department of Biotechnology, as major project-ll

DATE: **KRATIKA**

PLACE: DELHI

ACKNOWLEDGEMENT

By the grace of almighty, I would like to express the special thanks of gratitude to Prof. D. kumar, Head of Department of Biotechnology, Delhi technological University for giving me the opportunity to be in Biotechnology department of Delhi Technological University.

I express my profound sense of reverence of gratitude to my mentor Dr. Prakash Chandra, Department of Biotechnology, Delhi Technological University for his valuable guidance, congenial discussion, incessant help, calm, endurance, constructive criticism and constant encouragement throughout this investigation right from the imitation of work to the ship shaping of manuscript.

KRATIKA
2K15/IBT/05

CONTENTS

TOPIC	PAGE NO
1) Abstract	11
2) Chapter 1 Introduction	12-15
3) Chapter 2 Literature review	16-29
(a) Chapter 2.1 Bacteria	17
2.1.1 Bacterial colonies used	17-19
(b) 2.2 Chemicals used in silver nanoparticle synthesis	19-22
2.2.1 Mechanism	20-22
(c)2.3 Characterization	22-26
2.3.1 UV-VIS Spectrophotometer	22-23
2.3.2 FTIR	23-24
2.3.3 SEM	24-25
2.3.4 TEM	25-26
2.3.5 Ultra sonicator	26-27
(d)2.4 Antimicrobial activity	27-28
2.4.1 Disc diffusion test	27-28
(e) 2.5 Applications of silver nanoparticles	28-29
2.5.1 Medicine	28
2.5.2 Optics	29
2.5.3 Cancer	29
2.5.4 Drinking water purification	29
4) Chapter 3 Materials	30-32
3.1.1 Glassware's and tools	31-32
5) Chapter 4 Analytical Instrumentation	33-36
6) Chapter 5 Methodology	37-47
5.1.1 Chemical Synthesis	38-39
(a) 5.1 EXPERIMENT 1	38
(b) 5.2 EXPERIMENT 2	38-39

5.1.2 Bacteria's used 39-47
(a) 5.3 EXPERIMENT 3 40
(b) 5.4 EXPERIMENT 4 42
(c) 5.5 EXPERIMENT 5 42
(d) 5.6 EXPERIMENT 6 43
7) Chapter 6 Results and Discussion 48-71
6.1 FTIR 61-64
6.2 SEM 64-66
6.3 TEM 67
6.4 EDS 68
6.5 Antimicrobial activity 69-71
6.5.1 Disc Diffusion 69
9) Chapter 7 Conclusion 72-73
10) References 74-78

LIST OF FIGURES

1)	Reduction of $AgNO_3$ by $NaBH_4$	20
2)	Stabilization by Polyvinyl pyrrolidone	21
3)	UV-VIS Perkin Elmer Lambda 950	23
4)	Thermo Scientific Nicolet 380 FTIR	24
5)	Zeiss EVO18 Special Edition SEM	25
6)	Zeiss Libra 120 TEM	26
7)	Hielscher Ultrasound Technology	27
8)	Radwag Weighing Balance	34
9)	Magnetic Stirrer	35
10)	Eppendorf Mini Spin Centrifuge	35
11)	Eppendorf Centrifuge 5804 R	36
12)	Macflow Engineering Laminar Air Flow	36
13)	$NaBH_4$ solution	38
14)	*Shewanella putrefaciens*	39
15)	*Shewanella algae*	40
16)	Colour of all the solutions before reaction	46
17)	The color of solutions Sps and Sas respectively when reactions are set	47
18)	The colour of solutions Spp and Sap respectively on setting the reaction	47
19)	Color change on addition of different concentrations of $AgNO_3$	49
20)	UV-VIS spectrophotometer peaks of all the above concentration solutions	50
21)	Color change observed when $NaBH_4$ solution was added drop wise	50
22)	Colour change after reaction	51
23)	Maximum peaks obtained approximately at 420nm	51
24)	Spp, Sps, Sap, Sas peaks were going below 370nm	52
25)	Peak was obtained in the case of Sas and Chemical synthesis	52
26)	Color change observed in Sps and Sas in 24hrs	53
27)	Colour change observed in Spp and Sap in 24hrs	53
28)	Sps peak is obtained on at concentration of 7mM $AgNO_3$	54
29)	Sas peaks are not obtained at any concentration within range	54
30)	Spp peaks were also not obtained in this range	54

31) Sas peaks not obtained even after 48hrs. 55
32) Spp peak is observed in 9mM $AgNO_3$ sample at 374nm 55
33) Sap peaks are not obtained even in 48hrs 55
34) Sps colour changes 56
35) Sas remains the same as on the Day 1 56
36) The original reaction mixture of Sas and $AgNO_3$ solution was light Yellow which turned to dark brown after adding the $NaBH_4$ 2mM solution. 57
37) The colours of the Spp solutions have turned beginning from light pink to yellowish brown 57
38) the colour of Sap solutions had changed 58
39) Sas peaks are not obtained but few kinky points can be observed. 58
40) Sap peaks are not obtained even in 144hrs. 59
41) peaks are obtained in Sap after 168hrs two of the concentrations have been enlarged 59
42) Similarly in 3mM and 4mM AgNO3 reaction mixture the peaks are obtained. 60
43) Spp peaks are obtained at 420nm in 2 and 4mM AgNO3 60
44) The Infrared peaks observed for NaBH4, S. putrefaciens reaction mixture and S. putrefaciens supernatant (from top to bottom) as reducing agents. 61
45) FTIR results of S. putrefaciens pellets for 1mM AgNO3 reaction mixture. 62
46) FTIR results of S. putrefaciens supernatant for 5mM AgNO3 reaction mixture. 63
47) FTIR results of S. putrefaciens for 4mM AgNO3 reaction mixture. 63
48) SEM image of the sample which contains S. putrefaciens supernatant with 1mM AgNO3 solution was taken 65
49) SEM image of the S. putrefaciens pellet 1mM concentration shows the presence of the bacteria's in it along with the nanoparticles. 65
50) SEM image of S. putrefaciens supernatant 5mM concentration shows the presence of the silver nanoparticles at 200nm scale. 66

51) TEM images were taken at 50000x magnification to study the size and shape of the nanoparticles. (a) Spherical shaped nanoparticles (b) flower shaped 67
52) Energy dispersive X-ray spectroscopy was done for the bacterial reaction mixture, to observe strong signals of the silver. 68
53) Antimicrobial activity of silver nanoparticles against E.coli shows the zone of inhibition. 70
54) The zone of inhibitions at different concentrations of reaction mixtures. 70

LIST OF TABLES

1) Chemical synthesis, keeping $AgNO_3$ volume constant. 39
2) Color change observed against time 45
3) Change's observed in pH over time 45
4) Chemical synthesis, keeping $NaBH_4$ volume constant 49
5) Diameters of zone of inhibitions obtained are as follows 71

ABBREVIATIONS

1) UV-VIS spectroscopy – Ultraviolet visible spectroscopy
2) SEM - Scanning Electron Microscopy
3) TEM - Transmission Electron Microscopy
4) FTIR - Fourier Transform Infrared Spectroscopy
5) EDX - Energy Dispersive X-ray Spectroscopy
6) XPS - X-ray Photoelectron Spectroscopy
7) XRD - X-ray diffractometer
8) DLS - Dynamic light Scattering
9) AFM - Atomic Force Microscopy
10) S.a – Shewanella algae
11) S.p – Shewanella putrefaciens
12) E.coli – Escherichia coli
13) NP's – Nanoparticles
14) SERS - surface enhanced Raman spectroscopy
15) PVA - PolyVinylAlcohol
16) PVP - PolyVinylPyrrolidone
17) $AgNO_3$ - Silver nitrate
18) $NaBH_4$ -Sodium borohydride
19) HEPA - High Efficiency Particulate Arrestance
20) Spp – Shewanella putrefaciens pellet
21) Sps – Shewanella putrefaciens supernatant
22) Sap – Shewanella algae pellet
23) Sas – Shewanella algae supernatant

Green Synthesis and characterization of Silver Nanoparticles using Bacterial culture and its antimicrobial activity

Kratika

kratika.biotech@gmail.com

Delhi Technological University

ABSTRACT

Microorganisms are being used since a very long time as a reducing agent of metal ions due to the growing concern of environment-friendly and sustainable method. Metallic nanoparticles can be synthesized either by isolating metals inside the cell else exuding them into the extracellular media. Plenty of the microorganisms have already been used to synthesize nanoparticles of numerous metals such as gold, silver, titanium, palladium, platinum etc. The thesis includes the nanoparticle synthesis by two bacterial species ***Shewanella putrefaciens*** and ***Shewanella algae*** using the supernatant as well as the pellet of the cells to synthesize silver nanoparticles. Variants of the silver nitrates have been used to analyse the nanoparticle formation on the basis of concentration. The microbes are being used as both reducing as well as the capping agents.

The synthesis was done at different temperatures and the pH was monitored. UV-VIS spectroscopy was used to monitor the formation of silver nanoparticles quantitatively. UV-VIS peaks were obtained in the range of 380-460nm. After the silver nanoparticles are obtained Fourier Transform Infrared Spectroscopy is done to obtain the functional groups responsible for reduction of the silver nitrate. Scanning Electron Microscopy and Transmission Electron Microscopy results were obtained to confirm the size of the silver nanoparticles which was obtained to be 14-20nm. Lastly the antimicrobial activity was checked by the disc diffusion and well diffusion method against Escherichia coli DH 5α.

CHAPTER 1

INTRODUCTION

Lately, major importance has been given to research in nanotechnology field counting to its applications in versatile fields. This led to union of engineering sciences, chemical, physical, and biological which develop novel techniques in operating and controlling the study at the atomic level. The major reason of getting an utmost attention at the Nano-levels is its profound properties being exhibited by these materials at small size. Some changes are expressed in a noteworthy manner at Nano scale which is not much pronounced at micro-scale. One such example is surface area to volume ratio increases, which amends the catalytic, thermal and mechanical properties of the material. This change leads to increasing superiority of atoms present on the surface of the particle over those in the interior.

Gold and Silver being the most important inorganic nanoparticles as they have superior material properties with functional flexibility, such as potential tools for treating diseases as well as for medical imaging due to their size features and are also being used for cellular delivery due to their features like, rich functionality, capable of targeted drug delivery, better biocompatibility, wider availability and controlled release of drugs.

Conventionally only physical and chemical methods were used to synthesize nanoparticles. But use of undesirable chemicals in the synthesis protocol, creates environmental concerns such as contamination from precursor chemicals, toxic solvents used, high energy consumed and hazardous by-product formed. Problems like stability, crystal growth control and aggregation of the Nano particles are also depicted in other methods. Hence use of biological system like microbes, plants and fungi for various nanoparticles synthesis is emerging as a novel research field as the biological species have been proved to be free of these limits.

Microbial synthesis of nanoparticles has been a success forming nanoparticles of varied shapes and sizes with capable antibacterial activity. The microbial agent is a splendid opportunity for nanotechnology, as ample of microorganisms are available in the world can be used to study the synthesis. One such set of microbes is the Endophytes whose capability in biogenic synthesis of nanoparticles hasn't been studied completely yet. Bacon *et al.* has defined endophytes as "are microbes that form colonies in internal tissues of plants and do not cause any instantaneous, visible negative effects", while Strobel et al. suggests that the relation can vary from mutualistic to adjoining on pathogenic. Endophytes frequently encountered are fungi and bacteria, which co-exist with one another. Attempt was made to take off endophytic bacterium from medicinal plant and engage it in silver nanoparticles synthesis. These nanoparticles had been characterized and checked their antibacterial activity.

In a medical perspective, the pathogenic microorganisms have developed resistance against antibiotics which has been a major concern. This resistance mechanism, because of various genetic and enzymatic mutations in the pathogens which cause infectious diseases, has stirred researchers so as to design new antimicrobial agents to control infections. Metal nanoparticles are one of the potent ways in controlling pathogenic and antibiotic-resistant microorganisms.

Nanoparticles have applications in various areas such as, drug and gene delivery, cosmetics, medicine, diagnostic agents, coatings, biosensors, electronics, imaging, and environmental remediation. Among ample metal nanoparticles, silver nanoparticles have been widely studied because of its valuable properties in catalytic activity, conductivity, chemical stability and optical behavior. Silver nanoparticles also show broad spectrum bacteria and fungus lethal activity. Thus make useful as a disinfectant.

Due to the applications of silver nanoparticles in number of fields, the cost-effective methods have become the foremost need for the biosynthesis of silver nanoparticles. To be descriptive silver has been used since longtime in medical perspective for antimicrobial applications such as burn treatment, disinfection in water treatment, elimination of microorganisms on textile Fabrics, prevention of bacteria colonization on catheters, also found to prevent HIV from binding to host cells. Because of being less reactive compared to silver ions, Nano-silver is found more suitable for medical applications. The mechanism is as follows silver nanoparticles get attached to the surface of the cell membrane thus imposing bacterial effect, disrupts respiration and permeability of the cell. The theory also supports that silver nanoparticles besides interacting with the surface of a membrane also penetrate inside the bacteria.

To affirm that the synthesis has led to the right track, particle characterization is of utmost importance, because the physicochemical properties can have a notable impact on their biological properties. The characteristic attributes of nanomaterials such as shape, size, surface area, size distribution, solubility, aggregation, etc. need to be analyzed before checking toxicity or biocompatibility. Evaluation of the nanomaterial's synthesized, includes different analytical techniques such as Ultraviolet Visible Spectroscopy (UV-vis spectroscopy), Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM), Fourier Transform Infrared Spectroscopy (FTIR), Energy Dispersive X-ray Spectroscopy(EDX), X-ray Photoelectron Spectroscopy (XPS), X-ray diffractometer (XRD), Dynamic light Scattering (DLS), Atomic Force Microscopy (AFM), and so on.

CHAPTER 2

LITERATURE REVIEW

2.1 Bacteria (singular: **bacterium**) are relatively simple, single-celled (unicellular) organisms. Because their genetic material is not enclosed in a special nuclear membrane, bacterial cells are called **prokaryotes** (prō-KAR-e-ōts), from Greek words meaning prenucleus. Prokaryotes include both bacteria and Achaea.

Bacterial cells generally appear in one of several shapes. *Bacillus* (bah-SIL-lus) (rod like), illustrated in, *coccus* (KOK-kus) (spherical or ovoid), and *spiral* (corkscrew or curved)

Is among the most common shapes, but some bacteria are star shaped or square.

Individual bacteria may form pairs, chains, clusters, or other groupings; such formations are usually characteristic of a particular genus or species of bacteria. Bacteria are enclosed in cell walls that are largely composed of a carbohydrate and protein complex called *peptidoglycan.* (By contrast, cellulose is the main substance of plant and algal cell walls.) Bacteria generally reproduce by dividing into two equal cells; this process is called *binary fission.* For nutrition, most bacteria use organic chemicals, which in nature can be derived from either dead or living organisms. Some bacteria can manufacture their own food by photosynthesis, and some can derive nutrition from inorganic substances. Many bacteria can "swim" by using moving appendages called *flagella. [1]*

2.1.1 Bacterial colonies used in synthesis of silver nanoparticles

1) *Shewanella putrefaciens*: It is a Gram-negative pleomorphic bacterium. It has been isolated from marine environments, as well as from anaerobic sandstone in the Morrison Formation in New Mexico. *S. putrefaciens* is also a facultative anaerobe with the ability to reduce iron and manganese metabolically; that is, it can use iron and manganese as the terminal electron acceptor in the electron transport chain (in contrast to obligate aerobes which must use oxygen for this purpose). It is also one of the organisms associated with the odour of rotting fish, as it is a marine organism which produces trimethylamine (hence the species name putrefaciens, from putrid).

In both solid and liquid media, *S. putrefaciens* is often recognizable by its bright pink colour. On solid media, the colonies are round shaped, fast-growing, and pink. The organism is also fast-growing in liquid media, and there will give the liquid an overall pink hue. On blood agar plates, the colonies are typically convex and large, with a brown pigment, and cause "greening" of the agar around the colonies. *S. putrefaciens* are non-lactose fermenters on MacConkey agar. As with all *Shewanella*, this organism produces hydrogen sulfide on TSI.

Although it is very rare for it to act as a human pathogen, there have been cases of infections and bacteremia caused by *S. putrefaciens*. *[2] S. putrefaciens* is one of several species that have been shown to derive energy by reducing U (VI) to U (IV), which is thought to be important in making Uranium deposits. *[3]* In fact, strain CN32 is very metabolically versatile and is capable of reducing metals, metalloids, and even radionuclides in place of oxygen during anaerobic growth. This is known to include (but is not necessarily limited to) Fe(III)->Fe(II), Mn(IV)->(via Mn(III) intermediate)->Mn(II), V(V)->V(IV), Tc(VII)->Tc(V/IV) and U(VI)->U(IV)

2) *Shewanella algae*: It is a gram-negative, rod-shaped, motile bacillus with a single polar flagellum. *S.algae* is found in warm Marine environments throughout the world, and is isolated from seawater with 15-20ppt salinity *[4]. Shewanella algae* is of interest because of its potential to be a serious human pathogen. It is known to cause peritonitis and obstructive pneumonia, rupture of aortic aneurysm, ear infection *[5],* bacteraemia, rare occurrences of skin and soft tissue infections *[6],* bone and joint infections, and many others *[4].* In the past, its effects have often been attributed wrongly to *Shewanella putrefaciens*. There have been a rising number of disease cases attributed to *S. algae* in the last decade, thanks to correct classification. *Shewanella alga* is associated with bacterial peritonitis. The species is also of interest because of its potential to treat radioactive waste in groundwater. It has the ability to reduce uranium and plutonium by reducing soluble forms to insoluble forms. Patients experienced soft tissue infections, ear infection, or abdominal and biliary tract infections.

3) *Escherichia coli* DH 5α: This strain of E. coli is not a pathogen, and was developed for laboratory cloning use. This strain was developed by D. Hanahan as a cloning strain with multiple mutations that enable high-efficiency transformations. *[7]* E. coli is gram-negative bacillus bacteria. They reproduce by successive binary fission with a generation time of approximately 30 minutes with optimum growth occurring at 37 degrees centigrade. E. coli are facilitative aerobic bacteria and are capable of ATP synthesis via both aerobic respiration and, if oxygen is not present it is called fermentation. This particular strain can be identified and distinguished from other E. coli strains, by examining the genetic sequence of its 16s small ribosomal subunit, which has been fully sequenced *[8]*

2.2 CHEMICALS BEING USED IN SILVER NANOPARTICLE SYNTHESIS

1) Silver Nitrate: It is an inorganic compound with chemical formula AgNO3. This compound is a versatile precursor to many other silver compounds, such as those used in photography. It is far less sensitive to light than the halides. It was once called *lunar caustic* because silver was called *luna* by the ancient alchemists, who believed that silver was associated with the moon.
2) Sodium borohydride: It also known as sodium tetrahydridoborate and sodium tetrahydroborate, is an inorganic compound with the formula $NaBH_4$. This white solid, usually encountered as a powder, is a versatile reducing agent that finds wide application in chemistry, both in the laboratory and on a technical scale. It has been tested as pre-treatment for pulping of wood, but is too costly to be commercialized. *[9]*The compound is soluble in alcohols and certain ethers but reacts with water in the absence of a base.
3) Polyvinyl pyrrolidone: PVP can serve as a surface stabilizer, growth modifier, Nanoparticle dispersant, and reducing agent. Its role depends on the synthetic conditions. This dependence arises from the amphiphilic nature of PVP along with the molecular weight of the selected PVP. These characteristics can affect nanoparticle

growth and morphology by providing solubility in diverse solvents, selective surface stabilization, and even access to kinetically controlled growth conditions. This Perspective includes discussions of the properties of PVP-capped NPs for surface enhanced Raman spectroscopy (SERS), assembly, catalysis, and more. The contribution of PVP to these properties as well as its removal is considered. Ultimately, the NPs accessed through the use of PVP in colloidal syntheses are opening new applications, and the concluding guidelines provided herein should enable new nanostructures to be accessed facilely.

2.2.1 MECHANISM OF CHEMICAL SYNTHESIS BY $NaBH_4$

The borohydride anions were adsorbed onto silver nanoparticles and addition of PVP prevented the aggregation of particles. Chemical reduction of silver nanoparticles involves the reduction of a silver salt such as silver nitrate with a reducing agent like sodium borohydride in the presence of colloidal stabilizer. Sodium borohydride has been used with PolyVinylAlcohol (PVA), PolyVinylPyrrolidone (PVP) etc. Polyvinylpyrrolidone (PVP) is a polymer that binds strongly to the silver nanoparticle surface. It provides greater stability than citrate or tannic acid, but is more difficult to displace. PVP is used to protect the silver nanoparticles from growing and agglomerating.

Advantages of the in situ synthesis of Ag/PVP composites include that no additives(e.g., solvent, surface-active agent, or reducing agent of metallic ions) are used, and the stable silver nanocolloid solution can be directly prepared in high concentration sample by dissolving the Ag/PVP nanocomposites in water or organic solvent.

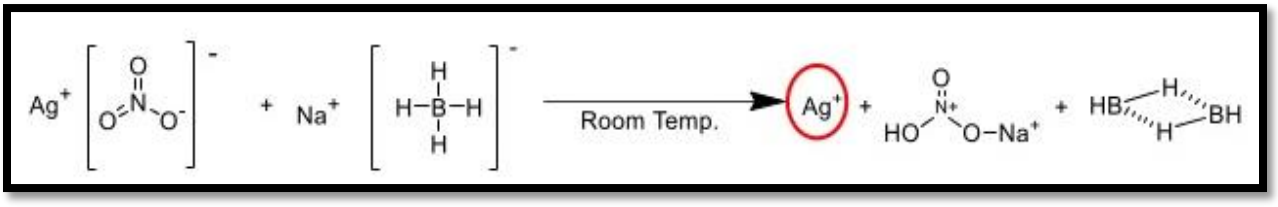


Fig 1: Reduction of $AgNO_3$ by $NaBH_4$

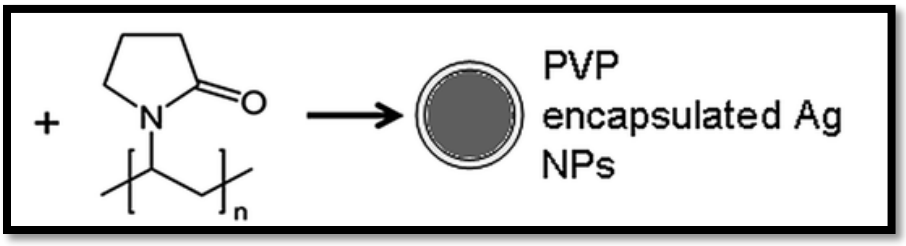


Fig 2: Stabilization by Polyvinyl pyrrolidone

The most common methods for nanoparticle synthesis fall under the category of wet chemistry, or the nucleation of particles within a solution. This nucleation occurs when a silver ion complex, usually $AgNO_3$ or $AgClO_4$, is reduced to colloidal silver in the presence of a reducing agent. When the concentration increases enough, dissolved metallic silver ions bind together to form a stable surface. The surface is energetically unfavourable when the cluster is small, because the energy gained by decreasing the concentration of dissolved particles is not as high as the energy lost from creating a new surface. *[10]* When the cluster reaches a certain size, known as the critical radius, it becomes energetically favourable, and thus stable enough to continue to grow. This nucleus then remains in the system and grows as more silver atoms diffuse through the solution and attach to the surface - *[11]* When the dissolved concentration of atomic silver decreases enough, it is no longer possible for enough atoms to bind together to form a stable nucleus. At this nucleation threshold, new nanoparticles stop being formed, and the remaining dissolved silver is absorbed by diffusion into the growing nanoparticles in the solution.

As the particles grow, other molecules in the solution diffuse and attach to the surface. This process stabilizes the surface energy of the particle and blocks new silver ions from reaching the surface. The attachment of these capping/stabilizing agents slows and eventually stops the growth of the particle. *[12]*

The most common capping ligands are tri-sodium citrate and polyvinyl pyrrolidone (PVP), but many others are also used in varying conditions to synthesize particles with particular sizes, shapes, and surface properties. *[13]*

There are many different wet synthesis methods, including the use of reducing sugars, citrate reduction, reduction via sodium borohydride, *[14]* the silver mirror reaction,*[15]* the Poly-ol process, *[16]* seed-mediated growth, *[17]* and light-mediated growth. *[18]* Each of these methods, or a combination of methods, will offer differing degrees of control over the size distribution as well as distributions of geometric arrangements of the nanoparticle. *[19]*

A new, very promising wet-chemical technique was found by Elsupikhe et al. (2015). *[20]* They have developed a green ultrasonically-assisted synthesis. Under ultrasound treatment, silver nanoparticles (AgNPs) are synthesized with κ-carrageenan as a natural stabilizer. The reaction is performed at ambient temperature and produces silver nanoparticles with face centered crystal structure without impurities. The concentration of κ-carrageenan is used to influence particle size distribution of the AgNPs.*[21]*

The benefit of using sodium borohydride is increased monodispersity of the final particle population. The reason for the increased monodispersity, when using $NaBH_4$ is that it is a stronger reducing agent than citrate. The impact of reducing agent strength can be seen by inspecting a lamer diagram which describes the nucleation and growth of nanoparticles.

2.3 DIFFERENT METHODS USED FOR CHARACTERIZATION OF SILVER NANOPARTICLES

2.3.1 UV-VIS Spectrophotometer- **Ultraviolet** and **visible** (**UV-Vis**) absorption **spectroscopy** is the measurement of the attenuation of a beam of light after it passes through a sample or after reflection from a sample surface. Absorption measurements can be at a single wavelength or over an extended spectral range.

Fig 3: UV-VIS Perkin Elmer Lambda 950

2.3.2 Fourier Transform Infrared Spectroscopy- FTIR or Fourier Transform Infrared Spectroscopy is one of the spectroscopy method which uses Infrared of the Electromagnetic spectrum. The principle is as follows, Infrared radiation passes through the sample. Some radiation is absorbed by the sample while the remaining radiation is passed through or transmitted.

The spectrum obtained depicts the molecular absorption and transmission; which forms the molecular fingerprints of the sample. These molecular fingerprints are different for different molecular structures. Thus this technique is useful for various types of analysis as follows:

1) It is used to ascertain the unknown materials.
2) The quality or consistency of sample is discerned.
3) Can discern the amount components in a mixture

Infrared Spectroscopy is being used since seven decades in the laboratory for the analysis of materials. The fingerprint of a sample depicted by the absorption peaks signifies the frequencies of vibrations between the bonds of the atoms forming the material. Different materials are formed by various permutations and combinations of atoms, thus giving no two compounds the exactly same infrared spectrum. Hence the resulting peaks determine the amount of the material present.

Fig 4: Thermo Scientific Nicolet 380 FTIR

2.3.3 Scanning Electron Microscopy: sectioning problems associated with a transmission electron microscope. It provides striking three-dimensional views of specimens. An electron gun produces a finely focused beam of electrons called the primary electron beam. These electrons pass through electromagnetic lenses and are directed over the surface of the specimen. The primary electron beam knocks electrons out of the surface of the specimen, and the secondary electrons thus produced are transmitted to an electron collector, amplified, and used to produce an image on a viewing screen or photographic plate. The image is called a *scanning electron micrograph.* This microscope is especially useful in studying the surface

structures of intact cells and viruses. In practice, it can resolve objects as close together as 10 nm, and objects are generally magnified 1000 to 10,000x.

Fig 5: Zeiss EVO18 Special Edition SEM

2.3.4 TRANSMISSION ELECTRON MICROSCOPY-

Transmission Electron Microscopy (TEM) is a vital characterization tool for directly imaging nanomaterials to obtain quantitative measures of particle or grain size, size distribution, and morphology. TEM images the transmission of a focused beam of electrons through a sample, forming an image in an analogous way to a light microscope. However, because electrons are used rather than light to illuminate the sample, TEM imaging has significantly higher resolution (by a factor of about 1000!) than light-based imaging techniques. Amplitude and phase variations in the transmitted beam provide imaging contrast that is a function of the sample thickness (the amount of material that the electron beam must pass through) and the sample material (heavier atoms scatter more electrons and therefore have a smaller electron mean free path than lighter atoms). Successful imaging of nanoparticles using TEM depends on the contrast of the sample relative to the background. Samples are prepared for imaging by drying nanoparticles on a copper grid that is coated with a thin layer of carbon.

Materials with electron densities that are significantly higher than amorphous carbon are easily imaged. These materials include most metals (e.g., silver, gold, copper and aluminum), most oxides (e.g., silica, aluminum oxide and titanium oxide), other particles such as polymer nanoparticles, carbon nanotubes, quantum dots, and magnetic nanoparticles. At Nano-Composite, we use a JEOL 1010 transmission electron microscope operating at an accelerating voltage of 100 keV and an AMT XR41-B 4-megapixel (2048 x 2048) bottom mount CCD camera. The camera's finite-conjugate optical coupler provides high resolution and flat focus with less than 0.1% distortion for magnifications as high as 150,000x. Images from the camera are saved as high-resolution TIFF files, and image processing software packages are used to quantify particle/grain size, size distribution and morphology.

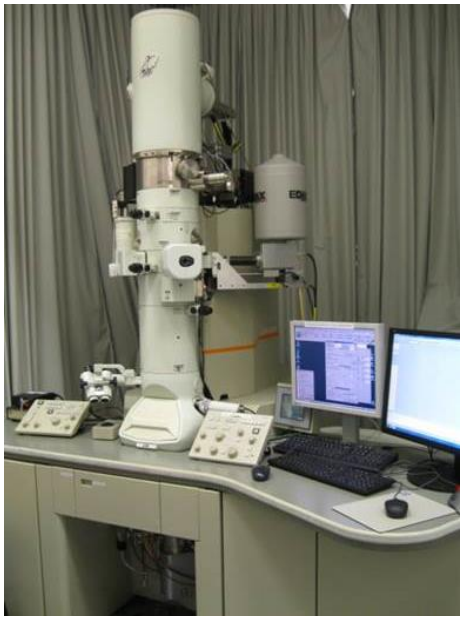


Fig 6: Zeiss Libra 120 TEM

2.3.5 ULTRA SONICATOR: Sonication is the act of applying sound energy to agitate particles in a sample, for various purposes. Ultrasonic frequencies (>20 kilo Hertz) are usually used, leading to the process also being known as ultra-sonication or ultra-sonication.

Fig 7: Hielscher Ultrasound Technology

2.4 ANTIMICROBIAL ACTIVITY OF METAL NANOPARTICLES

Nanoparticles (NPs) are increasingly used to target bacteria as an alternative to antibiotics. The primary reason why NPs are being considered as an alternative to antibiotics is that NPs can effectively prevent microbial drug resistance in certain cases. The rampant use of antibiotics has led to the emergence of numerous hazards to public health, such as superbugs that do not respond to any existing drug and epidemics against which medicine has no defence. The search for new, effective bactericidal materials is significant for combatting drug resistance, and NPs have been established as a promising approach to solve this problem. NPs not only can combat bacterial and microbial resistance themselves, but also can act as a "medium and carrier" of antibiotics.

2.4.1 Disc Diffusion Test

The **agar diffusion test** (**Kirby–Bauer antibiotic testing**, **KB testing**, or **disc diffusion antibiotic sensitivity testing**) is a test of the antibiotic sensitivity of bacteria. It uses antibiotic discs to test the extent to which bacteria are affected by those antibiotics. In this test, wafers containing antibiotics are placed on an agar plate where bacteria have been placed, and the plate is left to incubate. If an antibiotic stops the bacteria from growing or

kills the bacteria, there will be an area around the wafer where the bacteria have not grown enough to be visible. This is called a zone of inhibition. The size of this zone depends on how effective the antibiotic is at stopping the growth of the bacterium. A stronger antibiotic will create a larger zone, because a lower concentration of the antibiotic is enough to stop growth.

2.4 APPPLICATIONS OF SILVER NANOPARTICLES IN VARIOUS FIELDS

Silver nanoparticles possess unique properties which find myriad applications such as antimicrobial, anticancer, larvicidal, catalytic, and wound healing activities.

2.5.1 **Medicine** –

(a) Wound dressings, to treat various wounds in clinic, including burns, chronic ulcers, toxic epidermal necrolysis, and pemphigus. NSP-loaded wound dressings significantly reduced the healing time by an average of 3.35 days and increased bacterial clearance from infected wounds compared to silver sulfadiazine, with no adverse effects.

(b) Cardiovascular implants- Nano-composite with NSPs and diamond-like carbon as a surface coating for heart valves and stents, found that the surface of the Nano-composite showed anti-thrombogenic and antibacterial properties.

(c) Medical catheters are prone to bacterial infection, which can rapidly spread to the wound and its surrounding, and lead to serious complications. Because of their superior antibacterial properties and lack of observed toxicity, NSPs can decrease the incidence of bacterial infection and complications after surgery.

(d) Biodiagnosis- NSPs can be used for bio-diagnosis, where plasmonic properties of NSPs strongly depend on size, shape, and dielectric medium that surrounds it.

2.5.2 Optics- Silver nanoparticle optical properties also depend on the refractive index near the nanoparticle surface. As the refractive index near the nanoparticle surface increases, the nanoparticle extinction spectrum shifts to longer wavelengths (known as red-shifting). Practically, this means that the nanoparticle extinction

Peak location will shift to shorter wavelengths (blue-shift) if the particles are transferred from water (n=1.33) to air (n=1.00), or shift to longer wavelengths if the particles are transferred to oil (n=1.5).

2.5.3 Cancer- The extreme lethality of lung cancer arises due to lack of suitable diagnostic procedures for early detection of lung cancer and ineffective conventional therapeutic strategies. In course with desperate attempts to address these issues independently, a multifunctional Nano-therapeutic or diagnostic system is being sought as a favorable solution.

2.5.4 Drinking water purification- A ceramic water filter (CWF) is a simple device that can eliminate water-borne pathogens. CWFs are reported as effective in removing more than 99% of protozoa and 90-99.99% of bacteria from drinking water *[22-24].* However, a high removal of viruses is not achieved. AgNPs and silver nitrate (AgNO3, Ag+) are added to filters at all CWF factories to achieve higher pathogen removal due to their antimicrobial properties *[25,26]*

CHAPTER 3

3.1 MATERIALS REQUIRED

1) Silver Nitrate

2) Sodium Borohydride

3) Polyvinyl Pyrrolidone

4) Glucose

5) Peptone

6) Yeast Extract

7) Beef Extract

8) Sodium Chloride

9) Agar

10) Glycerol

11) 70% Ethanol

12) Distilled water

13) Cotton plugs

14) Tissue Roll

15) Sterile Discs

3.1.1 GLASSWARES AND TOOLS

1) Petri Plates
2) Conical flasks
3) Beakers
4) Measuring Cylinder

5) Inoculating loop
6) Spirit lamp
7) Spreader
8) Forceps
9) Aluminum foil
10) Micropipette and its tips

CHAPTER 4

ANALYTICAL INSTRUMENTATION

1) **Weighing balance -** is a digital balance used to measure small mass in the grams, milligrams and other small units of mass. The pan is confined from all the sides with the glass walls so that there is no inhibition from the dust or air, while measuring the sample. This is usually known as draft shield. The instrument should be calibrated before measuring the mass on pan.

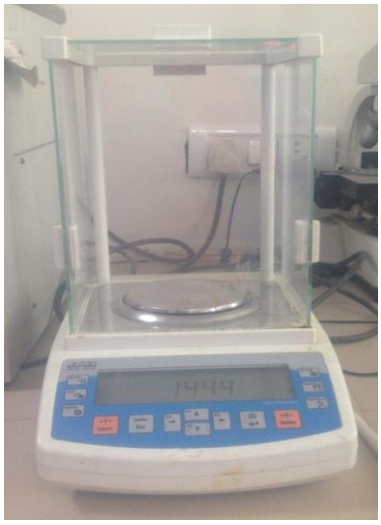


Fig 8: Radwag Weighing Balance

2) **Magnetic Stirrer –** It is also known as magnetic mixer which is a lab device that provides the magnetic field and when a bead is released in the liquid solution, due to the effect of magnetic field it starts to stir. The bead should be cleaned with 70% ethanol before using it in any solution.

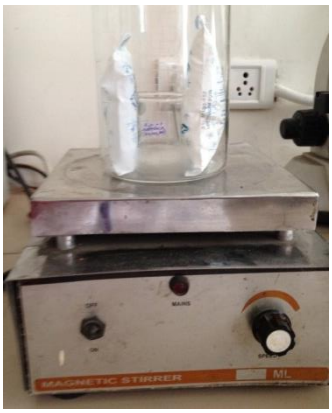


Fig 9: Magnetic Stirrer

3) **Mini Spin Centrifuge-** Mini Spin also known as bench-top or "personal centrifuges" is used for very small amount of samples. The 1.5-2ml eppendorf can be used to obtain the segregated supernatant and pellets of the sample.

Fig 10: Eppendorf Mini Spin Centrifuge

4) **High Speed Centrifuge:** The Centrifuge 580 R is a high speed centrifuge for sedimentation of denser particles at the bottom of the tube. Swing-bucket and fixed-angle rotors can be used to operate the centripetal acceleration on the particles inside the sample.

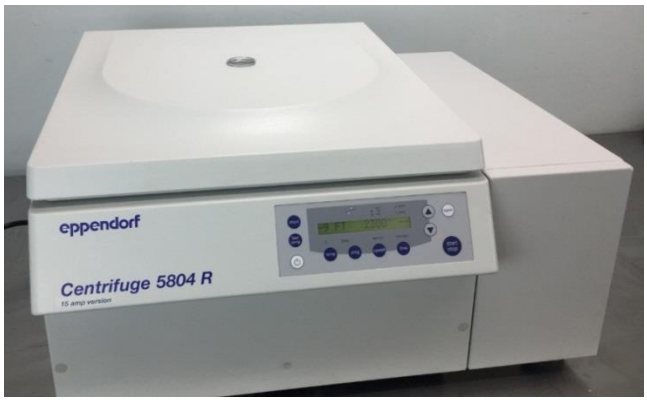


Fig 11: Eppendorf Centrifuge 5804 R

5) **Laminar Hood-** It provides the sterile working environment free from dust and other air-borne impurities by maintaining a unidirectional and constant flow of HEPA-filtered air over the working slab. The flow can be vertical, air drifting from top of the cabinet to the working slab else it can be horizontal, where filter is placed at the back of the working slab.

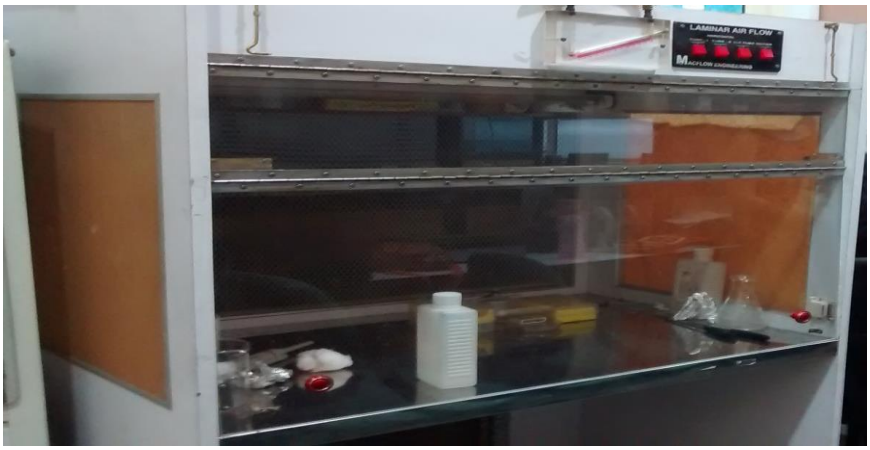


Fig 12: Macflow Engineering Laminar Air Flow

CHAPTER 5

RESEARCH METHODOLOGY

CHEMICAL SYNTHESIS

5.1 EXPERIMENT 1

- Stock solutions of all the reagents were made as:
- 15.132mg of $NaBH_4$ in 200ml of deionized water in the 250ml Erlenmeyer flask and stored at 4°C.
- 5 stock solutions of $AgNO_3$ in 100ml deionized water in the 250ml Erlenmeyer flask were prepared and all these flasks were covered with aluminum foil so that no light reaction could take place and stored at 4°C.
- Added 30ml of 2mM sodium borohydride ($NaBH_4$) in 50ml beaker.

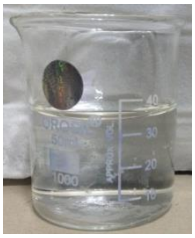


Fig13: $NaBH_4$ solution

5.2 EXPERIMENT 2

1) Added 10ml of 1mM $AgNO_3$ solution in a 50ml beaker and dripped $NaBH_4$ till the solution turned turbid.
2) Took the absorbance at 400nm. Results are as follows:

S. No.	$AgNO_3$ concentration	$AgNO_3$ solution	$NaBH_4$ solution	Absorbance 400
1.	1mM	10ml	4ml	1.818
2.	2mM	10ml	4ml	0.803
3.	3mM	10ml	3ml	0.513
4.	4mM	10ml	1ml	0.446
5.	5mM	10ml	1ml	0.809

Table1: Chemical synthesis, keeping $AgNO_3$ volume constant.

Bacteria's Used

1) ***Shewanella putrefaciens*** –

- Is a gram negative
- Pleomorphic bacteria
- Is a facultative anaerobe.
- On solid media, the colonies are round and fast-growing.
- The organism is also fast-growing in liquid media.

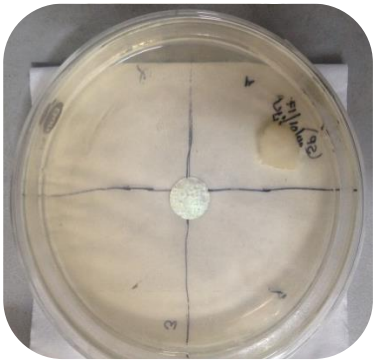


Fig 14: *Shewanella putrefaciens*

2) ***Shewanella algae*** –

- Is a gram-negative
- Rod shaped
- Motile bacillus with a single polar flagellum.
- They can reduce the metals as they have various electron donors like lactate, formate, pyruvate and H_2.

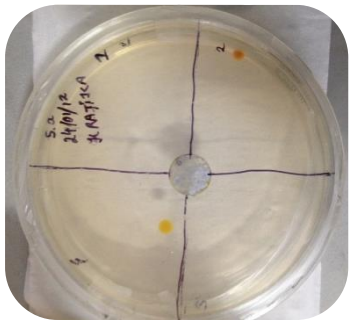
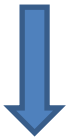


Fig 15: *Shewanella algae*

METHODOLOGY OF INTRACELLULAR AND EXTRACELLULAR SYNTHESIS

The protocol of both intracellular and extracellular synthesis as followed:

From the culture plate prepared the broth

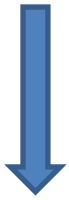


The media broth was centrifuged

Centrifugation

Pellet and supernatant is separately used

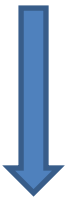
Intracellular Synthesis



Added to $AgNO_3$ solution

After the nanoparticles are obtained the characterization of the particles is done

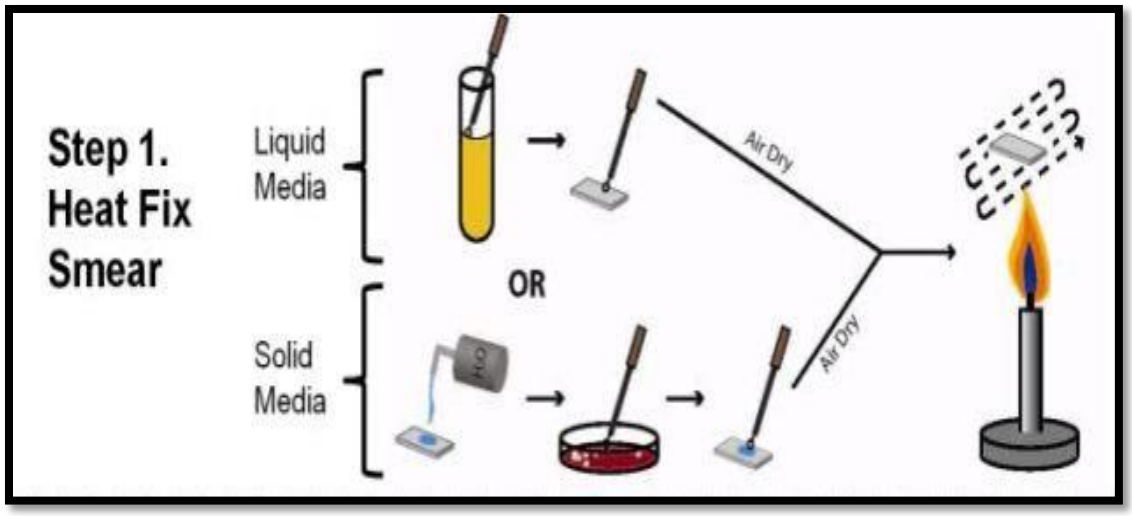
Extracellular Synthesis

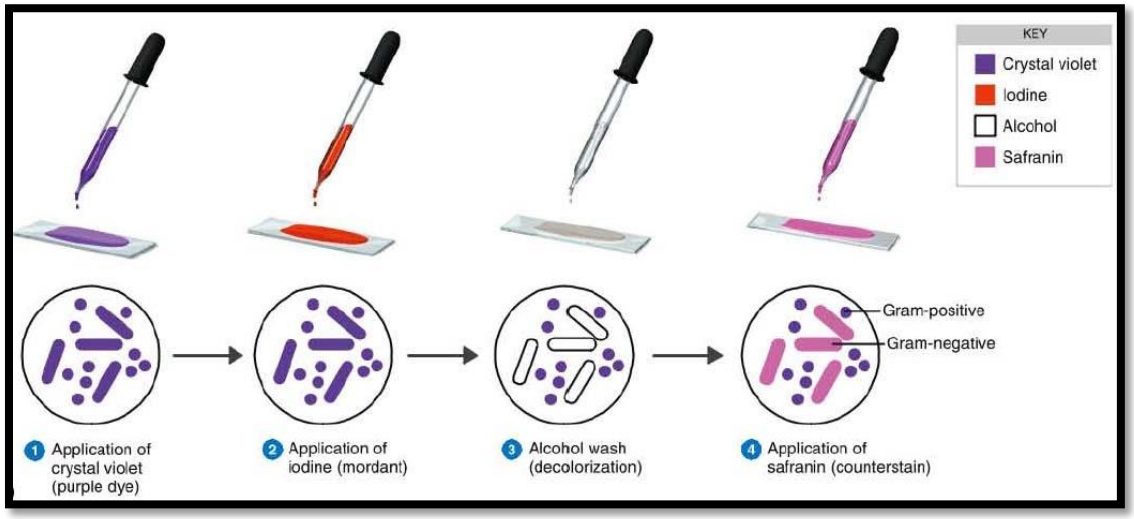


added to $AgNO_3$ solution

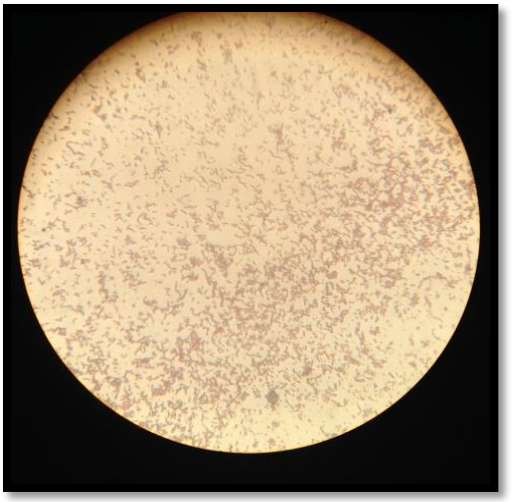
after the nanoparticles are obtained the characterization of the particles is done.

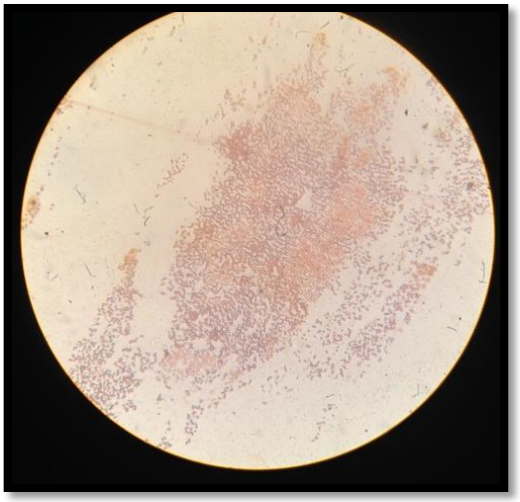
GRAM STAINING





RESULTS OF BACTERIAL GRAM STAINING





A- *Shewanella putrefaciens*

Pink Colour- gram negative

Structure- No fixed shape

B- *Shewanella algae*

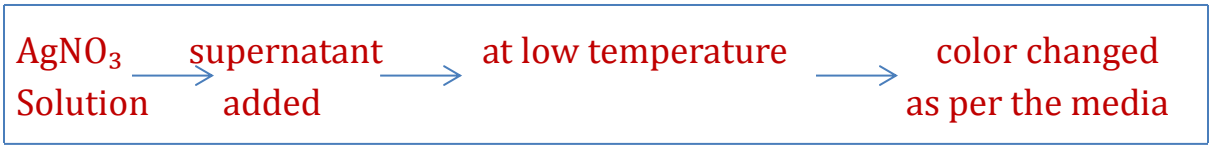
Pink colour- gram negative

Structure- Rod shaped

As when the gram staining test was conducted using the above given protocol of Gram staining, it was seen that the safranin color was retained. This phenomenon takes place in gram negative bacteria's as they have thin peptidoglycan cell wall which cannot retain the crystal violet color and gets decolorized when washed.

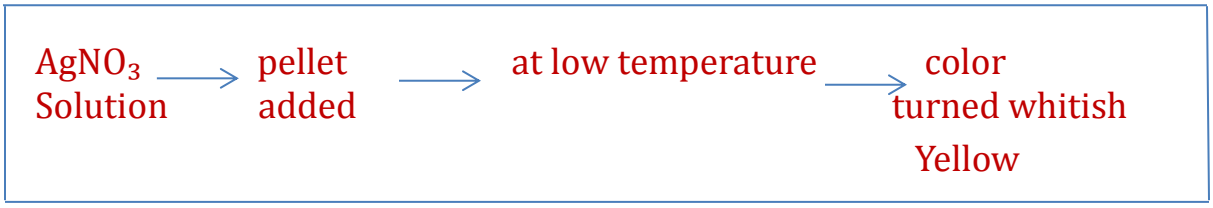
1) Shewanella putrefaciens

SET 1: supernatant used



2) Shewanella putrefaciens

SET 2: pellet used



5.3 EXPERIMENT 3

1) At Low Temperature, when the $AgNO_3$ (1mM) is kept at 4°C is used.
2) Five 50ml autoclaved beakers were taken and added 10ml of $AgNO_3$ solution.
3) Kept the beaker on magnetic stirrer, encircled with the icepacks.
4) Drop-wise added S. putrefaciens supernatant (Sps), S. putrefaciens pellet (Spp), S. algae supernatant (Sas), S. algae pellet (Sap) and $NaBH_4$ (2mM) to four different beakers respectively.
5) All these were kept in dark after the 3hrs of duration.

S. No.	0 min	5 min's	30 min's	1 hr	2 hr	3 hr
Sps	Transparent	Light Yellow	Light yellow	Light Yellow	Whitish yellow	Whitish yellow
Spp	Transparent	Yellowish White	Turbid Whitish Yellow	Whitish yellow	Turbid Whitish	turbid
Sas	Transparent	Transparent	Same	Same	Same	Same
Sap	Transparent	Turbid	Turbid	white	Clear	Clear
$NaBH_4$	Transparent	Brownish yellow	Same	Same	Same	Silver brown

Table 2: Color change observed against time

S.No.	0 min	5 min's	30 min's	1hr	2 hrs	3 hrs
Sps	pH 6	pH 7	pH 8	pH 8	pH 8	pH 8
Spp	pH 6	pH 7	pH 7	pH 7	pH 7	pH 7
Sas	pH 6	pH 6.5	pH 7	pH 7	pH 7	pH 7
Sap	pH 6	pH 6	pH 6	pH 6	pH 6	pH 6
$NaBH_4$	pH 6	pH 6	pH 6	pH 6	pH 6	pH 6

Table 3: Changes observed in pH over time

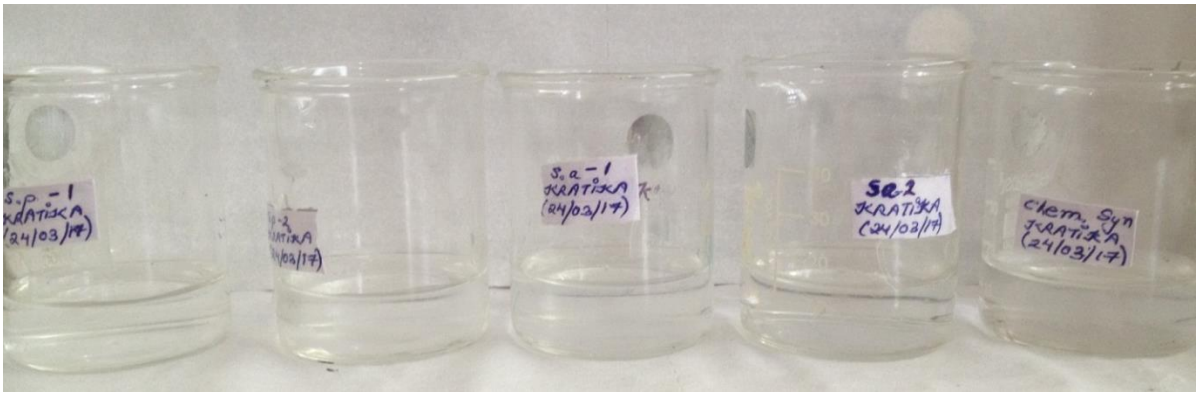


Fig 16: Colour of all the solutions before reaction

5.4 EXPERIMENT 4

1) The above reactions were again conducted but this time temperature was raised to 40°C.
2) UV-VIS spectrophotometer readings were taken for range 400-430nm.
3) The temperature was raised to 10°C in 10min's duration and was reached to 40°C.

5.5 EXPERIMENT 5

1) Now the above experiment was again conducted but at room temperature.

2) The supernatant and pellet was added to 30ml of $AgNO_3$ solution present in 50ml beaker.

3) Total supernatant added to 30ml $AgNO_3$ solution was 3ml, for both the species.

4) The pellet present in 1.5ml eppendorf was added to 30ml $AgNO_3$ solution.

5) Absorbance obtained was in the range 370-410nm.

5.6 EXPERIMENT 6

1) $AgNO_3$ was taken at different concentrations i.e. 2mM, 3mM, 4mM, 5Mm, 6Mm, 7Mm, 8Mm, 9Mm and 10mM.
2) 10ml of $AgNO_3$ of each concentration was taken in nine 50ml glass tubes for each set (i.e. Sps, Spp, Sas and Sap)
3) After adding 10ml samples to each tube kept these tubes in the open bright conditions.
4) Color change was observed.

DAY 1

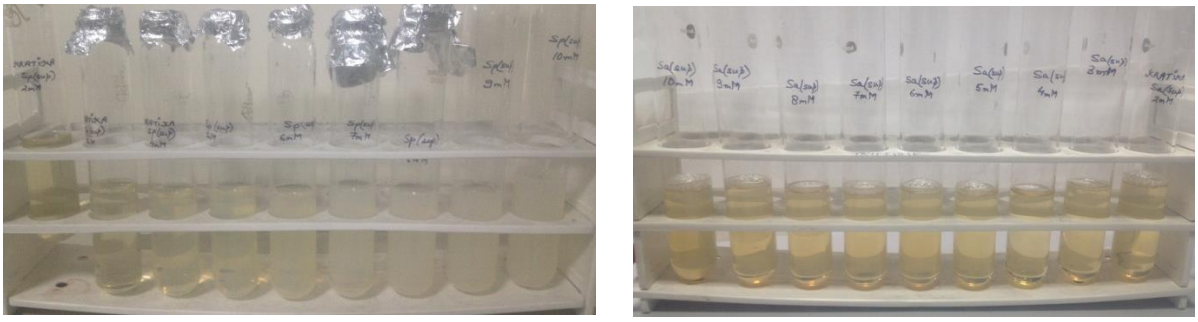


Fig 17: The colour of solutions Sps and Sas respectively when reactions are set.

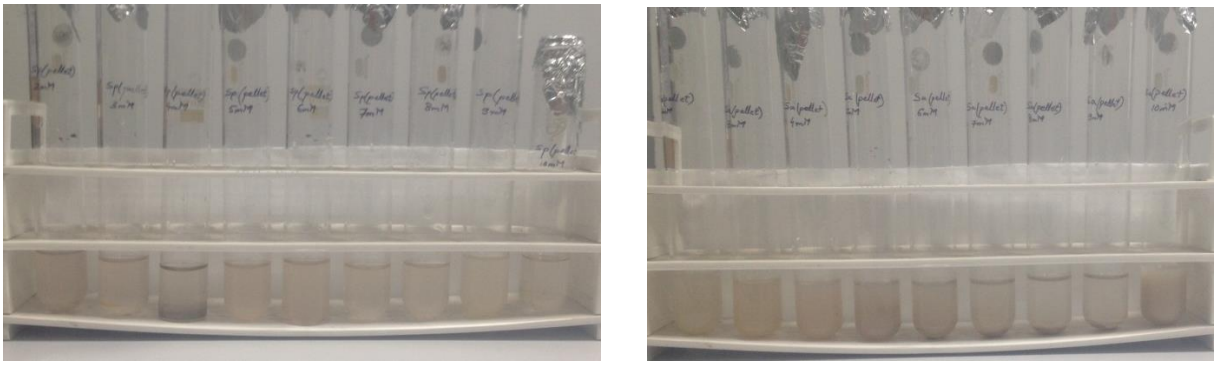


Fig 18: The colour of solutions Spp and Sap respectively on setting the reaction

CHAPTER 6

RESULTS AND DISCUSSION

5.1 RESULT:

1mM 2mM 3mM 4mM 5mM

Fig 19: color change on addition of different concentrations of $AgNO_3$

S.No.	$AgNO_3$ concentration	$NaBH_4$ solution	$AgNO_3$ solution	PVP solution	Absorbance400
1.	1mM	30ml	11ml	2 drops	0.873
2.	2mM	30ml	7ml	2 drops	1.108
3.	3mM	30ml	4ml	2 drops	1.770
4.	4mM	30ml	5ml	2 drops	1.276
5.	5mM	30ml	4ml	2 drops	1.357

Table 4: chemical synthesis, keeping $NaBH_4$ volume constant

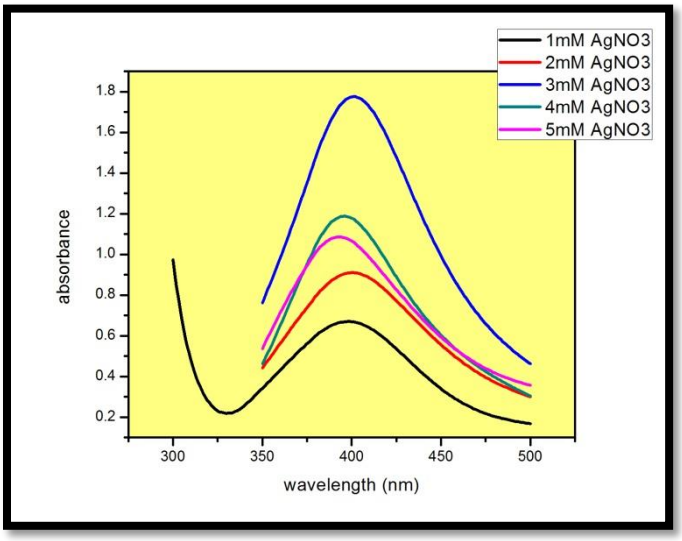
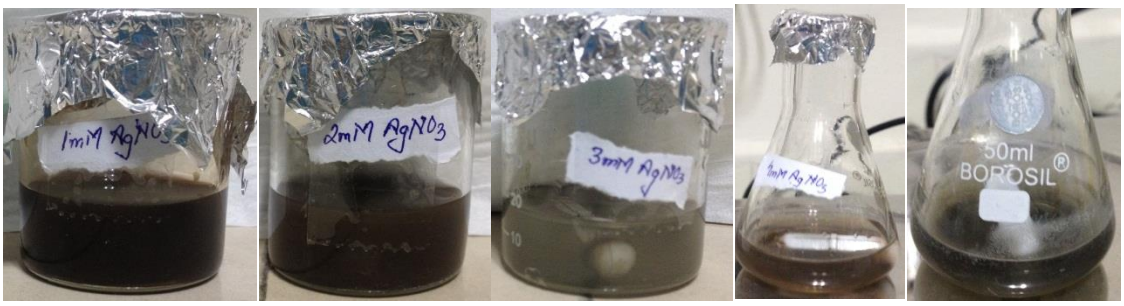


Fig20: UV-VIS spectrophotometer peaks of all the above concentration solutions

5.2 RESULT:



1mM 2mM 3mM 4mM 5mM

Fig21: Color change observed when $NaBH_4$ solution was added drop wise.

1) The color change was immediate.

5.3 RESULT: Colour change is visible

Fig 22: Colour change after reaction

1) The color change was obtained in the following samples as mentioned in table 5.

5.4 RESULT: Peaks are obtained at 420nm

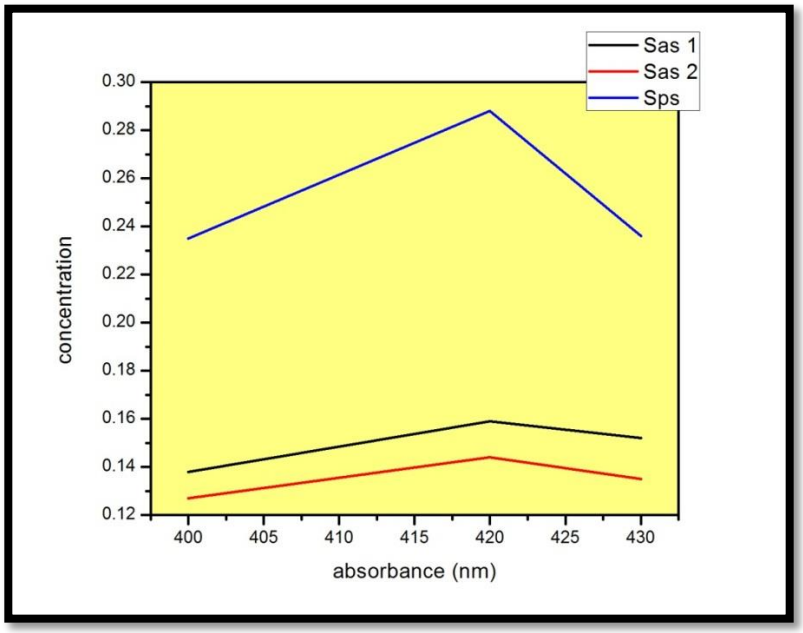


Fig 23: Maximum peaks obtained approximately at 420nm

1) As the peaks are obtained at 420nm thus, silver nanoparticles are formed.

5.5 RESULT: Peaks are not obtained

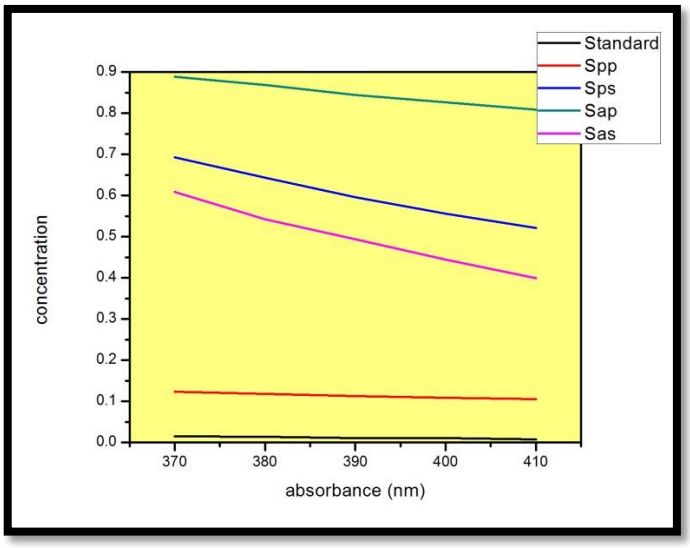


Fig 24**: Spp, Sps, Sap, Sas** peaks were going below 370nm

1) Experiment 7 was repeated this time S.a, S.p supernatant and chemical synthesis was done.
2) The absorbance was taken in the range of 380-430nm.

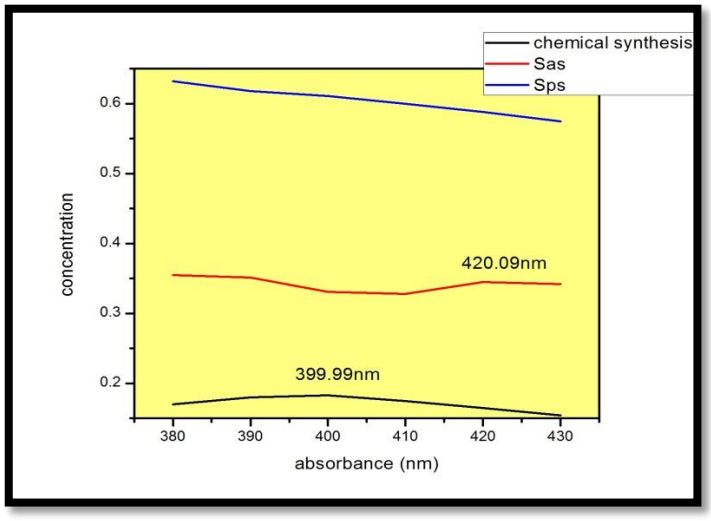


Fig 25: Peak was obtained in the case of **Sas** and Chemical synthesis

5.6 Results

DAY 2

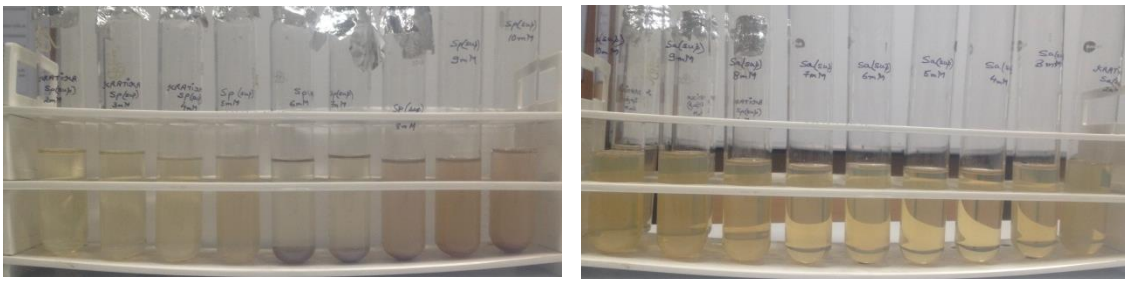


Fig 26: Colour change observed in Sps and Sas in 24hrs

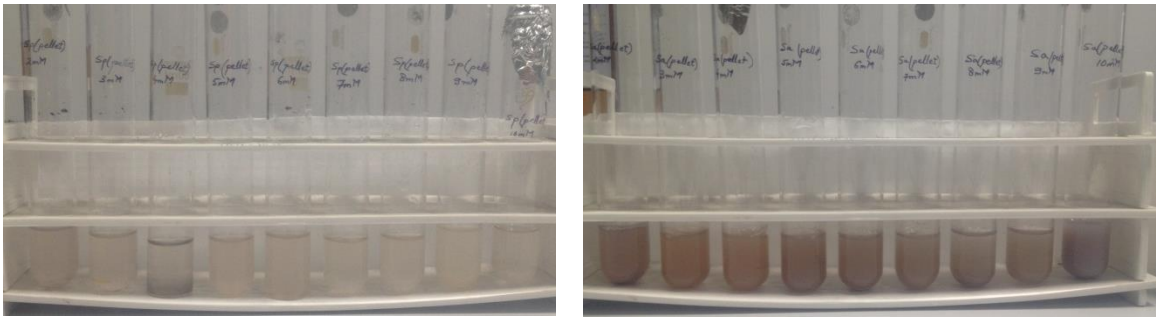


Fig 27: Colour change observed in Spp and Sap in 24hrs

1) The color change in 24hrs.
2) The absorbance obtained for these samples in 24hrs duration.
3) Absorbance was taken in the range of 380-430 nm.
4) Peaks were going below 380nm.
5) Absorbance readings of S.a pellet were going very high (i.e. beyond 2) thus the graph could not be drawn.

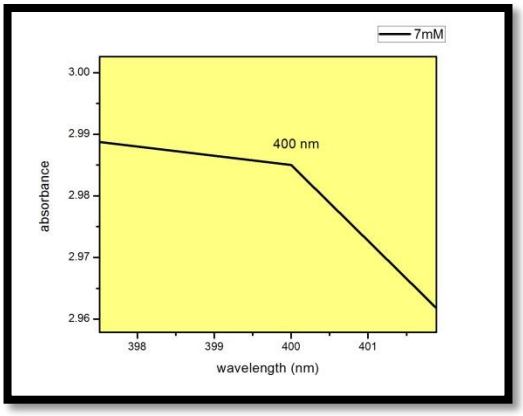


Fig 28: **Sps** peak is obtained at concentration of 7mM $AgNO_3$

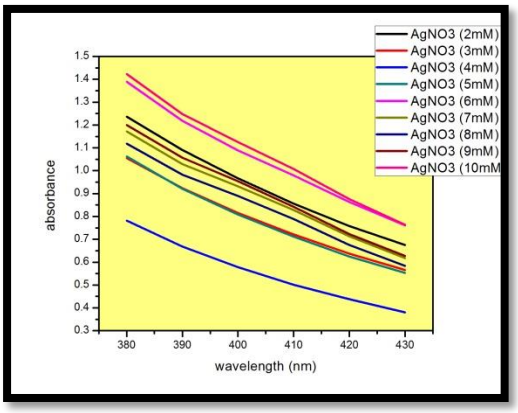


Fig 29: **Sas** peaks are not obtained at any concentration within range

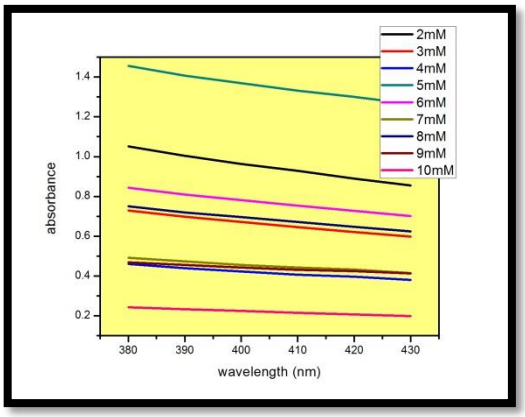


Fig 30: **Spp** peaks were also not obtained in this range

DAY 3

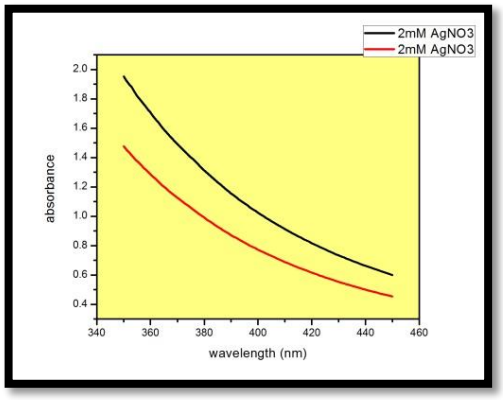


Fig 31: **Sas** peaks not obtained even after 48hrs

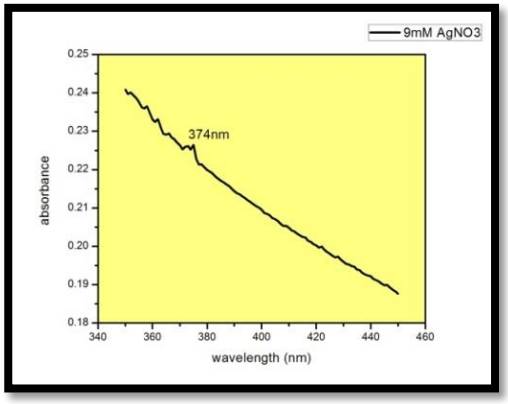


Fig 32: **Spp** peak is observed in 9mM $AgNO_3$ sample at 374nm

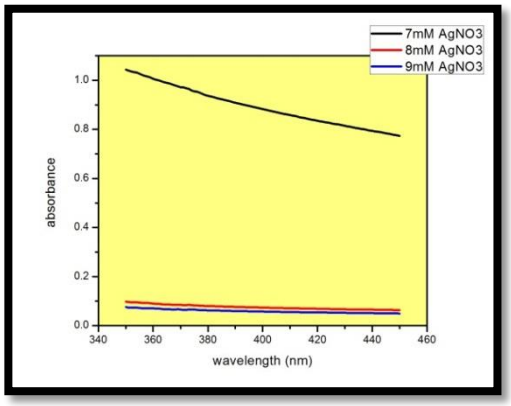


Fig 33: **Sap** peaks are not obtained even in 48hrs

DAY 6

Fig 34: Sps colour changes

1) As can be seen in the above picture the color of 2mM, 3mM, 4mM has not changed much since Day 1 but the color of 5Mm, 7mM and 8mM has turned turbid brown.
2) While 6mM solution has turned violet.
3) 9mM and 10mM solutions look brownish orange.
4) These results are obtained after 120hrs.

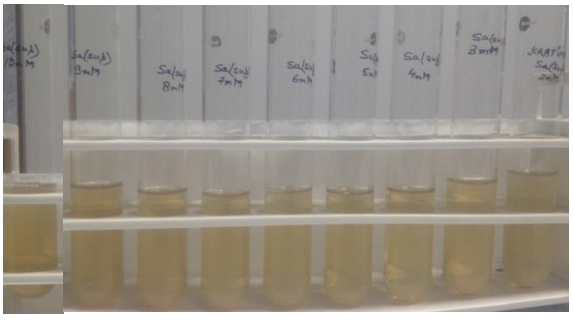


Fig 35: Sas remains the same as on the Day 1

1) As can be seen the color change cannot be observed, which can conclude to formation of no silver nanoparticles.
2) Thus and experiment was conducted in two above (i.e. Sas + 3mM and 5mM $AgNO_3$) solutions.

5.7 Sub-Experiment

1) Took 5mL of 3mM + Sas solution in a slant tube and to it added freshly prepared 2mM $NaBH_4$ solution, drop wise.
2) Similarly took 5mL of 5mM+ Sas solution in a slant tube and added to it freshly prepared 2mM $NaBH_4$ solution.
3) The chemical synthesis takes place and instantaneously the color changes to brown.
4) The results are as follows:

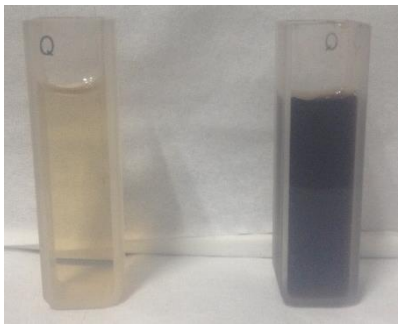


Fig 36: The original reaction mixture of Sas and $AgNO_3$ solution was light yellow which turned to dark brown after adding the $NaBH_4$ 2mM solution.

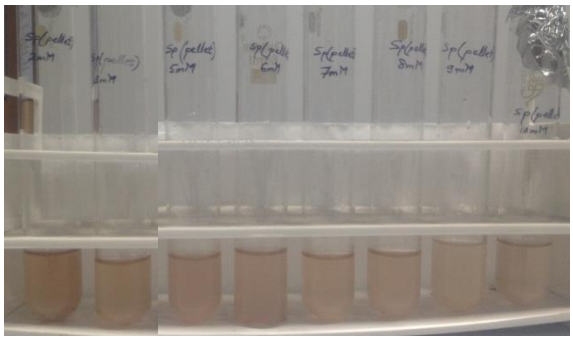


Fig 37: The colours of the Spp solutions have turned beginning from light pink to yellowish brown

1) 2mM, 3mM, 4mM, 5mM, 6mM and 7mM reaction mixtures have turned to pinkish white while the color of 9mM and 10mM solutions turn to yellowish brown.

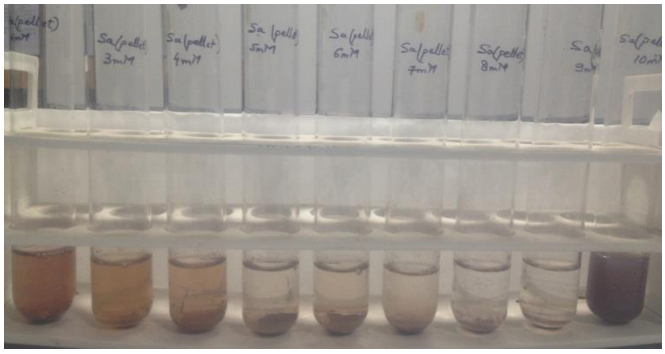


Fig 38: the colour of Sap solutions had changed

1) 2mM, 3mM and 4mM reaction mixtures seem to have changed the color to yellowish brown.
2) 5mM, 6mM, 7mM, 8mM and 9mM reaction mixture solution had turned turbid which gets settled on the bottom of the tubes.

RESULTS OF SPECTROPHOTOMETER

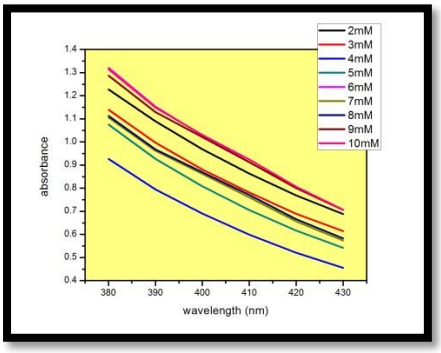


Fig 39: **Sas** peaks are not obtained but few kinky points can be observed.

Day 7

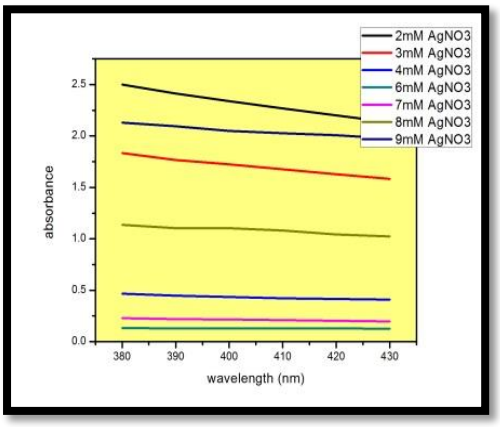


Fig 40**: Sap** peaks are not obtained even in 144hrs**.**

Day 8

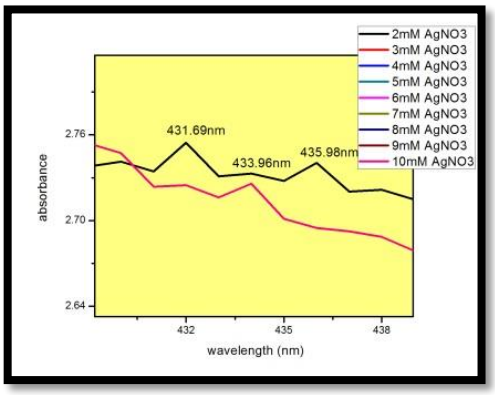


Fig 41: peaks are obtained in **Sap** after 168hrs two of the concentrations have been enlarged

1) In 2mM $AgNO_3$ reaction mixture the peaks are obtained at 431.69 and at 435.98nm.
2) In 10mM $AgNO_3$ reaction mixture the peak is obtained at 433.96nm.

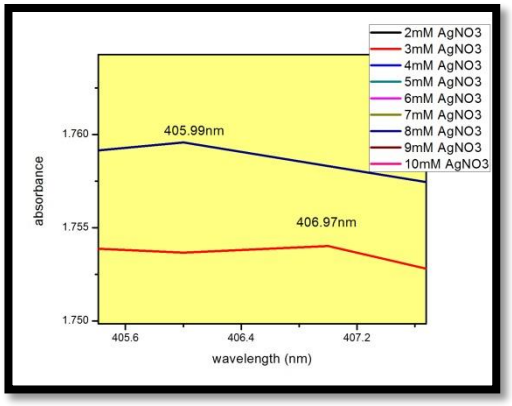


Fig 42: Similarly in Sap 3mM and 4mM $AgNO_3$ reaction mixture the peaks are obtained.

1) In 3mM $AgNO_3$ reaction mixture the peak is obtained at 406.97nm.
2) In 4mM $AgNO_3$ reaction mixture the peak is obtained at 405.99nm

DAY 9

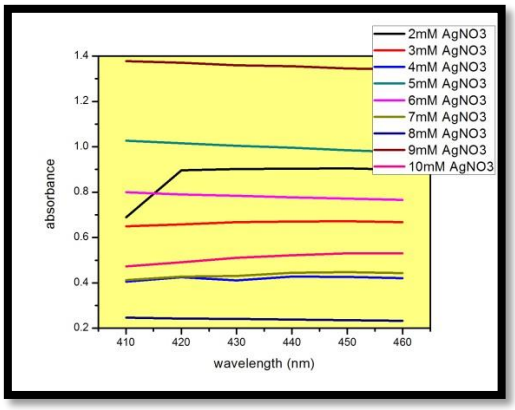


Fig 43**: Spp** peaks are obtained at 420nm in 2 and 4mM $AgNO_3$

DISCUSSION

The peaks were obtained in the range of 400-420nm for all the reaction mixtures with both the strains i.e S. algae as well as S. putrefaciens involving the intracellular as well as extracellular synthesis. Different $AgNO_3$ concentrations were used and it was found that even at various concentrations the peaks were obtained in the range specific for silver nanoparticles.

6.1 FOURIER TRANSFORM INFRARED SPECTROSCOPY (FTIR)

RESULTS

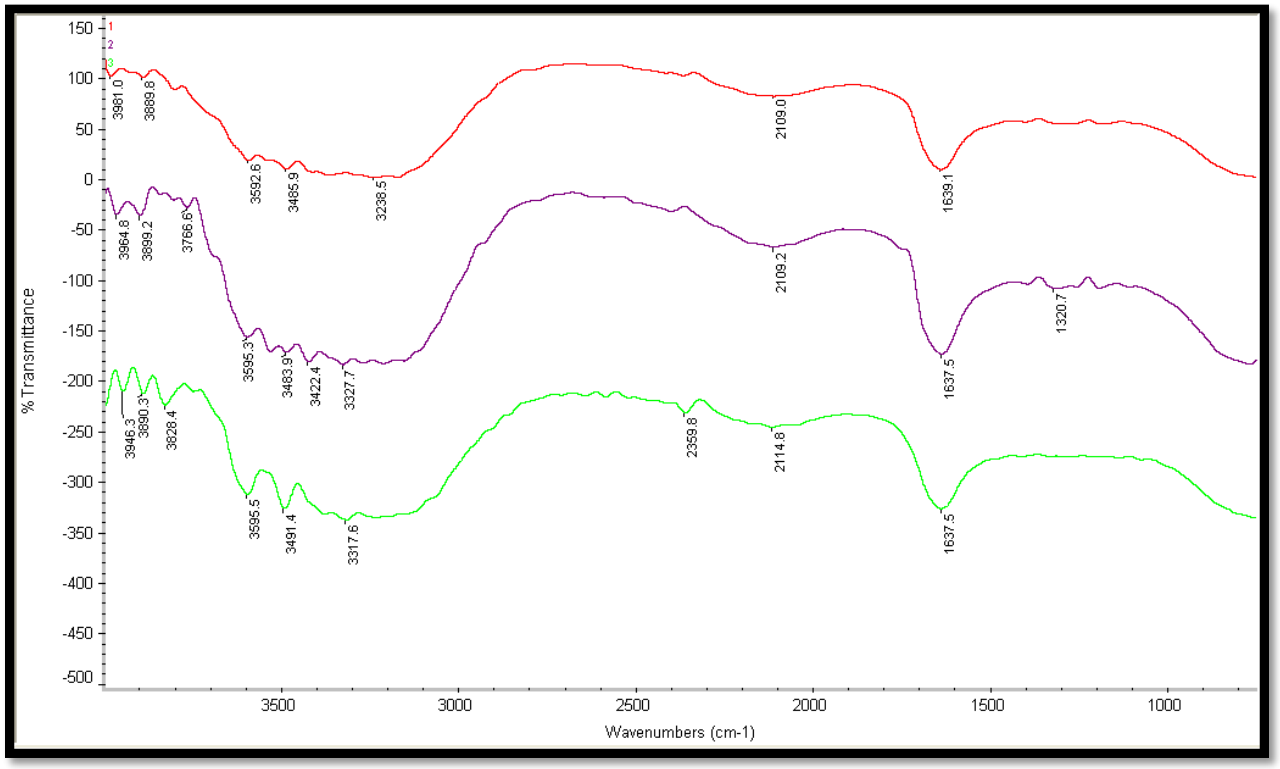


Fig 44: The Infrared peaks observed for $NaBH_4$, S. putrefaciens reaction mixture and S. putrefaciens supernatant (from top to bottom) as reducing agents.

DISCUSSION

The measurement by FTIR is done to get the best dynamic ranges for intense and weak bands. Infrared radiations are absorbed to get the peaks which define the wavenumbers. The stretching and bending of various functional groups are observed through the peaks, the stretching of the groups are usually obtained at higher wavenumber's while their corresponding bending peaks are obtained at lower frequencies.

1) The Peaks obtained in Sample 1, which consists of the $NaBH_4$ as the reducing agent are 3981.0, 3889.8, 3592.6, 3485.9, 3238.5, 2109.0 and 1639.1 cm^{-1}.
2) The Peaks of Sample 2 (i.e. *S. putrefaciens* supernatant reaction mixture) obtained are 3964.8, 3899.2, 3766.6, 3595.3, 3483.9, 3422.4, 3327.7, 2109.2, 1637.5, 1320.7 cm^{-1}.
3) The Peaks of *S. putrefaciens* culture supernatant obtained were 3946.3, 3890.3, 3828.4, 3595.5, 3491.4, 3317.6, 2359.8, 2114.8, 1637.5 cm^{-1}.

Here narrow strong bands of 1639.1 and 1637.5 cm^{-1} corresponds to the -C=O carbonyl group and alkenyl C=C stretch. The band of 1320.7 cm^{-1} is assigned to aromatic primary or secondary amine, CN stretch. 2109.0, 2114.8 cm^{-1} broad weak bands define the Cyanide ion, thiocyanate ion and terminal alkyne (monosubtituted) stretch. Whereas 3317.6 cm^{-1} is an alkyne C-H stretch while broad prominent peaks of 3238.5, 3485.9, 3327.7, 3422.4, 3483.9, 3491.4 and 3317.6 cm^{-1} corresponds to hydroxyl group, H-bonded –OH stretch. Phenols, -OH stretch is determined by 3592.6, 3595.3, 3595.5 cm^{-1}.

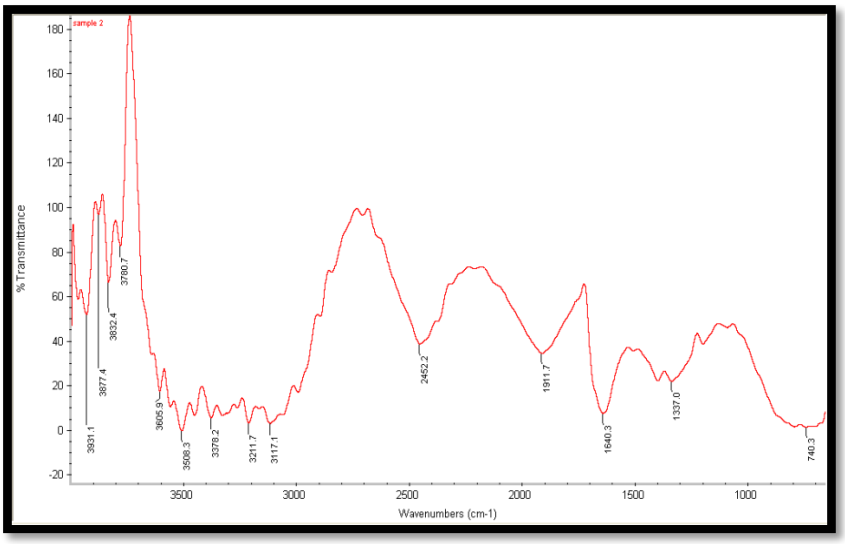


Fig 45: FTIR results of S. putrefaciens pellets for 1mM $AgNO_3$ reaction mixture.

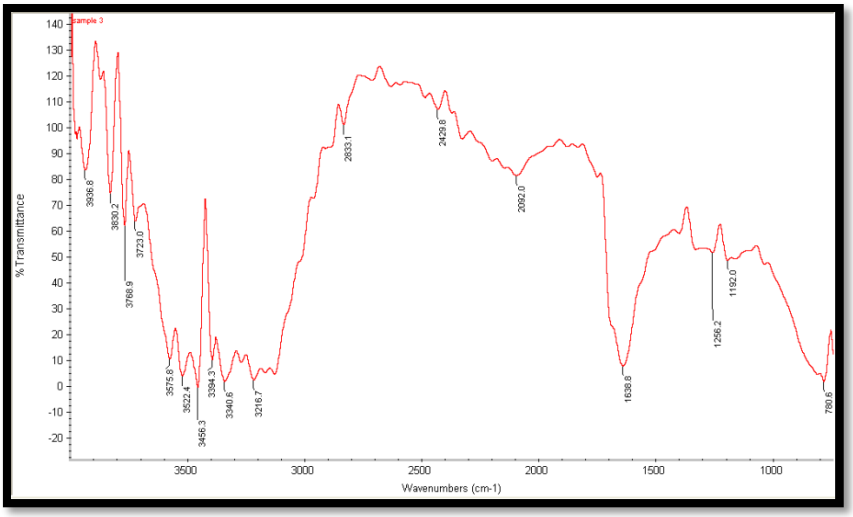


Fig 46: FTIR results of S. putrefaciens supernatant for 5mM $AgNO_3$ reaction mixture.

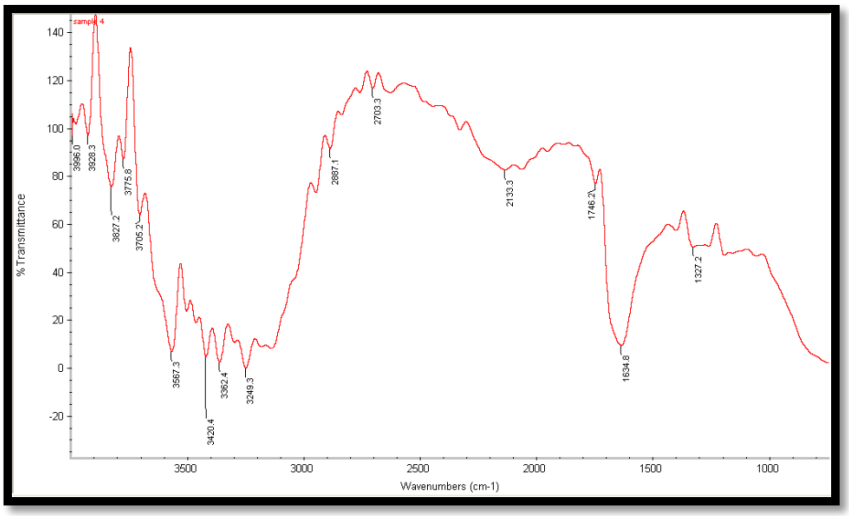


Fig 47: FTIR results of S. putrefaciens for 4mM $AgNO_3$ reaction mixture.

DISCUSSION

The most prominent broad and strong bands which are present at 3211.7, 3378.2, 3216.7, 3340.6, 3394.3, 3456.3, 3249.3, 3362.4, 3420.4 cm^{-1} corresponds to the hydroxyl group and –OH stretch.

The narrow strong peaks at 1640.3, 1638.8, 1634.8 cm^{-1} wavenumbers are assigned to alkenyl C=C stretch and organic nitrates. Broad weak peaks at 1337.0 and 1327.2 cm^{-1} depicts the functional groups of primary or secondary, -OH in plane bend and organic phosphates along with 1746.2 cm^{-1} which is a peak for alkyl carbonate shows the presence of carboxylic acid. 780.6 cm^{-1} explains the aliphatic C-Cl compound group frequency. 1192.0 cm^{-1} corresponds to the tertiary amine, CN stretch and 1256.2 cm^{-1} is assigned to aromatic primary amine, CN stretch which shows the presence of the proteins. The reported data tells that the silver nanoparticles get attached to the free amines or cysteine residues of sample proteins. Some other groups which are also present are as follows: 1911.7 and 2092.0 cm^{-1} is defined for aromatic combination bands, broad weak band at 2133 cm^{-1} assigned to terminal alkyne (mono-substituted). It is represented by weak bond because triple bond is not Polar. 2833.1 cm^{-1} corresponds to methyl oxy, Methyl ether and C-H stretch. 3508.3cm^{-1} characterises aromatic primary amine, N-H stretch. 3605.9, 3575.8 and 3567.3 cm^{-1} corresponds to phenols, -OH stretch.

6.2 SCANNING ELECTRON MICROSCOPE

RESULTS

Morphology of the nanoparticles is studied by Scanning Electron Microscope. S. putrefaciens supernatant of 1mM $AgNO_3$ concentration was processed under SEM to obtain the average size of the particles as 14.80nm as can be seen in fig 48. The SEM images of the three samples were taken and the average size of the nanoparticles obtained are mentioned in the description. All the three samples were of S. putrefaciens including two samples of supernatant of 1mM and 5mM while the other sample was from pellet reaction mixture of 1mM concentration.

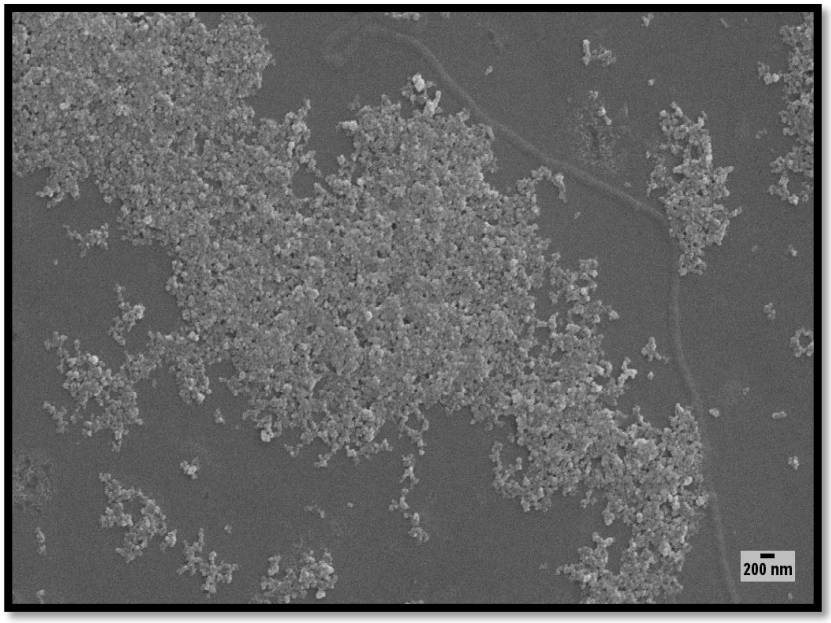


Fig 48: SEM image of the sample which contains S. putrefaciens supernatant with 1mM $AgNO_3$ solution was taken

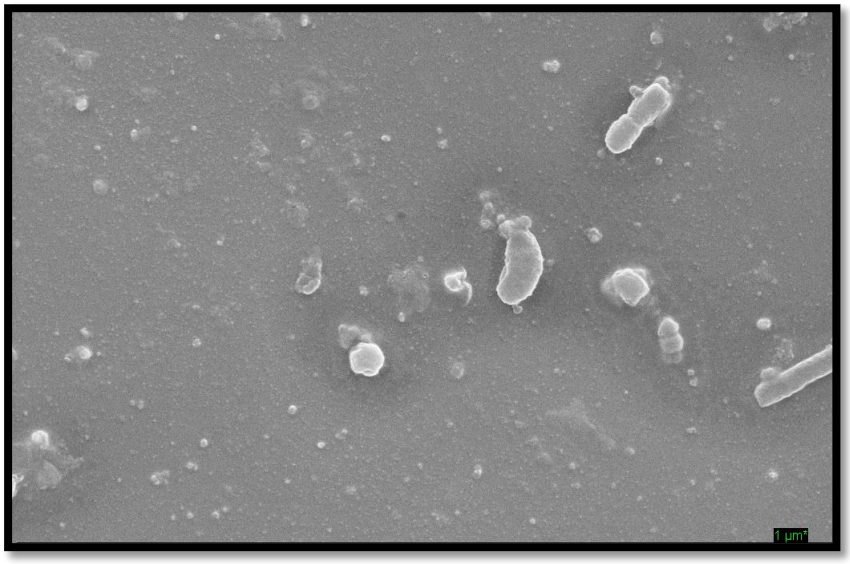


Fig 49: SEM image of the S. putrefaciens pellet 1mM concentration shows the presence of the bacteria's in it along with the nanoparticles.

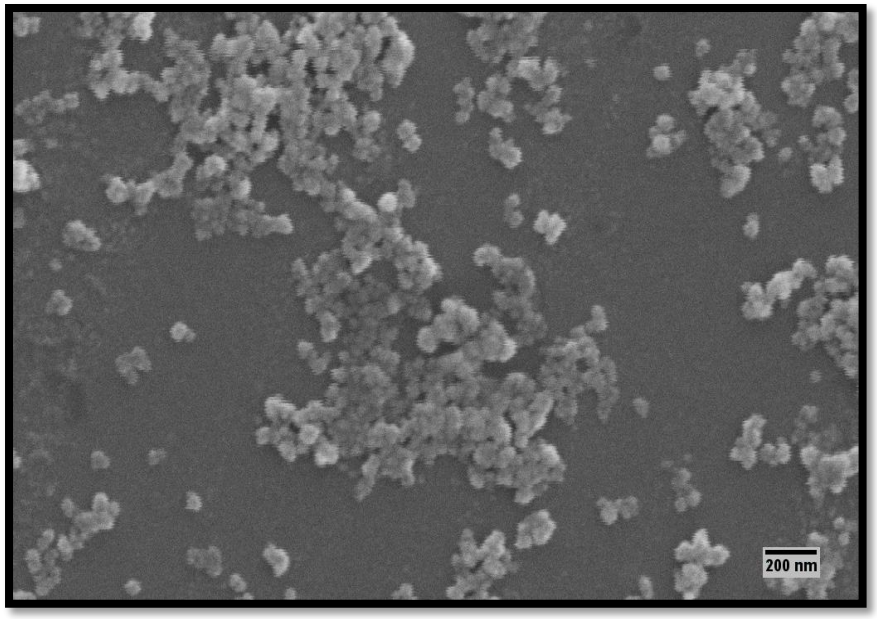


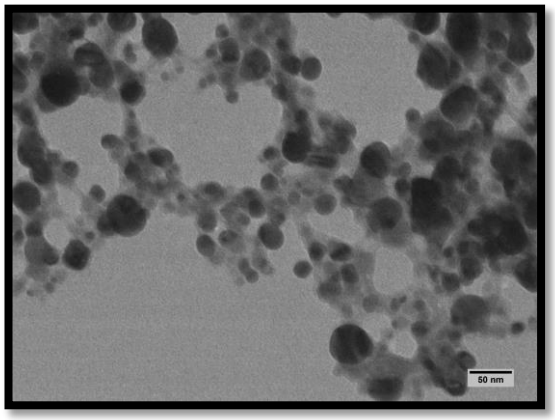
Fig 50: SEM image of S. putrefaciens supernatant 5mM concentration shows the presence of the silver nanoparticles at 200nm scale.

DISCUSSION

The SEM in figure 48 was conducted after 72hours of the reaction between 1mM of $AgNO_3$ and *Shewanella putrefaciens* supernatant which was kept at room temperature and normal daylight conditions were rendered. Since the images were taken after 72hrs the aggregation seen is excessively high. Similar conditions were given to other two reactions where in figure 49 the reaction between 1mM $AgNO_3$ and *Shewanella putrefaciens* pellets is set, as can be seen the culture contains the cells. In Figure 50 the reaction was conducted between 5 mM $AgNO_3$ and *Shewanella putrefaciens* supernatant, again the aggregation seen is very high. The average particle size was calculated for all the three SEM images which were obtained between 14-22 nm. The size of nanoparticles synthesized by the intracellular method were obtained approximately as of 20-22nm while it was seen when the concentration of silver nitrate was increased, the average size of the particles synthesized by supernatant reaction mixture was 19.24nm.

6.3 TRANSMISSION ELECTRON MICROSCOPE

RESULTS

(a) 

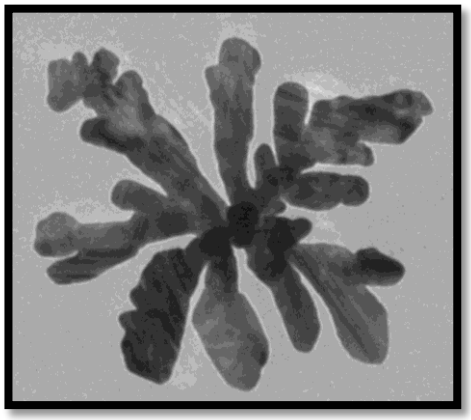
(b) 

Fig 51: TEM images were taken at 50000x magnification to study the size and shape of the nanoparticles. (a) Spherical shaped nanoparticles (b) flower shaped

DISCUSSION

Transmission electron microscope is used for the quantitative analysis of the nanoparticles, which measures the size, shape and morphology of the particles. *Shewanella putrefaciens* supernatant and 1mM $AgNO_3$ concentration reaction mixture was conducted for 72hours at approximately 35°C. This reaction was diluted in 15ml double distilled water and was sonicate by Ultra sonicator for 20min's. Then this sample was taken for TEM imaging. As can be seen the particles of varying sizes of spherical shapes were obtained as is visible in figure 51(a). The darker spheres are not found to be in direct contact with one another, which can prove as a good stabilizer. This can be an effect of the proteins secreted by the micro-organism. The flower shaped silver nanoparticles were also obtained on synthesis by 1mM $AgNO_3$ and *Shewanella putrefaciens* supernatant. The average particle size obtained was 17.58nm.

6.4 ENERGY DISPERSIVE X-RAY SPECTROSCOPY

RESULTS

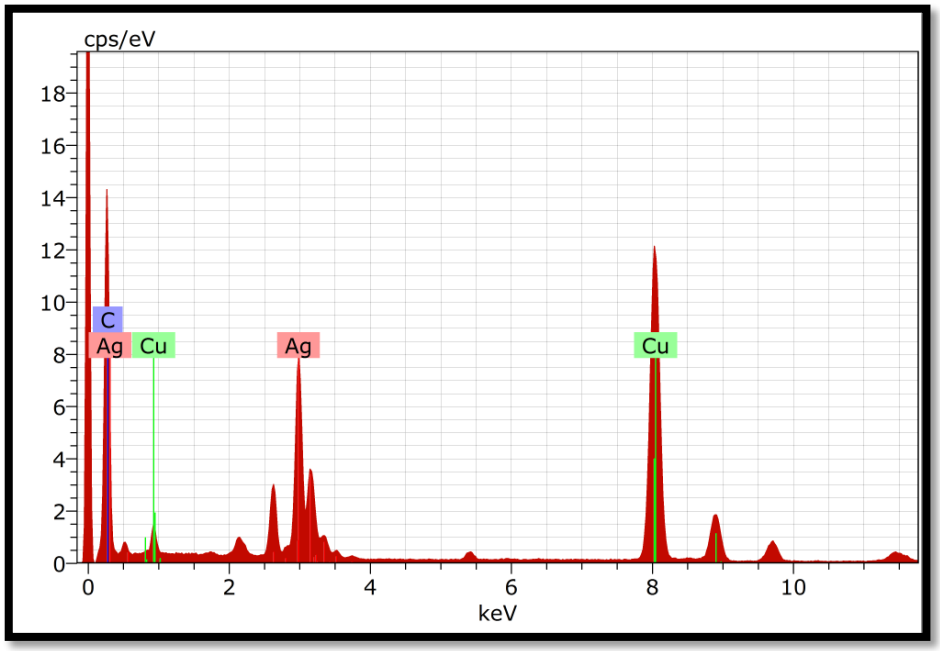


Fig 52: Energy dispersive X-ray spectroscopy was done for the bacterial reaction mixture, to observe strong signals of the silver.

DISCUSSION

EDS result confirms the presence of elemental silver nanoparticles synthesized by extracellular method. The peak at 3keV is the distinctive as it proves the presence of the silver nanoparticles in its elemental form. Apart from which silver peak is also present at 0-0.5keV.

6.5 ANTIMICROBIAL ACTIVITY

The antimicrobial activity of the silver nanoparticles was checked against *Escherichia coli* culture. The antibacterial activity is an important and an alternative to the antibiotics therefore checking the antimicrobial activity of the silver nanoparticles becomes a very important step.

6.5.1 Disc Diffusion Test

1) Isolated the colony from the culture plate and stirred it into the media broth and prepared the agar plates.
2) After 24hrs of its growth, the sterile discs were loaded with the different concentration reaction mixtures of S. putrefaciens and S. algae pellets.
3) Added 20µl of each sample on the 9mm sterile disc in sterile conditions.
4) After the samples were loaded, discs were left to dry. Marked six sections on the back side of the petri plates.
5) Take 20ml of the culture broth and with the help of the spreader spread it on the agar plates.
6) Place the loaded sterile discs on these agar plates, placing one in each section.
7) One was kept as Control; others were loaded with reaction samples.
8) Keep these disc loaded plates inversely in the incubator, at 37°C for 24hrs.
9) After which the zone of inhibition can be seen.

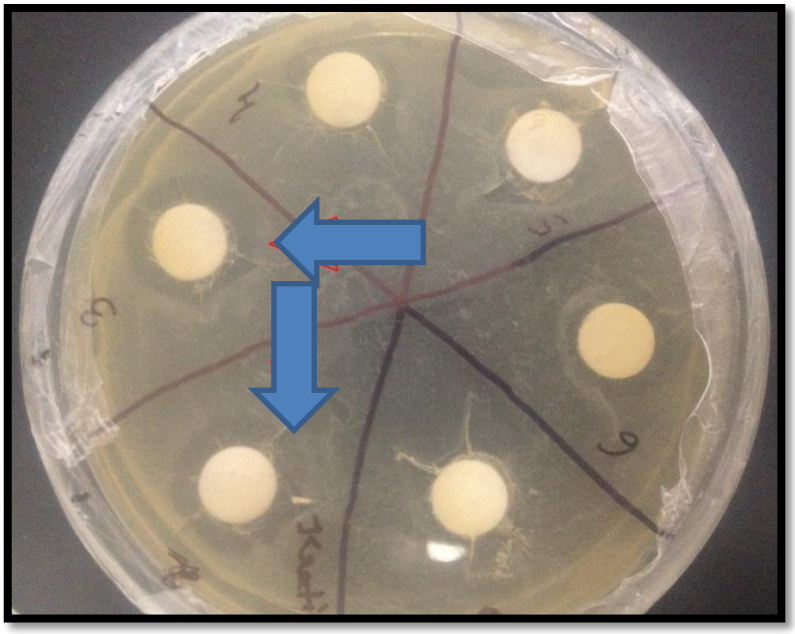


Fig 53: Antimicrobial activity of silver nanoparticles against *E.coli shows the zone of inhibition.C-control*, 2-2mM, 3-3mM, 4-4mM, 5mM and 6mM $AgNO_3$ as described below in table 5.

Fig 54: the zone of inhibitions at different concentrations of reaction mixtures.

RESULTS

In control the S. putrefaciens and S. algae supernatants were poured on discs, which did not show zone of inhibition. While the other discs loaded with reaction mixtures shows zone of inhibition but the diameters vary.

Table 5: Diameters of zone of inhibitions obtained are as follows:

S.No.	SAMPLE MIXTURE	DIAMETER(cm)
1	Chemical synthesis	1.6
2	Spp 2mM	1.7
3	Spp 3mM	1.7
4	Spp 4mM	1.7
5	Spp 5mM	1.5
6	Spp 6mM	1.6
7	Spp 7mM	1.5
8	Spp 8mM	1.5
9	Spp 9mM	1.9
10	Spp 10mM	1.4
11	S.p. supernatant 1mM	Not Obtained
12	Sap 2mM	1.2
13	Sap 3mM	1.2
14	Sap 4mM	1.7
15	Sap 5mM	1.6
16	Sap 6mM	1.5
17	Sap 7mM	1.5
18	Sap 8mM	1.5
19	Sap 9mM	1.6
20	Sap control	Not obtained

DISCUSSION: As per the above results antimicrobial activity gives the positive test against E. coli strain. When all the concentrations of *S. putrefaciens* and *S. algae* reaction mixtures were tested against E. coli culture, they were able to resist the growth of E. coli thus forming the zone of inhibition of different diameters. As can be seen the chemical synthesis creates 1.6 cm diameter of Zone of Inhibition, Thus when comparing the biological synthesis it gives comparatively better results than the chemically synthesized silver nanoparticles, as well as the controls show no Inhibition Zones.

CHAPTER 7

CONCLUSION

This work was done to test the green synthesis of silver nanoparticles synthesized by two bacterial strains i.e. *Shewanella putrefaciens* and *Shewanella algae.* Green synthesis is an eco-friendly method due to which it becomes reliable in medical and pharmaceutical applications. The silver nanoparticles were synthesized by extra-cellular as well as intra-cellular protocols to obtain the 14-22nm size silver nanoparticles. The formation of the silver nanoparticles was confirmed by various characterization techniques. UV-VIS spectrophotometry readings were the foremost method to detect the formation of the silver nanoparticles and to proceed in that particular way. FTIR graphs detected the presence of primary and secondary amines which are described to react with the silver nanoparticles, if the free amine groups are present in the sample of bacterial culture. As the broad and strong peaks of -OH stretch was obtained which suggests the banding of silver particles with oxygen of hydroxyl group. Scanning Electron Microscopy results helped to determine the average size of the silver nanoparticles. Transmission Electron Microscope results assessed the morphology of the synthesized silver nanoparticles, which came out as the spherical shaped as well as its flower shaped image was scanned. Energy dispersive X-ray spectroscopy results gave the standard peak at 3keV of silver which proves the formation of silver nanoparticles in the sample and lastly the antimicrobial activity of the synthesized silver nanoparticles were validated to obtain the resistance against the E. coli culture. Thus the silver nanoparticles formed by green synthesis become more economical and can be a good alternative to physical and chemical synthesis methods. These nanoparticles can prove its potential in different applications.

REFERENCES

1) Microbiology an introduction, *Tortora funke* case
2) *Pagani L, Lang A, Vedovelli C, et al. (May 2003).* "Soft tissue infection and bacteremia caused by *Shewanella putrefaciens*". *J. Clin. Microbiol.* ***41*** *(5): 2240–1.*
3) *Min, M.; Xu, H.; Chen, J.; Fayek, M. (2005). "*Evidence of uranium bio-mineralization in sandstone-hosted roll-front uranium deposits, northwestern China". *Ore Geology Reviews.* ***26*** *(3–4): 198.*
4) Holt, H.M., et al. *Shewanella algae* and *Shewanella putrifaciens*: clinical and microbiological characteristics. Clinical Microbiology and Infection, 2005. Volume 11: 347-352
5) Beleneva, Irina A., et al. Biochemical and pathogenic properties of the natural isolate of Shewanella algae from Peter the Great Bay, Sea of Japan. Journal of Invertebrate Pathology, 2009. Volume 102: 250-255.
6) Myung, D.S., et al. Primary Shewanella algae Bacteremia mimicking Vibrio Septicemia. Journal of Korean Medical Science, 2009. Volume 24(6): 1192-1194.
7) Taylor, R. G., Walker, D. C. and McInnes, R. R. (1993). E. coli host strains significantly affect the quality of small scale plasmid DNA preparations used for sequencing. Nucleic Acids Res. 21, 1677 -1678.
8) Singh, Om V and Steven P. Harvey. "Bacterial Communities." Sustainable Biotechnology. Dordrecht etc.: Springer, 2010. 169-71.
9) Peter Rittmeyer, Ulrich Wietelmann "Hydrides" in Ullmann's Encyclopedia of Industrial Chemistry 2002, Wiley-VCH, Weinheim.
10) Polte, Jörg. "Fundamental Growth Principles of Colloidal Metal Nanoparticles – a New Perspective."
11) Rama, Siva, Krishna Perala, and Sanjeev Kumar. "On the Mechanism of Metal Nanoparticle Synthesis in the Brust–Schiffrin Method."

12) Chenhui, Dingsheng Wang, Wen Zheng, and Qing Peng. "Growth and Assembly of mono-disperse Ag Nanoparticles by Exchanging the Organic Capping Ligands."
13) Johnston, Kathryn A., Ashley M. Smith, Lauren E. Marbella, and Jill E. Millstone. "Impact of As-Synthesized Ligands and Low-Oxygen Conditions on Silver Nanoparticle Surface Functionalization."
14) Dong, X.; Ji, X.; Jing, J.; Li, M.; Li, J.; Yang, W. (2010). "Synthesis of Triangular Silver Nano-prisms by Stepwise Reduction of Sodium Borohydride and Trisodium Citrate". J. Phys. Chem. C. **114** (5): 2070–2074.
15) Shan, Z.; Wu, J.; Xu, F.; Huang, F.-Q.; Ding, H. (2008). "Highly Effective Silver/Semiconductor Photo-catalytic Composites Prepared by a Silver Mirror Reaction". J. Phys. Chem. C. **112** (39): 15423–15428.
16) Wiley, B.; Sun, Y.; Xia, Y. Synthesis of Silver Nanostructures with Controlled Shapes and Properties. Accounts of Chemical Research Acc. Chem. Res. 2007, 40, 1067–1076
17) Pietrobon B., Mceachran M., Kitaev V. (2009). "Synthesis of Size-Controlled Faceted Pentagonal Silver Nanorods with Tunable Plasmonic Properties and Self-Assembly of These Nanorods". ACS Nano. **3**: 21–26
18) Tanimoto H., Ohmura S., Maeda Y. (2012). "Size-Selective Formation of Hexagonal Silver Nanoprisms in Silver Citrate Solution by Monochromatic-Visible-Light Irradiation". J. Phys. Chem. C. **116**: 15819–15825
19) Rycenga, M.; Cobley, C. M.; Zeng, J.; Li, W.; Moran, C. H.; Zhang, Q.; Qin, D.; Xia, Y. (2011). "Controlling the Synthesis and Assembly of Silver Nanostructures for Plasmonic Applications". Chemical Reviews. **111** (6): 3669–3712.
20) "Green sonochemical synthesis of silver nanoparticles at varying concentrations of κ-carrageenan" (PDF). Nanoscale Research Letters. springer.com. **10**. doi:10.1186/s11671-015-0916-1
21) "Green Sonochemical Route to Silver Nanoparticles". hielscher.com. Retrieved 2016-02-15

22) Brown, J. and M.D. Sobsey, Microbiological effectiveness of locally produced ceramic filters for drinking water treatment in Cambodia. J Water Health, 2010. 8(1): p. 1-10.
23) Van Halem, D., et al., Assessing the sustainability of the silver-impregnated ceramic pot filter for low-cost household drinking water treatment. Phys. Chem. Earth, 2009. 34(1-2): p. 36-42.
24) Lantagne, D.S., Investigation of the potters for peace colloidal silver impregnated ceramic filter. Report 1: Intrinsic Effectiveness. 2001.
25) Pradeep, T. and Anshup, Noble metal nanoparticles for water purification: A critical review. Thin Solid Films, 2009. 517(24): p. 6441-6478.
26) Lv, Y., et al., Silver nanoparticle-decorated porous ceramic composite for water treatment. Journal of Membrane Science, 2009. 331(1-2): p. 50-56.
27) Renata Dobrucka • Jolanta Długaszewska, " Antimicrobial activities of silver nanoparticles synthesized by using water extract of *Arnicae anthodium",* 2015 55(2):168–174
28) Swarnali Maiti et al, "Antimicrobial activities of silver nanoparticles synthesized from Lycopersicon esculentum extract", 2014
29) Akhilesh Kushwaha1 et al, "Isolation and identification of *E. coli* bacteria for the synthesis of silver nanoparticles: Characterization of the particles and study of antibacterial activity", 2015, 5(1):65-70
30) Vidhya Lakshmi Das, "Extracellular synthesis of silver nanoparticles by the Bacillus strain CS 11 isolated from industrialized area", (2014) 4:121–126
31) Kannan Badri Narayanan, Natarajan Sakthivel, "Biological synthesis of metal nanoparticles by microbes", (2010) 1–13
32) G. Singaravelu a et al. "A novel extracellular synthesis of monodisperse gold nanoparticles using marine alga, Sargassum wightii Greville", 57 (2007) 97–101
33) Kaushik N. Thakkar, "Biological synthesis of metallic nanoparticles", 6 (2010) 257–262
34) S. Minaeian, "Extracellular biosynthesis of silver nanoparticles by some bacteria", Vol 17, No . 66.

35) Yasuhiro Konishi, “Bioreductive deposition of platinum nanoparticles on the bacterium Shewanella algae”, 128 (2007) 648–653

36) M. Jannathul Firdhouse and P. Lalitha, “Biosynthesis of Silver Nanoparticles and Its Applications”, Volume 2015, Article ID 829526, 18 pages

37) K. M. M. Abou El-Nour, A. Eftaiha, A. Al-Warthan, and R. A. A. Ammar, “Synthesis and applications of silver nanoparticles,” Arabian Journal of Chemistry, vol. 3, no. 3, pp. 135–140, 2010.

38) R. Das, S. S. Nath, D. Chakdar, G. Gope, and R. Bhattacharjee, “Synthesis of silver nanoparticles and their optical properties,” Journal of Experimental Nanoscience, vol. 5, no. 4, pp. 357–362, 2010.

39) V. K. Sharma, R. A. Yngard, and Y. Lin, “Silver nanoparticles: green synthesis and their antimicrobial activities,” Advances in Colloid and Interface Science, vol. 145, no. 1-2, pp. 83–96, 2009.

40) N. Kulkarni and U.Muddapur, “Biosynthesis ofmetal nanoparticles: a review,” Journal of Nanotechnology, vol. 2014,Article ID 510246, 8 pages, 2014.

41) A. K.Mittal, Y. Chisti, and U. C. Banerjee, “Synthesis of metallic nanoparticles using plant extracts,” Biotechnology Advances, vol. 31, no. 2, pp. 346–356, 2013.

42) S. Prabhu and E. K. Poulose, “Silver nanoparticles: mechanism of antimicrobial action, synthesis, medical applications, and toxicity effects,” International Nano Letters, vol. 2, article 32, 2012.

43) K. Szczepanowicz, J. Stefa´nska, R. P. Socha, and P. Warszy´nski, “Preparation of silver nanoparticles via chemical reduction and their antimicrobial activity,” Physicochemical Problems of Mineral Processing, vol. 45, pp. 85–98, 2010.

44) N. T. P. Phong, N. H.Minh, N. V. K. Thanh, and D. M. Chien, “Green synthesis of silver nanoparticles and silver colloidal solutions,” Journal of Physics: Conference Series, vol. 187, no. 1, Article ID 012078, 2009.

45) S. Gurunathan, K. Kalishwaralal, R. Vaidyanathan et al., “Biosynthesis, purification and characterization of silver nanoparticles using Escherichia coli,” Colloids and Surfaces B: Biointerfaces, vol. 74, no. 1, pp. 328–335, 2009.

46) S.Minaeian, A. R. Shahverdi, A. S. Nohi, and H. R. Shahverdi, “Extracellular biosynthesis of silver nanoparticles by some bacteria,” Journal of Sciences (Islamic Azad University), vol. 17, no. 66, pp. 1–4, 2008.
47) S. Veeranna, A. Burhanuddin, S. Khanum, S. L. Narayan, and K. Pratima, “Biosynthesis and antibacterial activity of silver nanoparticles,” Research Journal of Biotechnology, vol. 8, no. 1, pp. 11–17, 2013.