2.1 Metal Nanoparticles

The nano size of material results in specific physicochemical characteristics different than those of the bulk materials or larger particles. This effect is mainly credited to high surface-area-to-volume ratio, which results in increased reactivity; hence, the nano scale materials are more advantageous than their bulk materials. Nanoparticles (NPs) have wide range of applications in areas such as health care, cosmetics, food, feed, environmental health, mechanics, optics, biomedical sciences, chemical industries, electronics, space industries, drug-gene delivery, energy science, optoelectronics, catalysis, single electron transistors, light emitters, nonlinear optical devices and photo-electrochemical applications (Colvin et al., 1994; Wang and Herron, 1991; Schmid, 1992; Hoffman et al., 1992; Hamilton and Baetzold, 1979; Mansur et al., 1995). Size, morphology, stability and properties (chemical and physical) of the metal nanoparticles are influenced strongly by the experimental conditions, the kinetics of interaction of metal ions with reducing agents and adsorption processes of stabilizing agent with metal nanoparticles. Hence, the design of a synthesis method in which the size, morphology, stability and properties are controlled has become a thrust area of research (Sharma et al., 2009). Nanoparticles can be broadly grouped into mainly two types, namely, organic nanoparticles which include carbon nanoparticles (fullerenes) while, some of the inorganic nanoparticles include magnetic nanoparticles noble metal nanoparticles (like gold and silver) and semi-conductor nanoparticles (like titanium oxide and zinc oxide).

There is a growing interest in inorganic nanoparticles i.e. of noble metal nanoparticles (Gold and silver) as they provide superior material properties with functional versatility. The metallic nanoparticles such as copper, titanium, magnesium, zinc, gold and alginate have a strong bactericidal potential owing to their large surface area to volume ratio (Gu et al., 2003). Inorganic nanomaterials have been widely used for cellular delivery due to their versatile features like wide availability, rich functionality, good compatibility and capability of targeted drug delivery and controlled release of drugs (Xu et al., 2006). The nanoparticles of functionalized, biocompatible, inert nanomaterials and noble metals are found to have potential applications in microelectronics (Li et al., 1999), optical devices (Kamat, 2002), catalysis, drug delivery system (Mann and Ozin, 1996), cancer diagnosis and therapy (Sengupta et al., 2005; Gao et al., 2004; Singh et al., 2008). Among the commercially
available nanosized materials, silver nanoparticles are by far the most used nano compounds (Ahmed et al., 2008) because of distinctive properties, such as good conductivity, chemical stability, catalytic, antibacterial activity, antifungal, anti-viral and anti-inflammatory (Sondi and Salopek-Sondi, 2004; Chen and Schluesener, 2008).

Silver nanoparticles (AgNPs) have attracted intensive research interest because of their important applications in antimicrobial, catalysis and surface-enhanced Raman scattering (Li et al., 2006; Chen et al., 2005; Setua et al., 2007). Silver has been used since ancient times for its microbicidal properties. Silver-based medical products ranging from topical ointments and bandages for wound healing to coated stents have been proven to be effective in retarding and preventing bacterial infections (Chen, 2007). AgNPs have applications in wound-healing, eye disease therapy, DNA processing and pharmaceuticals in addition to other relevant mainstream applications: electronics, optics, catalysis and Raman scattering (Tripathi, 2003; Zhang et al., 2005; Aroca et al., 2005; Jiang et al., 2005; Atiyeh et al., 2007). Improvements in the development of novel silver nanoparticles containing products are continuously sought. In particular, there is an increasing interest towards the exploitation of silver nanoparticles technology in the development of bioactive biomaterials, aiming at combining the relevant antibacterial properties of metal with peculiar performance of biomaterial.

2.2 Synthesis of silver nanoparticles

An important area of research in nanobiotechnology is the synthesis of NPs with different chemical compositions, sizes, morphologies and controlled dispersities. Nanotechnology involves the precise manipulation and control of atoms and molecules, the building blocks of all matter, to create novel materials with properties controlled at the nanoscale, billionths of a meter. Broadly, two production approaches are mainly used: the bottom-up and the top down (Horton and Khan, 2006). The bottom-up approach involves physically manipulating small numbers of basic building blocks, either individual atoms or more complex molecules, into structures typically using minute probes. Several physical and chemical methods have been used for synthesizing and stabilizing silver nanoparticles (Klaus-Joerger et al., 2001). At present, this technology is limited to low-volume, high-value applications such as high-
performance chip manufacture. The range of bottom-up techniques and areas of application are growing rapidly (Horton and Khan, 2006).

The top-down approach involves controlling physical processes. The conditions under which materials are grown, force atoms and molecules to move themselves as a unit to a desired location or structure. This approach is already used to create nanoparticles for various industrial applications, some of which are becoming feasible for high volumes and at lower cost. However, the technique is generally limited to production of simpler structures than the bottom-up approach. Here new processes are regularly being discovered or existing processes modified. More importantly, both approaches can work within both biological and nonbiological systems bridging important divides between the biological and nonbiological worlds (Horton and Khan, 2006). Different approaches used for synthesis of silver nanoparticles are shown in figure 2.1.

![Synthesis of Silver Nanoparticles Diagram]

**Fig. 2.1 Different approaches for silver nanoparticles synthesis**

### 2.2.1 Physical synthesis of silver nanoparticles

In physical processes, metal nanoparticles are generally synthesized by evaporation, condensation, arch discharge method and laser ablation, which could be carried out using a tube furnace at atmospheric pressure. The source material within a boat centred at the furnace
is vaporized into a carrier gas. Nanoparticles of various materials, such as Ag, Au, PbS and fullerene have previously been produced using the evaporation/condensation technique (Gurav et al., 1994; Kruis et al., 2000; Magnusson et al., 1999). It was verified that silver NPs could be synthesized via a small ceramic heater with a local heating area (Jung et al., 2006). The small ceramic heater was used to evaporate source materials. The evaporated vapour can cool at a suitable rapid rate, because temperature gradient in the vicinity of heater surface is very steep in comparison with that of a tube furnace. This makes possible the formation of small NPs in high concentration. The particle generation is very stable because temperature of heater surface does not fluctuate with time.

This physical method can be useful as a nanoparticles generator for long-term experiments for inhalation toxicity studies and as a calibration device for nanoparticle measurement equipment (Jung et al., 2006). The results showed that geometric mean diameter, geometric standard deviation and total number concentration of NPs increase with heater surface temperature. Spherical NPs without agglomeration were observed even at high concentration with high heater surface temperature. The nanoparticles synthesized by physical methods are of uniform distribution and do not require any solvent as compared to synthesized by chemical methods. The synthesis of silver NPs using a physical method require a large space for tube furnaces, consumes a large amount of energy to raise the temperature, several kilowatts power consumption and a lot of time to attain thermal stability (Kruis et al., 2000; Magnusson et al., 1999).

Size and shape of nanoparticles can be modified due to interaction with the laser light passing through (Mafune et al., 2003; Kazakevich et al., 2007; Mahfouz et al., 2008). Laser ablation method does not employ use of chemical reagents in solution, so it is preferred over other conventional method used for synthesis of metal colloids (Tsuji et al., 2002b). Silver nanoparticles (20-50 nm) were prepared by laser ablation in water with femto-second laser pulses at 800 nm (Tsuji et al., 2003). Silver NPs could be synthesized by laser ablation of metallic bulk materials in solution (Mafune et al., 2000; Kabashin and Meunier, 2003; Sylvestre et al., 2004; Dolgaev et al., 2002). The ablation efficiency and the characteristics of produced nano-silver particles depend upon many parameters including wavelength of laser impinging the metallic target, duration of laser pulses (in the femto, pico and nanosecond regime), laser fluence, ablation time duration and effective liquid medium,
with or without the presence of surfactants (Kim et al., 2005; Link et al., 2000; Tarasenko et al., 2006; Kawasaki and Nishimura, 2006).

One important advantage of laser ablation technique compared to other methods for production of metal colloids is the absence of chemical reagents in solutions. Therefore, pure and uncontaminated metal colloids for further applications can be prepared by this technique (Tsuji et al., 2002b). Silver nanospheroids (20-50 nm) were prepared by laser ablation in water with femto-second laser pulses at 800 nm (Tsuji et al., 2003). However, the formation efficiency for femto-second pulses was significantly lower than that for nanosecond pulses. The size of colloids prepared by femto second pulses was less dispersed than that of colloids prepared by nanosecond pulses. Furthermore, it was found that the ablation efficiency for femtosecond ablation in water was lower than that in air, while in the case of nanosecond pulses, the ablation efficiency was similar in both water and air.

Tien et al., (2008) used the arc discharge method to fabricate silver NPs suspension in deionized water with no added surfactants. In this synthesis, silver wires (Gredmann, 99.99%, 1 mm in diameter) were submerged in deionized water and used as electrodes. With a silver rod consumption rate of 100 mg/min, yielding metallic silver NPs of 10 nm in size and ionic silver obtained at concentrations of approximately 11 ppm and 19 ppm respectively. Siegel et al., (2012) demonstrated the synthesis of silver NPs by direct metal sputtering into the liquid medium. The method combining physical deposition of metal into propane-1, 2, 3-triol (glycerol) provides an interesting alternative to time-consuming and wet-based chemical synthesis techniques. Silver NPs possess round shape with average diameter of about 3.5 nm with standard deviation 2.4 nm. It was observed that the NPs size distribution and uniform particle dispersion remains unchanged for diluted aqueous solutions up to glycerol-to-water ratio 1:20.

**2.2.2 Chemical synthesis of silver nanoparticles**

The most popular chemical approaches widely used for synthesis of silver nanoparticles includes chemical reduction using a variety of organic and inorganic reducing agents, electrochemical techniques, photochemical reduction (Sharma et al., 2009), pyrolysis and radiolysis. Most of these methods are still in development stage and stability, aggregation of NPs, control of crystal growth, morphology, size and size distribution are the most common
problems associated with these methods. The most common approach for synthesis of silver NPs is chemical reduction by organic and inorganic reducing agents. Chemical synthesis of silver nanoparticles require mainly three components: (1) silver salt (e.g. AgNO$_3$) (2) a reducing agent (e.g. NaBH$_4$) and (3) a stabilizing or capping agent (e.g. polyvinyl alcohol) for controlling the size of nanoparticles and preventing their aggregation (Ledwith et al., 2007). Sodium citrate, ascorbate, sodium borohydride (NaBH$_4$), elemental hydrogen, polyol process, Tollens reagent, N, N-dimethylformamide (DMF) and poly (ethylene glycol)-block copolymers are commonly used reducing agents for reduction of silver ions (Ag$^+$) in aqueous or non-aqueous solutions. These reducing agents reduce Ag$^+$ and lead to the formation of metallic silver (Ag), which is followed by agglomeration and eventually lead to the formation of colloidal silver nanoparticles (Wiley et al., 2005; Evanoff and Chumanov, 2004; Merga et al., 2007).

The most common method for the synthesis of nanosized Ag particles is the reduction of AgNO$_3$ with NaBH$_4$, known as Creighton method. The synthesis procedure routinely yields ~10 nm particles of narrow size distribution (Creighton et al., 1979). Most reported examples of AgNPs obtained by chemical reduction were performed in aqueous media (Burda et al., 2005). However, the synthesis of metal nanoparticles in organic solvents has some advantages such as high yield and narrower size distribution with additional advantage, in some cases the solvent itself can act as reducing agent to obtain AgNPs (Sun, 2013).

Highly monodisperse spherical silver NPs was synthesized using the polyol process and a modified precursor injection technique (Kim et al., 2006). Silver nanoparticles with a controlled size were synthesized by Tollens method in which Ag (NH$_3$)$_2^+$ (as Tollens reagent) was reduced by an aldehyde (Yin et al., 2002). In the modified Tollens procedure, silver ions were reduced by saccharides in the presence of ammonia, leading to the formation of silver nanoparticles films (50-200 nm), silver hydrosols (20-50 nm) and silver NPs of different shapes (Kvitek et al., 2005). Silver NPs with controllable sizes were synthesized by reduction of [Ag (NH$_3$)$_2$]$^+$ with glucose, galactose, maltose and lactose (Panacek et al., 2006). Synthesis of starch-silver NPs was carried out with starch (capping agent) and β-D-glucose (reducing agent) in a gently heated system (Raveendran et al., 2003).

Protective agents are used to stabilize dispersive NPs during the process of metal nanoparticles preparation and to protect the NPs avoiding their agglomeration (Oliveira et al.,
Surfactants with functional groups like thiols, amines, acidic and alcohols stabilize particle growth by interaction with particle surfaces and protect them from sedimentation, agglomeration or losing their surface properties. Polymeric compounds such as poly (vinyl alcohol), poly (vinylpyrrolidone), poly (ethylene glycol), poly (methacrylic acid), and polymethylmethacrylate have been reported to be effective protective agents to stabilize NPs. Most of the chemical methods used for the synthesis of nanoparticles are too expensive and also involve the use of toxic, hazardous chemicals that are responsible for various biological risks. This enhances the growing need to develop environmentally friendly processes through green synthesis and other biological approaches.

*Photochemical method (irradiation)*

Ag NPs can also be successfully synthesized by using a variety of irradiation methods without the use of any reducing agent. Synthesis of silver nanoparticles by pulse electrolysis method was reported in ethylene glycol-water mixture using silver perchlorate (Jacob *et al.*, 2007). Silver nanoparticles of well defined shape and size were synthesized by laser irradiation of an aqueous solution of Ag salt (Sharma *et al.*, 2009) and as well as by X-ray irradiation of metal salt aqueous solutions in the absence of any stabilizer (Remita *et al.*, 2007). UV light irradiation method was employed for nanoparticles synthesis in a natural rubber matrix via photo reduction of film cast from natural rubber latex (NRL) containing silver salt (Abu Bakar *et al.*, 2007). Silver nanoparticles synthesis was also achieved by using microwave irradiation and this method is known to have a faster heating rate than conventional heating through conduction and convection containing (Sharma *et al.*, 2009). Fixed Frequency Microwave (2.45 GHz) as well as Variable Frequency Microwave (VFM) irradiations, both have been used for synthesis of silver nanoparticles. It has been reported that Variable Frequency Microwave (VFM) compared to the Fixed Frequency Microwave, provides more uniform heating and can lead to more homogeneous nucleation (Jiang *et al.*, 2006).

Gamma-irradiation synthesis has been also employed as one of the most promising methods to produce AgNPs (El-Batal *et al.*, 2013). As compared to conventional chemical/photochemical techniques, the radiochemical process can be performed to reduce Ag+ ions at the ambient temperature without using excessive reducing agents or producing
unwanted by-products of the reduction. Moreover, reducing agent can be uniformly distributed in the solution and AgNPs are produced in highly pure and stable form (Krkljess et al., 2007).

2.2.3 Biological synthesis of silver nanoparticles

The problem with chemical and physical methods of nanosilver production is that they are extremely expensive and also involve the use of toxic, hazardous chemicals, which may pose potential environmental and biological risks. It is an intensive need that the silver nanoparticles synthesized have to be handled by humans and must be available at cheaper rates for their effective utilization; thus, there is a need for an environmentally and economically feasible way to synthesize these nanoparticles. The quest for such a method has led to the need for biomimetic production of silver nanoparticles whereby, biological methods are used to synthesize the silver nanoparticles. Nature has devised various processes for the synthesis of small-scale inorganic materials. Biological systems provide a novel idea for the production of nano-materials (Bansal et al., 2011). Biological methods for the synthesis of AgNPs, employing microorganisms (Gaidhani et al., 2013; Singh et al., 2013) and plants (Ghosh et al., 2012; Salunkhe et al., 2014) have gained tremendous significance over physical and chemical procedures due to the use of nontoxic, biocompatible, environment friendly substrates and relatively easier synthesis process at ambient conditions (Shedbalkar et al., 2014; Thakkar et al., 2010). Biological synthesis of nanoparticles involves a natural phenomenon occurring in bacterial, fungal and plant biosystems, thereby generating biocompatible nanomaterials having therapeutic applications (Mohanpuria et al., 2008). Both unicellular and multicellular organisms are known to produce inorganic materials either by intra- or extracellular method (Mann, 1996). Biosynthesis of silver nanoparticles is a bottom-up approach that mostly involves reduction/oxidation reactions. The microbial enzymes or plant phytochemicals with antioxidant or reducing properties act on the respective compounds and give desired nanoparticles.

Several microorganisms from bacteria to fungi have been reported to synthesize inorganic materials either intra- or extracellularly and thus to be potentially utilized as eco-friendly nano-factories (Shankar et al., 2004; Mohanpuria et al., 2008). Jha et al., (2009) has explained the capacity of both bacteria and fungi making an exciting category of
microorganisms having naturally bestowed property of reducing/oxidizing metal ions into metallic/oxide nanoparticles thereby functioning as ‘mini’ nano-factories. Moreover, biomolecules act as natural stabilizers for such nanoparticles, thereby preventing not only aggregation over course of time but also an extra stabilization step as observed in chemical methods (Deepak et al., 2011; Gurunathan et al., 2009; Singh et al., 2013).

Many studies have shown that microorganisms both unicellular and multicellular have the ability to synthesize inorganic materials. The biological synthesis can be considered a bottom-up approach where nanoparticles formation occurs due to the reduction/oxidation of metallic ions via biomolecules such as enzymes, sugars and proteins secreted by the microorganism (Prabhu et al., 2012). Biological systems have a unique ability to control the structure phase and nano structural topography of inorganic crystals (Maribel Guzman et al., 2008). The rate of intracellular particle formation and the size of nanoparticles could be manipulated by controlling parameters such as pH, temperature, substrate concentration and exposure time to substrate (Gericke and Pinches, 2006). The use of microorganisms such as bacteria, yeast, fungi and actinomycetes has been described for the formation of nanoparticles and their applications (Sastry et al., 2003; Mandal et al., 2006). A variety of plants, bacteria, yeast and fungi have the ability to produce various kinds of nanoparticles. Some of the biological entities responsible for silver nanoparticles synthesis are listed in table 2.1

2.2.3.1 Plant mediated synthesis of silver nanoparticles

The use of plants as a production medium of silver nanoparticles has drawn attention because of its rapid, and presence of a variability of metabolites that may aid in reducing silver. The most important point ecofriendly, non-pathogenic, economical protocol and providing a single step technique for the biosynthetic processes. Plant-based synthesis of nanoparticles is faster, safer, works at low temperatures, requires only modest and environmentally safe components as compared to other approaches (Goodsell, 2004). In addition, the use of plants for nanoparticle synthesis offers a wide range of benefits over other biological synthesis methods because it does not require the maintenance of cell cultures and incorporates support for the large-scale synthesis of nanoparticles (Shankar et al., 2004). The advantages of using plants for the synthesis of nanoparticles include their availability, safety in handling is the active agent contained in these parts which makes the reduction and stabilization possible.
### Table 2.1 List of various biological entities in the production of nanoparticles

<table>
<thead>
<tr>
<th>Biological entity</th>
<th>Size</th>
<th>Extracellular/Intracellular</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Azadirachta indica</strong></td>
<td>50-100 nm</td>
<td>Extracellular</td>
<td>Shankar et al., (2004)</td>
</tr>
<tr>
<td><strong>Boerhaavia diffusa</strong></td>
<td>25-30 nm</td>
<td>Extracellular</td>
<td>Nakkala et al., (2014)</td>
</tr>
<tr>
<td><strong>Acalypha indica</strong></td>
<td>30-50 nm</td>
<td>Extracellular</td>
<td>Krishnaraj et al., (2010)</td>
</tr>
<tr>
<td><strong>Emblica Officinalis</strong></td>
<td>10-20 nm</td>
<td>Extracellular</td>
<td>Ankamwar et al., (2005)</td>
</tr>
<tr>
<td><strong>Fusarium oxysporum</strong></td>
<td>5-15 nm</td>
<td>Extracellular</td>
<td>Ahmad et al., (2003)</td>
</tr>
<tr>
<td><strong>Verticillium</strong></td>
<td>25± 12 nm</td>
<td>Intracellular</td>
<td>Mukherjee et al., (2001); Senapati et al., (2004)</td>
</tr>
<tr>
<td><strong>A. flavus</strong></td>
<td>5-30 nm</td>
<td>Extracellular</td>
<td>Naqvi et al., (2013)</td>
</tr>
<tr>
<td>MKY3 Yeast</td>
<td>2-5 nm</td>
<td>Extracellular</td>
<td>Kowshik et al., (2003)</td>
</tr>
<tr>
<td><strong>Cladosporium cladosporioides</strong></td>
<td>10-100 nm</td>
<td>Extracellular</td>
<td>Balaji et al., (2009)</td>
</tr>
<tr>
<td><strong>Fusarium oxysporum</strong></td>
<td>20-50 nm</td>
<td>Extracellular</td>
<td>Duran et al., (2005)</td>
</tr>
<tr>
<td><strong>Bacillus licheniformis</strong></td>
<td>50 nm</td>
<td>Extracellular</td>
<td>Kalishwaralal et al., (2008)</td>
</tr>
<tr>
<td><strong>Bacillus sp.</strong></td>
<td>10-15 nm</td>
<td>Intracellular</td>
<td>Nalenthiran et al., (2009)</td>
</tr>
<tr>
<td><strong>Pseudomonas aeruginosa</strong></td>
<td>30-40 nm</td>
<td>Extracellular</td>
<td>Jeevan et al., (2012)</td>
</tr>
<tr>
<td><strong>Lactobacillus sp.</strong></td>
<td>2-20 nm</td>
<td>Extracellular</td>
<td>Ranganath et al., (2012)</td>
</tr>
<tr>
<td><strong>Ochrobactrum sp.</strong></td>
<td>30-40 nm</td>
<td>Extracellular</td>
<td>Thomas et al., 2014</td>
</tr>
<tr>
<td><strong>Bacillus frigoritolerans</strong></td>
<td>10-30 nm</td>
<td>Extracellular</td>
<td>Singh et al., (2015)</td>
</tr>
</tbody>
</table>
### Table 1: Size and Location of Nanoparticles

<table>
<thead>
<tr>
<th>Biological entity</th>
<th>Size</th>
<th>Extracellular /Intracellular</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Vibrio alginolyticus</em></td>
<td>2-30 nm</td>
<td>Extracellular/Intracellular</td>
<td>Rajesh kumar <em>et al.</em>, (2013)</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>-</td>
<td>Extracellular</td>
<td>Gandhi and Khan (2016); Prabhusaran <em>et al.</em>, (2016)</td>
</tr>
<tr>
<td><em>Bacillus sp.</em></td>
<td>65-70 nm</td>
<td>Intracellular</td>
<td>Malarkodi <em>et al.</em>, (2013)</td>
</tr>
<tr>
<td><em>Lysinibacillus sphaericus</em></td>
<td>5-10 nm</td>
<td>Extracellular</td>
<td>Gou <em>et al.</em>, (2015)</td>
</tr>
<tr>
<td><em>Bacillus sp.</em></td>
<td>77-92 nm</td>
<td>Extracellular</td>
<td>Elbeshehy <em>et al.</em>, (2015)</td>
</tr>
</tbody>
</table>

Ecofriendly plant extracts contain biomolecules, which act as both reducing and capping agents that form stable and shape-controlled nanoparticles. Main compounds which affect reduction and capping of the nanoparticles are biomolecules such as phenolics, terpenoids, polysaccharides, flavones, alkaloids, proteins, enzymes, amino acids and alcoholic compounds are already present in plants. However, quinol and chlorophyll pigments, linalool, methyl chavicol, eugenol, caffeine, theophylline, ascorbic acid and other vitamins have also been reported (Sharma *et al.*, 2009; Kesharwani *et al.*, 2009; Huang *et al.*, 2007; Sathishkumar *et al.*, 2009; Mallikarjuna *et al.*, 2011; Vilchis-Nestor *et al.*, 2008; Kasthuri *et al.*, 2009; Bindhu and Umadevi, 2013). Plant crude extracts contain novel secondary metabolites such as phenolic acid, flavonoids, alkaloids and terpenoids, which are mainly responsible for the reduction of ionic metal into bulk metallic nanoparticles (Aromal and Philip, 2012). Primary and secondary metabolites are constantly involved in redox reactions required to synthesize eco-friendly nanoparticles. Biosynthesis reactions can be modulated to transform the shape and size of nanoparticles by using different metal concentrations and amounts of plant extract in the reaction medium (Chandran *et al.*, 2006; Dubey *et al.*, 2010).

A large number of plants capable of silver nanoparticles synthesis have been reported. The first report of using living plant system for the synthesis of metallic nanoparticles by using *alfalfa* sprouts was published in 2003 (Gardea-Torresdey *et al.*, 2003). *Alfalfa* roots
have the capability of absorbing Ag from agar medium and transferring them into the shoots of the plant in the same oxidation state. In the shoots, these Ag atoms arranged themselves to form nanoparticles by joining themselves and forming larger arrangements. Green synthesis of AgNPs by using plant extracts containing phytochemical agents has attracted considerable interest. Most of the plant parts like leaves, roots, latex, bark, stem and seeds are being used for nanoparticles synthesis (Kharissova et al., 2013).

Extracellular nanoparticles synthesis, which utilizes extracts from individual, leaves rather than entire plants may prove to be more inexpensive due to easier downstream processing. The extracellular silver nanoparticles synthesis by aqueous leaf extract is quick, simple, economical process and the most commonly used method. This environment friendly approach is more biocompatible and cost-efficient and includes the capability of supporting larger synthesis (Arunachalam et al., 2013; Mittal et al., 2015). A lot of studies have been reported on the synthesis of silver nanoparticles by leaf extract. Variety of plants has been used as a source of leaf extract for the synthesis of silver nanoparticles. *Carica papaya* (Jain et al., 2009); *Morinda tinctoria* (Vanaja and Annadurai, 2012); geranium leaf (Shankar et al., 2003); *Azadirachta indica* (Shankar et al., 2004); *Camellia sinensis* (green tea); henna leaves (Kasthuri et al., 2009); *Nelumbo nucifera* (SanthoshKumar et al., 2011); *Acalypha indica* (Krishnaraj et al., 2010); *Sorbus aucuparia* (Dubey et al., 2010); *Coriandrum sativum* (Sathyavathi et al., 2010); *Argemone Mexicana* (Singh et al., 2010); *Allium cepa* (Saxena et al., 2010); *Ocimum* leaf extract (Mallikarjun et al., 2011) are some of the reported plants that has been explored for the synthesis of silver nanoparticles.

Farooqui et al., (2010) investigated the synthesis of silver nanoparticles employing *Clerodendrum Inerme* leaf extract and synthesized nanoparticles from three different leaf conditions- fresh leaves, sun-dried leaves and hot-air oven dried leaves. They observed that silver nanoparticles synthesized using fresh leaves were smallest in size. Photo-luminescent silver nanoparticles of varying sizes were synthesized by using *Parthenium* leaf extract at a temperature of 100 °C as well as at room temperature (Sarkar et al., 2010). They also observed that particles size varies with the reaction temperature and reaction time. Saikia et al., (2015) described the green synthesis of silver nanoparticles using *Asiatic Pennywort* and *Bryophyllum* leaf extracts as reducing and capping agents. Vanaja and Annadurai, (2012) synthesized silver nanoparticles by using *Morinda tinctoria* leaf extract and also reported
photocatalytic activity of silver nanoparticles. Although, currently there are no reports on very large quantity of synthesis of silver nanoparticles, some prototypes have been designed in continuous flow reactors similar to chemical methods. The use of plant product from *Cinnamomum camphora* leaf has been used to synthesize silver nanoparticles in a continuous flow tubular microreactor (Huang et al., 2008). Velusamy et al., (2015) described the synthesis of monodispersed and spherical silver nanoparticles using *Azadirachta indica* and colloidal silver nanoparticles synthesis has been reported by using *Opuntia ficus-indica* (Gade et al., 2010). Philip, (2009) reported mushroom mediated green chemistry approach for the synthesis of gold, silver and gold-silver nanoparticles. *Acorus calamus* was also used for the synthesis of silver nanoparticles to evaluate its antioxidant, antibacterial as well as anticancer effects (Nakkala et al., 2014). *Boerhaavia diffusa* plant extract was utilized as a reducing agent for green synthesis of 25 nm spherical silver nanoparticles (Nakkala et al., 2014).

Shankar et al., (2003) reported that *Geranium* leaf takes around nine hours in completing 90 % reaction compared to the 24 to 124 hours necessary for other reactions reported earlier. Singhal et al., (2011) reported the biosynthesis of silver nanoparticles using *Ocimum sanctum* leaf extract within 8 min of reaction time. The cubic shape silver nanoparticles were synthesized by using *Carica papaya* (Jain et al., 2009) and spherical shaped silver nanoparticles using *Paederia foetida L.* leaf extract as a reducing as well as stabilizing agent (Mollick et al., 2012). Dwivedi and Gopal, (2011) investigated biosynthesis of silver nanoparticles by utilizing leaf extract of an obnoxious weed *Chenopodium album* and reported synthesis of spherical shape silver nanoparticles.

Recently, other parts of the plants have also been explored for the synthesis of silver nanoparticles. Green synthesis of silver nanoparticles using fruit extract of *Embllica officinalis* (Ramesh et al., 2015) and *Cucumis sativus* (Roy et al., 2015) has been reported. They also analyzed the photo catalytic and antibacterial activity of synthesized nanoparticles. The spherical shaped silver nanoparticles were also synthesized by using dried fruit body extract of the plant *Tribulus terrestris* (Gopinatha et al., 2012). Banerjee and Narendhirakannan, (2011) synthesized silver nanoparticles by using *Syzygium cumini* seed extract as reducing agent and also reported that green synthesized silver nanoparticles using *Syzygium cumini* seed have greater antioxidant activity as compared to seed extract of *Syzygium cumini.*
Silver nanoparticles were also synthesized by using *Citrus limon* extract and observed that the alkaline environment is favourable for the biosynthesis of silver nanoparticles using *Citrus limon* (Mohapatra *et al*., 2015). Satyavani *et al*., (2011) reported biosynthesis of silver nanoparticles using stem derived callus extracts of bitter apple (*Citrullus colocynthis* L.) and analyzed the antimicrobial activity of the prepared nanoparticles. Sathish kumar *et al*., (2009) investigated biosynthesis of nanoparticles utilizing the bark extract and powder of *Cinnamomum zeylanicum*. They reported that pH played a major role in size control of the particles and also bark extract are more effective silver nanoparticles producer than the powder. Stem extract of *Breynia rhamnoides* was reported as reducing agent for Au and AgNPs (Gangula *et al*., 2011). A green rapid biogenic synthesis of silver nanoparticles (AgNPs) by using *Terminalia chebula* (*T. chebula*) aqueous extract was demonstrated (Acharya *et al*., 2009). Sinha and Paul, (2015) synthesized silver nanoparticles by using aqueous extract of *Andrographis paniculata*. Krishnaraj *et al*., (2010) studied the rapid synthesis of silver nanoparticles using aqueous leaves extract of *A. indica* and evaluated its antibacterial activity.

Flavonone and terpenoid components of leaf broth are being predicted to stabilize the formation of nanoparticles in comparison to high molecular weight proteins of fungal biomass (Shankar *et al*., 2004). Flavanoids and polyphenols present in *R. damascena* extract were responsible for accelerated reduction and capping (Ghoreishin *et al*., 2011). The polyol components and water soluble heterocyclic components are mainly responsible for reduction of silver ions (Ag⁺) as well as stabilization of nanoparticles. Information regarding the activity of reductase in nanoparticles fabrication is well illustrated (Anil Kumar *et al*., 2007). Colloidal silver nanoparticles were synthesized by an easy green method using thermal treatment of aqueous solutions of silver nitrate and natural rubber latex extracted from *Hevea brasiliensis*. The silver nanoparticles presented diameter ranging from 2 nm to 10 nm and had spherical shape with face centred cubic (fcc) crystalline structure (Ramyal and Subapriya, 2012). In a recent report, these nanoparticles have been synthesized on irradiation using an aqueous mixture of *Ficus carica* leaf extract (Ulug *et al*., 2015). Now, scientists are trying to combine different options together. It was reported that the symbiotic biological systems such as *Geranium* leaf combined with endophytic fungus *Colletotrichum* sp. can synergize the outcome of the reaction. In fact, plants contain biomolecules which are able to stabilize unstable particles whereas fungi secrete enzymes for reduction (Shankar *et al*., 2003).
The nontoxic phytochemicals including flavonoids and phenols have unique chemical power to reduce and also effectively wrap nanoparticles, thus preventing their agglomeration. In addition, the synthesis of nanoparticles using plants offers several advantages such as utilization of safer solvents, decreased use of dangerous reagents and milder response conditions. In comparison to bacteria and fungi, green synthesis by using plants appears to be faster for synthesis of silver nanoparticles. Phenolic compounds possess hydroxyl and carboxyl groups, which are able to bind to metals (Ahmad et al., 2010). The synthesis by using plant extracts is less time-consuming but produces polydispersed AgNPs due to involvement of multiple components like flavonoids, terpenoids and polyphenols in the reduction of silver ions (Ghosh et al., 2012; Salunkhe et al., 2014). In addition, physical requirements for their synthesis including pressure, energy, temperature and constituent materials are trivial. The size distribution of nanoparticles in general is an important issue as nanoparticles exhibit different physical and chemical properties depending on their shape and size. Synthesis methods that generate uniformly sized and shaped nanoparticles are therefore being pursued.

2.2.3.2 Fungal mediated synthesis of silver nanoparticles

Fungi have been of greater interest in biological production of metallic nanoparticles due to their tolerance ability, metal bioaccumulation ability, high binding capacity and intracellular uptake (Murali et al., 2003; Sastry et al., 2003). Fungi can produce larger amounts of nanoparticles because they can secrete larger amounts of proteins, which are directly related to higher productivity of nanoparticles (Mohanpuria et al., 2008). A number of fungal species grow fast and therefore culturing and keeping them in the laboratory are very simple (Castro-Longoria et al., 2012). The easiness of fungi scale-up is another advantage of utilizing them in nanoparticles synthesis (e.g. utilizing a thin solid substrate fermentation technique). Fungi mediated synthesis of metal nanoparticles is ecofriendly and economical.

Fungi are able to synthesize metal nanoparticles by reducing enzyme intracellularly or extracellularly. Intracellular synthesis of silver nanoparticles has been reported by using Verticillium sp. (Mukherjee et al., 2001) and Coriolis versicolor (Sanghi and Verma, 2009). The Verticillium sp. fungal biomass when exposed to aqueous AgNO₃ solution resulted in the
intracellular formation of silver nanoparticles, while *Fusarium oxysporum* biomass resulted in the extracellular silver nanoparticles (Senapati *et al*., 2004). The first fungus-mediated synthesis of the silver nanoparticles synthesis was carried out utilizing *Verticillium* and Ag NPs were formed below the surface of the fungal cells (Mukherjee *et al*., 2001). Silver nanoparticles were accumulated on the surface of cell wall of *Aspergillus flavus*, when challenged with silver nitrate solution (Vighneshwaran *et al*., 2007). In most cases, the synthesis of extracellular NPs is published, though biomass has usually exposure to metallic ion solutions (Ahmad *et al*., 2002).

Since fungi are very effective secretors of extracellular enzymes, therefore achieving vast production of enzymes is feasible (Castro-Longoria *et al*., 2012). Extracellular synthesis of nanoparticles using cell filtrate could be beneficial over intracellular synthesis, the fungi being extremely good microbes for extracellular process and also environmental friendly (Husseiny *et al*., 2007). Intracellularly synthesized nanoparticles require additional downstream processing steps such as ultrasound treatment, reaction with suitable detergents to release the nanoparticles from cells during their purification (Kalimuthu *et al*., 2008). Extracellular synthesis is advantageous as the synthesized nanoparticles will not bind to the biomass (Balaji *et al*., 2009; Duran *et al*., 2005) and it is therefore possible to extend this approach for the biosynthesis of nanomaterials over a range of chemical compositions such as oxides, nitrides and so forth.

First report of extracellular biosynthesis of silver nanoparticles was documented using *Fusarium oxysporum* and the synthesized particles were in size range between 5-15 nm (Ahmad *et al*., 2003). Extracellular synthesis of silver nanoparticles using various fungal species such as *Aspergillus fumigatus* (Bhainsa and D’Souza, 2006); *Phaenerochaete chrysosporium* (Vighneshwaran *et al*., 2006); *Fusarium solani* (Ingle *et al*., 2009); *F. acuminatum* (Ingle *et al*., 2008); *T. asperellum* (Mukherjee *et al*., 2008); *P. Fellutatum* (Kathiresan *et al*., 2009); *C. cladosporoides* (Balaji *et al*., 2009); *Fusarium acuminatum* (Ingle *et al*., 2008) and *Penicillium fellutanum* (Kathiresan *et al*., 2009) have been reported. The extracellular synthesis of stable AgNPs using the fungus *Penicillium brevicompactum* WA 2315 was also reported (Shaligram *et al*., 2009). The nanoparticles were produced by incubating silver ions with the fungus filtrate for 72 hr. Fungus like white rot fungus is
nonpathogenic and contributes to the mass production of Ag NPs (Vigneshwaran et al., 2006).

Abd El-Aziz et al., (2012) described the extracellular biosynthesis and characterization of silver nanoparticles using Aspergillus niger strain KSU-12 isolated from soil. This is reported that secreted enzymes are responsible in the reduction process (Ahmad et al., 2003). Sadowski et al., (2008) have reported synthesis of silver nanoparticles using two Aspergillus niger strains. Silver nanoparticles (5-25 nm) synthesized using Aspergillus fumigatus were mainly spherical in shape while some others having occasionally triangular shapes (Bhainsa and D’Souza, 2006). Pyramidal and hexagonal shaped silver particles were synthesized by the white rot fungus Phanerochaete chrysosporium and reported that silver nanoparticles accumulate on the surface of its cell wall (Vigneshwaran et al., 2006). Polydisperse and spherical shaped silver nanoparticles were produced extracellularly by filtrate of Cladosporium cladosporioides biomass (Balaji et al., 2009). The bioreduction of the silver ions occurred on the surface of the cells and proteins might have critical role in the formation and stabilization of the synthesized NPs. Penicillium genus has been utilized for green synthesis of silver NPs (Sadowski et al., 2008). A marine fungus, Penicillium fellutanum (Kathiresan et al., 2009) and Penicillium sp. J3 (Maliszewska et al., 2009) were able to synthesize silver nanoparticles extracellularly. Monali et al., (2009) has reported the synthesis of AgNP using Alternaria alternata (Fr.) Keissler (MTCC-6572).

Korbekandi et al., (2013) also demonstrated the bioreductive synthesis of silver NPs using F. oxysporum and reported that silver NPs were of spherical shape, single (25-50 nm) or in aggregates (100 nm) and attached to the surface of biomass. In contrast with the previous studies, it is claimed that the nanoparticles production in F. oxysporum is intracellular by engulfing the NPs in vesicles, transporting and excreting of them through exocytosis outside the cells (Korbekandi et al., 2013). Ingle et al., (2008) described the potential ability of Fusarium acuminatum Ell. and Ev. (USM-3793) cell extracts in biosynthesis of silver NPs. The NPs produced within 15-20 min and were spherical with a broad size distribution in the range of 5-40 nm with average diameter of 13 nm. The stable silver NPs could be achieved by using Aspergillus flavus (Vigneshwaran et al., 2007). These NPs were found to be stable in water for more than 3 months with no significant aggregation.
because of surface binding of stabilizing materials secreted by the fungus (Vigneshwaran et al., 2007). A nitrate-dependent reductase enzyme might act as the reducing agent.

Sanghi and Verma, (2009) investigated the ability of Coriolus versicolor in formation of monodisperse spherical silver NPs under alkaline conditions. It was indicated that alkaline conditions might be involved in bioreduction of silver ions, water hydrolysis and interaction with protein functionalities. This was reported that glucose was necessary for the reduction of silver NPs and S-H of the protein played an important role. It was suggested that proteins, organic acids and polysaccharides released by C. cladosporioides were responsible for formation of spherical crystalline silver NPs. Another important factor considered for selection of synthesis method is the reaction rate. First report of rapid synthesis was using Aspergillus fumigatus that allowed obtaining monodispersed Ag NPs within 10 minutes (Bhainsa and D’Souza, 2006). These investigations confirmed the suitability and the potential of using fungi for mass production of nanoparticles. Recently, a rapid method for synthesizing small (1-7 nm) monodisperse AgNPs has been described by electrochemically active biofilm (EAB) using sodium acetate as an electron donor (Kalathil et al., 2011).

Despite these impressive results, the use of fungi for AgNPs synthesis is still limited and the detailed mechanism still not well elucidated. Previous reports have shown that a large number of active substances secreted by fungi played important roles as reducing agents and capping agents in the reaction (Bharde et al., 2006). The mechanism of nanoparticles production using fungi is different. Fungi secrete large amounts of enzymes which are used to reduce silver ions that induce the formation of metal nanoparticles (Mandal et al., 2006). The main hypothesis about mechanism of fungi-based nanoparticles synthesis is that, the NPs are formed on the surface of mycelia, not in the solution. It was suggested that in the first step Ag⁺ ions are adsorbed on the surface of fungal cells due to electrostatic interaction between negatively charged carboxylate groups in enzymes present in the cell wall of mycelia and positively charged Ag ions. Finally, the silver ions are then reduced by the enzymes present in cell wall, leading to the formation of silver nuclei (Mukherjee et al., 2001). The extracellular enzymes like naphthoquinones and anthraquinones are said to facilitate the reduction. However, the exact mechanism of the formation of silver nanoparticles is yet to be elucidated.
2.2.3.3 Bacterial synthesis of silver nanoparticles

Plant mediated synthesis is affected by geographical and seasonal variations (Singh et al., 2013). But, such types of variations do not affect bacterial synthesis of nanoparticles, although regular maintenance of culture and sterile conditions for nanoparticles synthesis is required (Salunkhe et al., 2014). The use of bacterial strain in the bio-manufacturing process has advantage that ease of handling than the fungal sources (Morones et al., 2005). One major advantage of using bacteria as nanoparticles synthesizers is that they can be easily modified using genetic engineering techniques for over expression of specific enzymes, apart from the ease of handling (Kalimuthu et al., 2008; Mukherjee et al., 2008; Parikh et al., 2008). Mehrbod et al., (2009) stated that biological methods based on microbes such as bacteria are able to absorb/accumulate metals and can be used in the reduction of metal ions and thus for the synthesis of nanoparticles. The first evidence of synthesizing silver nanoparticles was established in 1984 using the microorganism Pseudomonas stutzeri AG259, a bacterial strain that was originally isolated from silver mine (Haefeli et al., 1984; Zhang et al., 2005). Some bacteria are able to survive in an extreme silver-rich environment, which might be the possible explanation for accumulation of nanosilver (Klaus et al., 1999). The accumulation of AgNPs inside the cells of Pseudomonas stutzeri AG259 was reported first time in 1999 (Klaus et al., 1999). The synthesis of magnetic nanoparticles has been reported by using magnetotactic bacteria (Roh et al., 2001). In some studies, silver-resistant bacterial strains were employed for nanoparticles synthesis (Parikh et al., 2008; Prakash et al., 2011). Pugazhenthiran et al., (2009) have reported that the silver resistant Bacillus spp. when cultured with silver nitrate synthesize silver nanoparticles in the periplasmic space of cell. Sintubin et al., (2009) has reported that both gram-positive and gram-negative bacteria have been used to synthesize silver nanoparticles, i.e. Lactobacillus sp., Pediococcus pentosaceus, Enterococcus faecium and Lactococcus garvieae.

Several bacteria (gram-negative and gram-positive) have been screened for the synthesis of silver nanoparticles. Microbial syntheses of nanoparticles in both intracellular and extracellular form are observed efficiently from variety of bacterial strains. The variety of bacterial strains includes Bacillus licheniformis (Pugazhenthiran et al., 2009), Bacillus subtilis (Saifuddin et al., 2009), Staphylococcus aureus (Nanda and Saravanan, 2009), Brevibacterium casei (Kalishwaralal et al., 2010), E. coli DH5α (Du et al., 2007), Aeromonas
sp. SH10 (Mouxing et al., 2006), Actinobacter sp. (Bharde et al., 2005), Acinetobacter calcoaceticus (Singh et al., 2013; Gaidhani et al., 2013; Wang et al., 2012), Bordetella sp. (Thomas et al., 2012), Enterobacter aerogenes (Karthik and Radha, 2012), Escherichia coli (Gurunathan et al., 2009), Geobacter sulfurreducens (Law et al., 2008), Gluconobacter roseus (Krishnaraj and Berchmans, 2013), Idiomarina sp. (Seshadri et al., 2012), Klebsiella pneumoniae (Duraisamy and Yang, 2013), Morganella sp. (Parikh et al., 2011; Ramanathan et al., 2011), Pseudomonas aeruginosa (Srivastava and Constanti, 2012; Kumar and Mamidyala, 2011; Otaqsara, 2011), Pseudomonas stutzeri AG259 (Klaus et al., 1999), Rhodobacter sphaeroides (Bai et al., 2011), Rhodopseudomonas palustris (Chun-Jing and Hong-Juan, 2010), Salmonella typhimurium (Ghorbani, 2013), Shewanella oneidensis MR-1 (Debabov et al., 2013), Stenotrophomonas maltophilia (Oves et al., 2013), Vibrio alginolyticus (Rajeshkumar et al., 2013), Xanthomonas oryzae (Narayanan and Sakhivel, 2013).

Although silver nitrate salt is mostly used for AgNPs production in bacteria, other silver-containing nanoparticles like silver sulfide (Ag$_2$S) and silver oxide (Ag$_2$O) nanoparticles have also been reported (Debabov et al., 2013; Dhoondia and Chakraborty, 2012). These nanoparticles of various shapes such as spherical, disk, cuboidal, hexagonal, triangular, etc., have been synthesized using cells, culture supernatant or aqueous cell-free extract (Klaus et al., 1999; Oves et al., 2013; Singh et al., 2013; Srivastava and Constanti, 2012). The recovery is usually done by high-speed centrifugation (10,000-20,000 rpm) of the solution containing nanoparticles. These nanoparticles are collected as a pellet, which can be redispersed in the desired solvent. Minaeian et al., (2008) used fresh culture of the strains (Bacillus subtilis, Lactobacillus acidophilus, Klebsiella pneumoniae, Escherichia coli, Enterobacter cloacae, Staphylococcus aureus and Candida albicans) for nanoparticle synthesis. The biosynthesized silver nanoparticles have the size range of 50-100 nm.

### 2.2.3.3.1 Extracellular synthesis of nanoparticles

Extracellular synthesis of nanoparticles occurs outside the bacterial cell. Extracellular methods of synthesis are advantageous over intracellular synthesis due to the ease of recovery of nanoparticles from the solution. Extracellular synthesis of silver nanoparticles can occur by either by cell biomass or by cell free culture supernatant.
Synthesis using biomass

Some bacteria produce extracellular AgNPs when exposed to silver salts. There are two possible routes for the synthesis of silver nanoparticles using biomass: (i) bacteria may release some biomolecules into the external medium for the reduction of silver ions (Ag\(^+\)) to AgNPs (ii) silver ions may be reduced into nanoparticles inside the cell and secreted outside. Synthesis of silver nanoparticles has been observed by both live (Mahdieh et al., 2012) as well as dried bacterial biomass (Zhang et al., 2005). Silver nanoparticles synthesized by this method may remain attached to the bacterial cell wall (Parikh et al., 2011) and may be recovered by mild sonication for recovery. Parikh et al., (2011) suggested the genus dependent extracellular synthesis of AgNPs, where production of crystalline AgNPs was observed in the genus Morganella and closely related members of the Enterobacteriaceae family (Escherichia coli, Salmonella typhimurium, Klebsiella pneumoniae and Serratia marcescens) were unable to synthesize AgNPs. However, later AgNP synthesis using E. coli biomass was also reported (Muthukumarasamy et al., 2012; Zaki et al., 2011). Monodispersed silver NPs were successfully prepared with reduction of \([\text{Ag (NH}_3\text{)}_2]^+\) by Aeromonas sp. SH10 and Corynebacterium sp. SH09 in the presence of small quantities of NaOH (Fu et al., 2006). It was speculated that \([\text{Ag (NH}_3\text{)}_2]^+\) first reacted with -OH to form Ag\(_2\)O, which was then metabolized independently and reduced to silver NPs by the biomass.

Synthesis using culture supernatant

The supernatant is comprised of nutrient media components and organic molecules secreted by the bacteria during growth, it may prove as an ideal source for reduction of silver ions to AgNPs. For the synthesis of silver nanoparticles by biomass the optimum concentration that has been patented is 1 mM (Ahmad et al., 2003; Kalimuthu et al., 2008). But higher concentrations of silver ions had been reported for the synthesis of silver nanoparticles by the crude culture supernatant (Gurunathan et al., 2009). Kalishwaralal et al., (2008) reported the extracellular synthesis of highly stable silver nanoparticles (40 nm) by reduction of aqueous Ag\(^+\) ions with the culture supernatant of Bacillus licheniformis in 24 hr. Moreover, well-dispersed silver nanocrystals (50 nm) were synthesized using B. licheniformis (Kalimuthu et al., 2008). In case of Bacillus flexus, spherical and triangular shaped silver NPs (12-65 nm)
were successfully biosynthesized. The NPs were stable in aqueous solution for five-month period stored at room temperature in the dark condition.

Jeevan et al., (2012) reported the extracellular biosynthesis of silver nanoparticles by culture supernatant of *Pseudomonas aeruginosa*. Sunkar and Nachiyar, (2012) reported the biogenesis of antibacterial silver nanoparticles using the endophytic bacterium *Bacillus cereus* isolated from *Garcinia xanthochymus*. Extracellular synthesis of spherical silver nanoparticles of homogeneous composition by utilizing the γ-proteobacterium *S. oneidensis* MR-1, was reported (Suresh et al., 2010). Ranganath et al., (2012) reported extracellular synthesis of silver nanoparticles using probiotic bacteria *Lactobacillus* sp. in size range of 2-20 nm. Ranganathan and Ranganathan, (2012) revealed synthesis of AgNP using probiotic bacteria *B. Linens* and tested for antimicrobial activity against multi drug resistant bacterial strains. AgNPs were synthesized by using culture supernatant of *Exiguobacterium* sp. KNU1 (Dhawal and Dae, 2013) and probiotic bacteria *B. Linens* (Ranganathan and Ramachandran, 2012). A bacterial isolate *Ochrobactrum* sp. from marine water collected from Calicut beach was used for the synthesis of silver nanoparticles (Thomas et al., 2014).

Cell-free culture supernatants of five psychrophilic bacteria *Phaeocystis antarctica*, *Pseudomonas proteolytica*, *Pseudomonas meridiana*, *Arthrobacter kerguelensis*, *Arthrobacter gangotriensis* and two mesophilic bacteria *Bacillus indicus* and *Bacillus cecembensis* have been used to biosynthesize silver NPs (6-13 nm). These NPs were stable for 8 months in the dark. The synthesis and stability of silver NPs appeared to depend on the temperature, pH, or the species of bacteria from which the supernatant was used. It was observed that the *A. kerguelensis* supernatant could not produce silver NPs at the temperature where *P. Antarctica* could synthesize silver NPs. Therefore, this study provided important evidence that the factors in the cell-free culture supernatants which facilitated the synthesis of silver NPs varied from bacterial species to species (Shivaji et al., 2011). Lengke et al., (2007) has also reported the formation of extracellular silver nanoparticles by photoautotrophic cyanobacterium *Plectonema boryanum*.

A novel approach, combination of culture supernatant of *B. subtilis* and microwave irradiation in water has been employed for the synthesis of silver nanoparticles. The use of this combination approach leads to biosynthesis of mono dispersed silver nanoparticles (5-50 nm) and the use of microwave radiation increase the rate of reaction (Saifuddin et al., 2009).
Microwave radiations provide uniform heating around the nanoparticles and could assist formation of nanoparticles without aggregation. The major disadvantage of this method is AgNPs synthesized from culture supernatant may embed in the organic matrix of media components, which could hinder their colloidal dispersion, characterization, recovery and hence, potential application.

Biosynthesis of silver nanoparticles using microorganisms is rather slow. However, finding microorganisms to synthesize Ag nanoparticles rapidly is an important aspect. The culture supernatants of different bacteria from Enterobacteriaceae are potential candidates for the rapid synthesis of silver nanoparticles. Rapid biosynthesis of metallic silver nanoparticles using the culture supernatants of Klebsiella pneumonia, Escherichia coli and Enterobacter cloacae (Enterobacteriaceae) has been reported (Shahverdi et al., 2007). The synthesis process was quite fast and silver nanoparticles were formed within 5 min of the silver ion coming into contact with the cell filtrate. It was reported that nitroreductase enzymes might be responsible for bioreduction of silver ions. Huang et al., (1996) reported the synthesis of silver nanoparticles (AgNPs) by irradiation of aqueous AgNO₃ solution with UV light of wavelength in the presence of poly (N-vinylpyrrolidone) [PVP] as a stabilizer.

In another study, effect of different visible-light irradiation and liquid mixing process on the formation of silver nanoparticles from silver nitrate using the culture supernatant of Klebsiella pneumoniae was investigated. It was also reported that visible-light emission could significantly increase synthesis of silver nanoparticles (1-6 nm) by culture supernatants of K. pneumoniae and evenly dispersed silver nanoparticles of uniform size and shape in the range of 1-6 nm were synthesized (Mokhtari et al., 2009). Wei et al., (2012) reported the synthesis of circular and triangular crystalline silver NPs (14.6 nm) by solar irradiation of cell-free extracts of Bacillus amyloliquefaciens and silver nitrate (AgNO₃). Light intensity, extract concentration and NaCl addition affected the synthesis of silver NPs. Another report focused on the extracellular synthesis of metallic bio-nanoparticles of silver using a reduction of aqueous Ag⁺ ion with the culture supernatants of Staphylococcus aureus under bright conditions for 5 minutes (Nanda and Saravanan, 2009). Recently, bacterial cell supernatant of Pseudomonas aeruginosa was used for the reduction of gold ions resulting in extracellular biosynthesis of gold nanoparticles (Husseiny et al., 2007). Usha et al., (2010) demonstrated the synthesis of metal oxide nanoparticles by a Streptomyces sp.
Synthesis using cell-free extract

Cell-free extract (CFE) of bacteria for extracellular AgNPs synthesis has been adopted for the synthesis of silver nanoparticles due to the problems associated with the use of biomass and cell-free supernatant (Singh et al., 2013). Bacterial biomass is resuspended in sterile distilled water for a specific time ranging from 1 to 3 days for CFE preparation. CFE, collected after centrifugation and/or membrane filtration is further challenged with silver salt for AgNPs production after optimal incubation (Singh et al., 2013). Solar/microwave irradiation and combination of CFE has been employed to increase the rate of synthesis of AgNPs (Boopathi et al., 2012; Wei et al., 2012). This method ensures complete removal of bacterial biomass and media components through repeated washings and enables the synthesis of nanoparticles only through organic biomolecules released by cells in aqueous solution due to starvation conditions or by autolysis. Major advantage of this method is that no downstream processing is required for recovery of nanoparticles.

2.2.3.3.2 Intracellular synthesis

For intracellular synthesis, bacterial cells are added to the culture medium containing silver salt and incubated at appropriate conditions for growth. To avoid the hindrance by media components, grown cells may be resuspended in sterile distilled water before challenging with silver salt. Intracellular method of synthesis requires additional steps to recover the accumulated nanoparticles from cells and therefore, is less preferred. Ultrasonication of bacterial cells is the most common technique to recover AgNPs (Kalishwaralal et al., 2010a, b). Besides this, heat treatment like autoclaving and the use of detergents and salts can also be employed to lyse the cells (Fesharaki et al., 2010; Sneha and Yun, 2013).

Periplasmic deposition of triangular and hexagonal nanoparticles was reported in P. stutzeri (Klaus et al., 1999). The size of intracellular AgNPs depends on the culture medium used to grow the cells (Srivastava et al., 2013). Reduction of metal ions by bacterial cells to its nano form is one of the survival strategies to render the toxic metal ions nontoxic (Klaus et al., 1999). Acinetobacter has been shown to exhibit biocompatibility with its own metal nanoparticles; however, exposure to the corresponding metal salt decreases their cell count (Wadhwani et al., 2014). This could be because of the bacterial self biomolecules that cover the surface of nanoparticles, which make them nontoxic to their host. P. stutzeri AG259
detoxified silver through its precipitation in the periplasmic space and its reduction to elemental silver with a variety of crystal typologies, such as hexagons and equilateral triangles, as well as three different types of particles: elemental crystalline silver, monoclinic silver sulfide acanthite (\( \text{Ag}_2\text{S} \)) and an undetermined structure (Klaus et al., 1999).

The periplasmic space limited the thickness of crystals, but not their width, which could be rather large (100-200 nm) (Klaus-Joerger et al., 2001). Larger particles were formed when \( P. \text{stutzeri AG259} \) when challenged with high concentrations of silver ions during culturing, resulted in intracellular formation of silver nanoparticles ranging in size from a few nm to 200 nm (Klaus-Joerger et al., 2001; Klaus et al., 1999). Silver nanocrystals of different compositions were successfully synthesized by \( \text{Pseudomonas stutzeri AG259} \) (Klaus et al., 1999). Kalishwaralal et al., (2008) reported the synthesis of well dispersed silver nanoparticles (50 nm) by using the bacterium \( \text{Bacillus licheniformis} \). Nalenthiran et al., (2009) investigated biosynthesis of silver nanoparticles by \( \text{Bacillus sp.} \) and reported that most of the silver deposited as seed like particles between the outer membrane and the plasma membrane.

Monodispersed and stable silver nanoparticles were also successfully synthesized with bioreduction of \([\text{Ag} (\text{NH}_3)_2]^+\) using \( \text{Aeromonas sp. SH10} \) and \( \text{Corynebacterium sp. SH09} \) (Mouxing et al., 2006). It was speculated that \([\text{Ag} (\text{NH}_3)_2]^+\) first reacted with -OH to form \( \text{Ag}_2\text{O} \), which was then metabolized independently and reduced to silver nanoparticles by the biomass. \( \text{Lactobacillus} \) strains, when exposed to silver ions resulted in biosynthesis of nanoparticles within the bacterial cells (Nair and Pradeep, 2002; Korbekandi and Iravani, 2012). Nair and Pradeep, (2002) reported that common \( \text{Lactobacillus} \) strains found in buttermilk assisted the growth of microscopic gold, silver and gold-silver alloy crystals of well-defined morphology. Korbekandi and Iravani, (2012) demonstrated the green biosynthesis of silver NPs using \( \text{Lactobacillus casei} \) subsp. at room temperature. The biosynthesized NPs were almost spherical, single (25-50 nm) or in aggregates (100 nm) attached to the surface of biomass or were inside and outside of the cells. The bioreduction of metal ions and stabilization of the silver NPs were confirmed to occur by an enzymatic process. Silver NPs were formed on the surface of cytoplasmic cell membrane, inside the cytoplasm and outside the cells, possibly due to the bioreduction of metal ions by enzymes present on the cytoplasmic membrane and within the cytoplasm. The nucleation of silver
nanoparticles occurred on the cell surface through sugars and enzymes in the cell wall, and then the metal nuclei were transported into the cell where they aggregated and grew to larger-sized particles.

Some bacteria have the ability to produce both extra and intracellular AgNPs simultaneously. These include *Lactobacillus* sp. (Nair and Pradeep, 2002), *Aeromonas* sp. SH10 (Mouxing *et al.*, 2006), *Vibrio alginolyticus* (Rajesh *kumar et al.*, 2013) and cyanobacteria such as *Plectonema boryanum* UTEX 485 (Lengke *et al.*, 2007). Kalimuthu *et al.*, (2008) has used *B. licheniformis* for both intra and extra-cellular synthesis of silver nanoparticles. Lengke *et al.*, (2007) has investigated the formation of extracellular and intracellular silver nanoparticles by bacteria *Pseudomonas stulzeri, Escherichia coli, Vibrio cholerae, Pseudomonas aeruginosa, Salmonells typlus and Staphylococcus currens*. Kumar *et al.*, (2013) reported intracellular and extracellular synthesis of silver nanoparticles using marine bacteria *Vibrio alginolyticus* with spherical shapes 50-100 nm.

Piperitone (a natural product which show an inhibitory effect on nitro reduction activity of Enterobacteriaceae) could partially inhibit the bioreduction of silver ions to silver NPs by different strains of Enterobacteriaceae including *K. pneumoniae*. As a result of this control experiment, nitroreductase enzymes might be responsible for bioreduction of silver ions. However, there may be some other kind of mechanism available for synthesis silver nanoparticles, since heat-inactivated extracts also mediated the formation of silver NPs, enzymatic reactions are likely not involved in silver NPs formation (Wei *et al.*, 2012).

### 2.2.3.4 Yeast mediated synthesis of silver nanoparticles

As compared to other microorganisms such as bacteria and fungi, there are a few reports describing the use of yeasts to produce metal nanoparticles (Kowshik *et al.*, 2003). The unicellular algae *Chlamydomonas reinhardttii* was used as a model system to investigate the role of cellular proteins in the synthesis of AgNPs (Barwal *et al.*, 2011). The cell free extract (*In vitro*) of *C. reinhardttii* and *In vivo* cells produced AgNPs of size range 5 to 15 nm and 5 to 35 nm respectively. Silver nanoparticles in the size range of 2-5 nm were synthesized extracellularly by a silver-tolerant yeast strain MKY3, when challenged with 1 mM/L soluble silver in the log phase of growth (Kowshik *et al.*, 2002). Prakasham *et al.*, (2012) reported silver nanoparticles production by the green chemistry approach using an isolated marine actinomycetes strain *Streptomycyes albidoflavus*. The Biological synthesis of Ag, Au and Ag
shell-Au core nanoparticles were performed by using single cell protein *Spirulina platensis* and seaweed *Sargassum wightii* (Singaravelu *et al.*, 2007).

**Mechanisms of silver nanoparticles synthesis**

Microorganisms, such as bacteria and fungi play an important role in the remediation of toxic metals through the reduction of metal ions (Kalishwaralal *et al.*, 2008). Several studies have reported that NADH- and NADH-dependent enzymes are important factors in the biosynthesis of metal nanoparticles (Mukherjee *et al.*, 2002; Ahmad *et al.*, 2003; Duran *et al.*, 2005). Widely accepted mechanism for the synthesis of silver nanoparticles is the presence of enzyme “Nitrate reductase” (Anil Kumar *et al.*, 2007; Kalimuthu *et al.*, 2008). Under alkaline conditions the ability of enzyme responsible (not only nitrate reductase) for the synthesis of silver nanoparticles increases (Sanghi and Verma, 2009). Vaidyanathan *et al.*, (2010) optimized the synthesis of nitrate reductase by the organism *B. licheniformis* and optimization led to enhanced synthesis of silver nanoparticles. The synthesis was found to be dependent on the enzyme activity. There are other ways to biosynthesize silver nanoparticles without the presence of enzymes. It has been reported that dried cells of *Lactobacillus* sp. A09 can reduce silver ions by the interaction of silver ions with the groups on the microbial cell wall (Fu *et al.*, 2000). It was suggested that the phytochemicals are involved directly in the reduction of ions and formation of silver nanoparticles (Jha *et al.*, 2009). The reduction seems to be initiated by electron transfer from the NADH by NADH-dependent reductase as electron carrier.

Fungi mediated reduction also involves nitrate reductase. The nitrate reductase was also found to be responsible for the synthesis of silver nanoparticles by *Fusarium oxysporum*. However *Fusarium moniliforme* did not produce nanoparticles either intracellularly or extracellularly even though they had intracellular and extracellular reductase in the same fashion as *Fusarium oxysporum* (Duran *et al.*, 2005). Anilkumar *et al.*, (2007) has demonstrated *In vitro* synthesis of silver nanoparticles using α-NADPH-dependent nitrate reductase and phytochelatin from the organism *Fusarium oxysporum*. Instead of fungi culture, isolated proteins from them have also been used successfully in nanoparticles formation. The reduction of Ag⁺ ions has occurred by the action of nitrate reductase enzyme and quinine in extracellular electron transfer (Gade *et al.*, 2008). The use of a specific
enzyme in *In vitro* synthesis of nanoparticles is important because this would eliminate the downstream processing required for the use of these NPs in homogeneous catalysis and other applications such as non-linear optics. The biggest advantage of this protocol based on purified enzyme was the development of a new approach for green synthesis of nanomaterials over a range of chemical compositions and shapes without possible aggregation.

The biochemical and molecular mechanisms of AgNPs biosynthesis remain poorly characterized and should be investigated to further optimize the process. For instance, characterization of biochemical mechanisms underscored the importance of phytochemicals, which may mediate biosynthesis. Plant extracts may act both as reducing and capping agents in AgNPs synthesis. The reduction of Ag$^+$ ions by combinations of biomolecules found in these extracts such as enzymes/proteins, amino acids, polysaccharides and vitamins is environmentally benign (Collera *et al*., 2005). The mechanism of silver nanoparticles production by fungi is said to follow the following steps: trapping of Ag$^+$ ions at the surface of fungal cells and the subsequent reduction of silver ions by enzymes present in the fungal system (Mukherjee *et al*., 2001). The extracellular enzymes like naphthoquinones and anthraquinones are said to facilitate the reduction. However, the exact mechanism of the formation of silver nanoparticles is yet to be elucidated. Studies with *Fusarium oxysporum* has shown that the reduction of silver ions occurs by a nitrate-dependent reductase and a shuttle quinone extracellular process (Duran *et al*., 2005). In *Bacillus licheniformis*, the enzyme found at the membrane is called as respiratory enzymes (Rey *et al*., 2004).

### 2.3 Characterization

The application of AgNPs is highly dependent on the chemical composition, shape, size and monodispersity of particles (Bansal *et al*., 2010). The characterization of nanoparticles is important to understand nanoparticles synthesis and applications. The characterization is performed using a variety of different techniques such as transmission electron microscopy (TEM) and scanning electron microscopy (SEM), atomic force microscopy (AFM), dynamic light scattering (DLS), X-ray photoelectron spectroscopy (XPS), powder X-ray diffractometry (XRD), Fourier transform infrared spectroscopy (FTIR), and UV-Vis spectroscopy (Choi *et al*., 2007; Yoosaf *et al*., 2007; Hutter and Fendler, 2004; Sun *et al*., 2000; Vilchis-Nestor *et al*., 2008; Yeo *et al*., 2003; Zhang *et al*., 2004; Zhang *et al*., 2004).
These techniques are used for determination of different parameters such as particle size, shape, crystallinity, elemental composition and surface area.

The analytical techniques such as TEM, scanning electron microscopy (SEM) or atomic force microscopy (AFM) are used to measure the size and shape of metal nanoparticles. For measuring the aggregation state of particles, the effective size of particles is measured in solution such as dynamic light scattering (DLS) or analytical disc centrifugation. However, due to the unique optical properties of silver nanoparticles, a great deal of information about the physical state of nanoparticles can be obtained by analyzing the spectral properties of silver nanoparticles in solution. Although chemical synthesis aids the size control over the synthesis of nanoparticles, size control can also be achieved in biological methods.

For instance, the morphology and particle size could be determined by TEM, SEM and AFM. The advantage of AFM over traditional microscopes such as SEM and TEM is that AFM measures three-dimensional images so that particle height and volume can be calculated. Furthermore, dynamic light scattering is used for determination of particles size distribution. Moreover, X-ray diffraction is used for the determination of crystallinity, while UV-Vis spectroscopy is used to confirm sample formation by showing the plasmon resonance.

### 2.3.1 UV-Visible absorption spectroscopy

UV-Vis spectroscopy refers to absorption spectroscopy or reflectance spectroscopy in the ultraviolet-visible spectral region. Absorbance spectroscopy is used to determine the optical properties of a solution. UV-Vis spectroscopy has been routinely used not only in analytical chemistry for the quantitative determination of different analytes, such as highly conjugated organic compounds, transition metal ions and biological macromolecules, but also, in recent years high-precision and high-energy spectrophotometers are used to measure absorption on solid samples including semiconductors, films, glass and absorbing materials. This absorption spectroscopy uses electromagnetic radiations between 190 nm to 800 nm and divided into the ultraviolet (190-400 nm) and visible (400-800 nm) regions. A light is send through the
sample solution and the amount of absorbed light is measured. When the wavelength is varied and the absorbance is measured at each wavelength. The absorbance can be used to measure the concentration of a solution.

The basic principle of the UV-Vis spectroscopy is based on the Beer-Lambert law. The Beer-Lambert law provides relation of concentration and light intensity as shown in equation 2.1

\[- \log \frac{I}{I_0} = A = \varepsilon c l\]  

(2.1)

Where, \(\varepsilon\) is the molar absorptivity or molar extinction coefficient, \(A\) is measured absorbance and \(c\) is the concentration of the analyte and \(l\) is path length.

Light is a form of energy and absorption of light by matter causes the energy content of the molecules (or atoms) to increase. The total potential energy of a molecule is given by equation

\[E = h\nu, \quad \nu = c/\lambda\]  

(2.2)

UV-visible absorptions in organic molecules occur as a result of transition of valence electrons between molecular orbitals. Photons of UV and visible light have enough energy to cause transitions between the different electronic energy levels in some molecules and atoms. The energy required to move an electron from a lower energy level to a higher energy level is the wavelength of light absorbed.

UV-Vis spectroscopy is the prime characterization technique to analyze the nanoparticles synthesis (Denney and Sinclair, 1988). The optical measurement of UV-visible spectrophotometer has different absorbance peak like 410 nm, when *Nerium obander* plant extract treated with aqueous 1 mM silver nitrate solution (Subbaiya *et al.*, 2014). The absorption spectrum of spherical silver nanoparticles presents a maximum wavelength between 420 and 450 nm (Ninganagouda *et al.*, 2013; Sunkar and Nachiyar, 2012). In case of *Azadirachta indica* mediated iron nanoparticles peaks was found through UV -visible spectroscopy at the range of 216- 265 nm as indication of suitable surface Plasmon resonance with high band intensities and (Monalisa and Nayak, 2013). The frequency and width of the
surface plasmon absorption depends on the size and shape of the metal nanoparticles (Mukherjee et al., 2002).

2.3.2 X-ray Diffraction (XRD)

X-ray diffraction is a conventional technique for the determination of crystallographic structure and morphology. X-ray diffraction has been used to determine the crystal structure of solids, including lattice constants and geometry, identification of unknown materials, orientation of single crystals, defects, etc (Wang, 2000). There is increase or decrease in intensity with the amount of constituent. In a variety of X-ray spectroscopic techniques, XRD is a primary tool for completely resolving the tertiary structures of crystalline materials at the atomic scale (Cantor and Schimmel, 1980; Sapsford et al., 2011). X-ray diffraction analysis with various nanoparticles has been studied by various research workers to find the high crystallinity of the prepared sample (Yelil et al., 2012).

This technique is used to establish the metallic nature of particles, information on translational symmetry, size of the unit cell from peak positions and information on electron density inside the unit cell, namely where the atoms are located from peak intensities. The XRD technique mainly depends on the diffraction of X-ray radiation, which is the reflection of a collimated beam of X-rays incident on the crystalline planes of an examined specimen according to Bragg's law (Cantor and Schimmel, 1980). Diffraction occurs only when the wavelength of the wave motion is of the same order of magnitude as the repeat distance between scattering centers. The directions of diffracted X-rays provide information about the atomic arrangements and hence the phase formation and crystal structure can be confirmed by x-ray diffraction studies. Bragg’s law mathematical expression and is represented in equation

\[ 2d \sin \theta = n \lambda \] ................. (2.3)

where, d= Interplanar spacing, \( \theta \)= diffraction angle, \( \lambda \)= wavelength of X-ray, \( n \)= order of diffraction

A typical diffractometer consists of a source of X-ray and for the detection of diffracted X-rays, a detector. A crystalline solid is a unit cell, where atoms are ordered in a particular repeated pattern referred with its inter-atomic spacing comparable to wave length of X-rays (0.5 to 2.5Å) (Cullity, 1978). Hence crystals are the best gratings for the diffraction
of X-rays. Phases can be identified from the d-spacing in a sample using the standard JCPDS powder diffraction file data provided by the International Center for Diffraction Data (ICDD) and the reflections can be indexed with Miller indice. However, there is no more complete destructive interference at θ±dθ, if size of the diffracting crystal is little tiny, which broadens the peak corresponding to diffracted beam in proportion to the size of small crystal. This broadening of the peak can be used for the determination of crystallite size. The average size of the nanoparticles can be estimated by using the Debye-Scherrer equation:

\[ CS = \frac{0.94 K}{\beta \cos \theta}, \]  

(2.4)

Where, CS is the crystalline size, β line broadening at Full Width Half Maxima (FWHM)

Full width at half maximum in radius = FWHM x /180 = 1.5406 x 10^-10, \( \cos \theta = \) Bragg angle and Cu K radiation =1.5406 Å.

Typically, XRD, based on wide-angle elastic scattering of X-rays is a tool for characterizing crystalline size, shape and lattice distortion by long-range order, but is limited to disordered materials (Caminade et al., 2005; Sapsford et al., 2011; Zanchet et al., 2001). Difficulty in growing crystals and the ability of getting results only from single conformation/binding state of the sample limit the applications of XRD technique (Cao, 2004; Sapsford et al., 2011; Zanchet et al., 2001). Another disadvantage of XRD is the low intensity of diffracted X-rays, particularly for low atomic number materials compared with electron diffractions (Cao, 2004). A recent X-ray diffraction study reported a new approach using femtosecond pulses from a hard-X-ray free-electron laser for structure determination, which may benefit structure determination of macromolecules that do not yield sufficient crystal size for using conventional radiation sources or are not sensitive to radiation damage (Chapman et al., 2011).

2.3.3 Microscopic techniques

SEM and TEM are mainly used for morphological studies of nanoparticles. Many researchers used these techniques to establish that the synthesized nanoparticles were more or less uniform in size and shape (Shobha et al., 2014).

2.3.3.1 Scanning Electron Microscopy (SEM)
Scanning electron microscope analysis is employed to determine the size, shape and morphology of synthesized nanoparticles. SEM is a surface imaging method in which the incident electron beam scans across the sample surface and interacts with the sample to generate signals reflecting the atomic composition and topographic detail of the specimen surface (Hall et al., 2007; Johal, 2011). SEM gives high resolution images of the surface of a sample. The scanning electron microscope works on the same principle as an optical microscope, but optical microscope uses light sources and glass lenses to illuminate specimens to produce magnified images, whereas SEM uses beams of accelerated electrons and electrostatic or electromagnetic lenses to generate images of higher resolution. This technique is based on the much shorter wavelengths of electrons than visible light photons. The electrons can be accelerated by an electric potential and wavelength can be made shorter than one of photons. This makes the SEM capable of magnifying images up to 2, 00,000 times.

For SEM characterization, nanoparticles solution should be first converted into a dry powder, which is then mounted on a sample holder followed by coating with a conductive metal, such as gold using a sputter coater. The sample is then scanned with a focussed fine beam of electrons (Jores et al., 2004). The incident electrons cause emissions of elastic scattering of electrons referring to backscattered electrons, inelastic scattering of electrons named low-energy secondary electrons and characteristic X-ray light called cathodoluminescence, from the atoms on the sample surface or near-surface material (Johal, 2011). Among these emissions, detection of secondary electrons is the most common mode in SEM and can achieve resolution smaller than 1 nm (Johal, 2011). The surface characteristics of the sample are obtained from the secondary electrons emitted from the sample surface.

Scanning electron microscopy (SEM) analysis offer several advantages in morphological, size analysis and provides morphological examination of sample with direct visualization. The size, size distribution and shape of nanomaterials can be directly acquired from SEM; however, this technique is time consuming, costly and do not provide information about size distribution (Molpeceres et al., 2000). Moreover, the nanoparticles must be able to withstand vacuum and electron beam may damage or alter the characteristics of the nanomaterials (Bootz et al., 2004; Hall et al., 2007). In addition, nonconductive samples can acquire charge by an electron beam and insufficient deflection of the electron beam, which
can results into faults in images. Coating an ultrathin layer of electrically conducting material onto the biomolecules is often required for this sample preparation procedure (Hall et al., 2007; Suzuki, 2002). A cryogenic freezing method is often required in EM, to image surface groups attached to NPs, the size of nanomaterial cannot be investigated in physiological conditions (Hall et al., 2007).

2.3.3.2 Transmission Electron Microscopy (TEM)

TEM is the most frequently used analysis method for characterizing nanomaterials in a range of scientific fields, in both physical and biological sciences. TEM provides direct images and chemical information of nanomaterials at a spatial resolution down to the level of atomic dimensions (1 nm) (Patri et al., 2006; Wang, 2001). TEM operates on different principle than SEM, yet it often brings same type of data. An incident electron beam is transmitted through an ultra-thin specimen, during which the incident electrons interacting with specimen are transformed to unscattered electrons, elastically scattered electrons or inelastically scattered electrons (Williams and Carter, 2009). The magnification of TEM is mainly determined by the ratio of distance between objective lens and the specimen and the distance between objective lens and its image plane (Williams and Carter, 2009).

The scattered or unscattered electrons are focused by a series of electromagnetic lenses and then projected on a screen to generate an electron diffraction, amplitude-contrast image, a phase-contrast image or a shadow image of varying darkness according to the density of unscattered electrons (Williams and Carter, 2009). An image is formed from the interaction of electrons transmitted through the specimen; the image is magnified and focused onto an imaging device, such as a fluorescent screen, on a layer of photographic film, or to be detected by a sensor such as a CCD camera. In addition to high spatial resolution of TEM that enhances the morphological and structural analyses of nanomaterials, a wide variety of analytical techniques can be coupled with TEM for different applications; for example, chemical analyses of electron energy loss spectroscopy and energy dispersive X-ray spectroscopy can quantitatively investigate the electronic structure and chemical composition of nanomaterials respectively (Patri et al., 2006; Tiede et al., 2008; Wang, 2001).
The sample preparation for TEM is complex and time consuming because of its requirement to be ultra thin for the electron transmittance. The nanoparticles dispersion is deposited onto support grids or films. Overall, both TEM and SEM can reveal the size and shape heterogeneity of nanomaterials, as well as the degrees of aggregation and dispersion. TEM has advantages over SEM in providing better spatial resolution and capability for additional analytical measurements (Hall *et al.*, 2007). There are certain drawbacks accompanying the advantages of TEM (Williams and Carter, 2009). A very high vacuum and thin sample section are required for electron-beam penetration in TEM measurement (Hall *et al.*, 2007). Sample destruction and measurement in unnatural/non-physiological conditions are common to all EM techniques. High resolution imaging enables examination of a minute part of the specimen over a certain period of time and results in poor statistical sampling. The extensive preparation of thin specimens increases the risk of altering sample's structure and makes TEM analysis a very time consuming process. Another big concern is that TEM specimens can be damaged or even destroyed by intense, high-voltage electron beams.

### 2.3.4 Fourier Transform Infrared [FTIR] spectroscopy

FTIR is the analytical technique used to qualify and quantify compounds utilizing infrared absorption of molecules. It is used to determine the nature of associated functional groups and structural features of biological extracts with nanoparticles. FTIR measures infrared intensity vs. wavelength of light, vibrational technique involved is the interactions of photons with species in a sample that results in energy transfer to or from the sample via vibrational excitation or de-excitation is exploited for characterization. These vibrational frequencies provide the information of chemical bonds in the detecting samples. It deals with the vibration of chemical bonds in a molecule at various frequencies depending on the elements and types of bonds. Absorption occurs when the energy of beam of light (photons) are transferred to the molecule. The excitation of molecules results in to transfer of molecule to higher energy state. The energy transfer takes place in different forms like molecular bond vibrations, electron ring shifts, rotations and translations. IR is mostly concerned with vibrations and stretching. On absorbing infrared energy, bonds between atoms in the molecule stretch and bend consequently creating the infrared spectrum. These absorption frequencies represent excitations of vibrations of chemical bonds and thus are specific to the
type of bond and the group of atoms involved in the vibration. The energy corresponding to these frequencies correspond to the infrared region of electromagnetic spectrum.

The infrared source emits a broad band of different wavelength of infrared radiation. The IR radiation is passed through an interferometer that modulates the infrared radiation. The interferometer carries out an optical inverse Fourier transform on the entering IR radiation. The altered IR beam passes through the gas sample, where it is absorbed to various extents at different wavelengths by the various molecules present. Finally a detector detects the intensity of the IR beam, which is a liquid-nitrogen cooled MCT (Mercury-Cadmium-Telluride) detector. The detected signal is digitised and Fourier transformed by the computer to get the IR spectrum of the sample gas. The calculated spectra clearly reflect the well-known dependence of nanoparticle optical properties. The green synthesized silver nanoparticles by employing various leaf extract was analysed using Fourier Transform Infrared [FTIR] Spectroscopy showed characteristic peaks (Murugan et al., 2014). In Fourier transform infrared spectroscopy, after absorbing electromagnetic radiation the frequency of vibration of a bond increases leading to transition between ground state and several excited states. The FT-IR measurement can be utilized to study the presence of protein molecule in the solution as the FTIR spectrum in the 1400 cm\(^{-1}\)-1700 cm\(^{-1}\) region provides information about the presence of -CO- and -NH- groups (Banwell and McCash, 1996).

Infrared radiation from the source is collected and collimated (made parallel) before it strikes the beam splitter. One half of the radiation is transmitted by the beam splitter ideally and other half is reflected. Transmitted and reflected beams strike mirrors, reflecting the two beams back to the beam splitter. Thus, first one half of the infrared radiation that finally goes to the sample gas has been reflected first from the beam splitter to the moving mirror and then back to the beam splitter. The remaining other half of the infrared radiation going to the sample has first gone through the beam splitter and then reflected from the fixed mirror back to the beam splitter. The interference occurs when these two optical paths are reunited at the beam splitter because of the optical path difference caused by the moving mirror (Thermo Nicolet Corporation, 2001).

2.3.5 Dynamic light scattering (DLS)

Several physicochemical characteristics of nanomaterials including hydrodynamic size, shape, structure, aggregation state and biomolecular conformation can be explored using
radiation scattering techniques (Inagaki et al., 2013; Sapsford et al., 2011). Dynamic Light Scattering or Photon Correlation Spectroscopy is one of the most popular methods used to determine the size of nanoparticles in the colloidal solution. DLS can probe the size distribution of small particles, molecules or polymers at the scale from submicron down to one nanometer in solution or suspension using a monochromatic light source, e.g. a laser (Patri et al., 2006; Sapsford et al., 2011). The photon correlation spectroscopy (PCS) represent the most frequently used technique for accurate estimation of the particle size and size distribution based on DLS (DeAssis et al., 2008). DLS is widely used to determine the size of brownian nanoparticles in colloidal suspensions in the nano and submicron ranges. On incidence of a monochromatic light beam, such as a laser, onto colloidal solution of nanoparticles in brownian motion causes a Doppler Shift, where the light hits the moving particle and further changes the wavelength of incoming light. The change is solely related to the size of nanoparticles. The principle of DLS is to monitor the temporal fluctuation of the elastic scattering intensity of light i.e. Rayleigh scattering, induced from the brownian motion of the particles/molecules of a size much smaller than the incident light wavelength, at a fixed scattering angle (Brar and Verma, 2011; Sapsford et al., 2011). The intensity fluctuation trace comprises a mixture of constructive and destructive interferences of the scattered light, through which the particle size can be derived from analysis of the motion-dependent autocorrelation function using the Stokes-Einstein equation (Brar and Verma, 2011; Pons et al., 2006b; Sapsford et al., 2011).

A short experimental time and increased accuracy due to automated system are the major advantage of this system. The main strengths of DLS is its non invasive manner, short experiment duration (in minutes), accuracy in determining the hydrodynamic size of monodisperse samples, capabilities of measuring diluted samples, analyzing samples in a wide range of concentrations, detecting small amounts of higher molecular weight species, along with lower apparatus costs and more reproducible measurement than other methods (Brar and Verma, 2011; Filipe et al., 2010; Lim et al., 2013). However, some limitations of DLS are difficulty in correlating size fractions with a particular composition of aggregated materials, interference of dust particles in the scattering intensity and a relatively small range of particle or molecule size (1 nm-3 μm) (Bootz et al., 2004; Brar and Verma, 2011; Filipe et al., 2010). In addition, DLS has limited utility for analysis of samples with heterogeneous
size distributions and resolving the dimensions of a mixed sample population varying in size less than a factor of three. DLS is unsuited to accurately measuring the sizes of non-spherical nanomaterials because spherical nature of particles is already assumed in the analysis (Boottz et al., 2004; Brar and Verma, 2011; Filipe et al., 2010; Uskokovic, 2012). As compared to TEM, size obtained from DLS is slightly higher. As DLS provides the information of inorganic particles along with any coating material and solvent layer attached to particles, which moves under the influence of brownian motion, whereas TEM provides the information regarding the size of inorganic particles.

2.3.6 Zeta potential

The nature and intensity of surface charge of nanoparticles is very important as it determines their interaction with the biological environment as well as their electrostatic interaction with bioactive compounds. The physical mechanism that is used to stabilize most aqueous colloidal systems is electrostatic repulsion. The charged colloidal particles are resulting in their mutual repulsion at extended distances. In other cases, the colloidal particles may already carry specific groups that are covalently bound to their surfaces and are ionizable. They carry a net positive or negative charge, or are neutral, depending on the pH of the surrounding aqueous solvent. Hence, the pH of suspension will strongly influence the net charge of the colloidal particles and therefore their stability against aggregation. The colloidal stability is analyzed through zeta potential of nanoparticles. This potential is an indirect measure of the surface charge. It corresponds to the potential difference between outer Helmholtz plane and the surface of shear. The magnitude of zeta potential gives an indication of potential stability of colloidal system. If the particles in suspension have a large negative or positive zeta potential then they will tend to repel each other which in results lower the tendency to flocculate. However, if the particles have low zeta potential values then there is no force to prevent the particles aggregation.

Zeta potential is usually determined by measuring the velocity of charged species towards the electrode in the presence of an external electric field across the sample solution (Pons et al., 2006 b; Sapsford et al., 2011). The zeta potential with a value of ±30 mV is generally chosen to infer particle stability, through which the absolute value greater than 30 mV indicates a stable condition, whereas a low zeta potential value of less than 30 mV
indicates a condition towards instability, aggregation, coagulation or flocculation (Sapsford et al., 2011). The zeta potential of a nanoparticles is commonly used to characterise the surface charge property of nanoparticles (Couvreur et al., 2002). It reflects the electrical potential of particles and is influenced by the composition of particle and medium in which it is dispersed. Nanoparticles with a zeta potential above (+/-) 30 mV have been shown to be stable in suspension, as the surface charge prevents aggregation of the particles. The zeta potential can also be used to determine whether a charged active material is encapsulated within the centre of the nanocapsule or adsorbed onto the surface.

The zeta potential of colloidal dispersions is routinely measured by using the technique of micro electrophoresis. A voltage is applied across a pair of electrodes at either end of a cell containing the particle dispersion. The charged particles are attracted to the oppositely charged electrode and their velocity is measured and expressed in unit field strength as their mobility. Dispersion gives possibility of controlling the electrostatic interactions in dispersion, and hence controls the stability of emulsion or dispersion (Zetasizer Nano Series Technical Note MRK654-01, 2011). The extent of surface hydrophobicity can be predicted from the values of zeta potential. The zeta potential can also provide information regarding the nature of material encapsulated within the nanocapsules or coated onto the surface (Pangi et al., 2003).

Among the methods of evaluating zeta potential, the technique of electrophoretic light scattering (ELS), which can simultaneously measure the velocities of many charged particles in liquid is most commonly used (Doane et al., 2011; Xu, 2008). However, it still suffers the electro-osmotic effect that reduces precision and reproducibility of the measurement (Weiner et al., 1993). Although measuring the zeta potential of suspended particles after dilution reduces difficulty of light penetration into the sample solution. It is worth noting that zeta potential is a property sensitive to environmental changes including pH and ionic strength (Weiner et al., 1993; Xu, 2008). Therefore, a precise, repeatable zeta potential measurement in a diluted solution cannot reflect the true value in a concentrated suspension (Xu, 2008).

2.4 Antimicrobial activity of silver nanoparticles

A burden on the economy of the developing countries is rising due to the production cost of traditional antibiotics. The emergence and spread of antibiotic resistance pathogen is an
alarming concern in clinical practice. Many organisms such as MRSA, HIV-1, Hepatitis-B virus and ampicillin resistant *E. coli* are difficult to treat. Medicinal chemists are desperately trying to develop new compounds that can kill strains such as MRSA (methicillin, or multiple-resistant *Staphylococcus aureus*) and *E. coli* O157. The effect of silver nanoparticles was found to be more on *S. aureus* and *E. coli* but less on *B. subtilis* (Ruparelia et al., 2008). Increasing resistance of microorganisms against traditional antibiotics and high production cost has raised the need for continued development in the field of antibiotic. There is a need of a cheap, broad-active agent that can be used against a variety of pathogens. The Ag NPs have been found to be effective against many viruses and bacteria. The recent development in nanotechnology has provided tremendous impetus in this direction due to its capacity of modulating metals into nanosizes and various shapes, which drastically changes the chemical, physical and optical properties and their use. The high surface area to volume ratio with unique physicochemical properties of noble metal nanoparticles makes them a suitable antimicrobial agent (Weir et al., 2008). The biological synthesis method of metal and metal oxide nanoparticles have been explored since few years. The production cost of nano materials is too less than chemical antibiotics. It has also been demonstrated that the rate of acquiring resistance to the nanoparticles is slower as compared to conventional antibiotics (Muhling et al., 2009).

Among all, silver nanoparticles have proved to be the most effective antimicrobial agent against bacteria, viruses and other eukaryotic micro-organisms (Gong et al., 2007). Ag NPs are attractive because they are non-toxic to the human body at low concentrations and have broad spectrum antibacterial actions (Baker et al., 2005). Silver has long been known to exhibit a strong toxicity to a wide range of micro-organisms (Liau et al., 1997). Owing to antimicrobial property, silver-based compounds have been used extensively in many bactericidal applications (Gupta and silver, 1998; Nomiya et al., 2004). This is reported that silver nanoparticles are not only antibacterial against gram-positive bacteria such as *Staphylococcus aureus* and *Streptococcus pneumoniae* but also against gram-negative bacteria like *Escherichia coli* and *Pseudomonas aeruginosa*.

Silver in the forms of metallic silver, silver sulfadiazine and silver nitrate has been used for burn wound treatment and disinfection of catheters, dental instruments and bacterial infection control (Becker and Wilson, 1980). After discovery of antibiotics in the 1940s silver
become out of favour to treat bacterial infections (Bumpus et al., 1985). AgNPs among other metallic NPs have proven to be the most lethal against viruses, bacteria and other eukaryotic microorganisms (Sharma et al., 2008). The respiratory chain and cell division are affected by AgNPs, which finally lead to cell death (Klasen and Burns, 2000). The antimicrobial activity of AgNPs is inversely related to size and shape (Pal et al., 2007). The high specific surface-to-volume ratio of silver nanoparticles increases their contact with microorganisms, promoting the dissolution of silver ions, thereby improving biocidal effectiveness. The ability of silver nanoparticles to release silver ions is a key to their bactericidal activity (Stobie et al., 2008). Amalgamation of AgNPs with antibiotics such as penicillin G, erythromycin, amoxicillin and vancomycin resulted in improved and synergistic antimicrobial effects against gram-positive and gram-negative bacteria (Shahverdi et al., 2007; Fayaz et al., 2010).

The antimicrobial activity of silver nanoparticles has been investigated against fungus, yeast, microbial biofilms yeast, gram negative and positive bacteria (Kim et al., 2007; Sondi and Salopek-Sondi, 2004; Abdeen et al., 2014; Gaidhani et al., 2013; Manivasagan et al., 2013; Singh et al., 2013). There are various methods to evaluate the inhibition of microbes in response to exposure to AgNPs. These include determining the (i) zone of inhibition by disk diffusion and agar well diffusion method, (ii) minimum inhibitory concentration (MIC) by broth macrodilution and microdilution assay, (iii) minimum bactericidal concentration (MBC), (iv) growth pattern and (v) time-kill curve (Priyadarshini et al., 2013; Zhang et al., 2014). Although diffusion techniques are mostly preferred, these are labour-intensive and calculation disparities between these methods make it more difficult to compare the various published data (Allahverdiyev et al., 2011). MIC and MBC are easy to assess despite different concentration units such as micrograms per millilitre, milligrams per litre, or parts per million and provide accurate information with respect to susceptibility of a microorganism.

Silver nanoparticle is reported as an effective antimicrobial agent against a broad spectrum of gram negative and gram positive bacteria (Burrell et al., 1999; Yin et al., 1999) including antibiotic resistant strains (Wright et al., 1998; Percival et al., 2007). Shirley et al., (2010) reported the significant antibacterial activity of silver nanoparticles against various pathogenic bacteria of gram positive (S. aureus, S. epidermidis) and gram negative strains (E. coli, S. typhi, Pseudomonas aeruginosa, Klebsiella pneumonia, Proteus vulgaris) using well
diffusion technique. Many researchers used *Escherichia coli* as a model for gram negative bacteria and proved that AgNPs may be used as an antimicrobial agent (Feng et al., 2003).

The antimicrobial activity of synthesized nanoparticles was reported against *Pseudomonas aeruginosa, Staphylococcus aureus, Aspergillus flavus* and *Aspergillus niger* (Govindaraju et al., 2010). Mirzajani et al., (2011) also investigated the antibacterial activity of silver nanoparticles on *S. aureus* PTCC1431 and suggested that concentration of silver nanoparticles above 8 μg/ml resulted in release of muramic acid (MA) into the medium which causes cell wall distraction. Some studies observed that biologically synthesized silver nanoparticles exhibited a potent antibiofilm formation activity that was tested *In vitro* on biofilms formed by *P. aeruginosa* and *S. epidermidis* during 24 hr treatment. Silver nanoparticles treatment resulted in more than 95 % inhibition in biofilm formation (Kalishwarlal et al., 2010). Antibacterial activity of silver nanoparticles synthesized from psychrophillic bacteria were analyzed against *Arthrobacter kergulensis, A. gangotriensis* and *B. indicus*. Minimum concentration of silver nanoparticles at which they arrest the bacterial growth was 2 μg/ml (Shivaji et al., 2011).

Small nanoparticles with a larger surface area to volume ratio provide a more efficient means for antibacterial activity even at a very low concentration. Silver nanoparticles of different shapes (spherical, rod-shaped, truncated and triangular nanoparticles) were developed by synthetic routes. Truncated triangular silver nanoparticles were found to display the strongest anti-bacterial activity could be due to their larger surface area to volume ratios. Jun et al., (2007) investigated antimicrobial activity of silver nanoparticles against yeast, *E. coli* and *S. aureus* and reported that nanoparticles were most effective against *E. coli*, least growth-inhibitory effect observed against *S. aureus*. The growth inhibition effect observed was directly related to concentration of nanoparticles. In other study, low concentration of silver nanoparticles was found to exhibit microbicidal effect on yeast and *E. coli*, However, on *Staphylococcus aureus* (gram positive bacteria) the antibacterial effect of silver nanoparticles was mild (DVM et al., 2007).

Thirumurugan et al., (2009) reported that silver nanoparticles exhibited discrete antibacterial activity against clinically isolated seven pathogenic bacteria at a concentration of 5 μg/ml. Silver nanoparticles (NPs), exhibiting very strong bactericidal activity against
both gram-positive and gram-negative bacteria including multi-resistant strains can be considered as potential antifungal agent (Panacek et al., 2006). Hernandez-Sierra et al., (2008) used nanoparticles of silver, zinc oxide and gold of an average size of 25 nm, 125 nm and 80 nm respectively to demonstrate bacteriostatic and bactericidal effects against S. mutans. Findings showed that nanoparticles of silver as compared with those of gold (MIC on average 197 μg/ml) and zinc oxide (MIC on average 500 ± 306.18 μg/ml) required a lower concentration (MIC on average 4.86 ± 2.71 μg/ml) to inhibit development of the S. mutans strains leading to consideration that silver particles may be most effective for controlling S. mutans.

Silver nanoparticles possess strong antibacterial effects against 12 species of bacteria including multi-resistant bacteria like methicillin-resistant Staphylococcus aureus (MRSA), as well as multidrug-resistant Pseudomonas aeruginosa, ampicillin-resistant E. coli O157:H7 and erythromycin-resistant S. pyogenes (Shahverdi et al., 2007; Sondi and Salopek-Sondi, 2004). Extracellular silver nanoparticles synthesized using Bacillus flexus were proved to have antibacterial effect on clinically isolated multidrug resistant (MDR) E. coli, B. subtilis, S. pyogenes and P. aeruginosa (Priyadarshini et al., 2013). Silver nanoparticles generated extracellularly by Bacillis megaterium (NCIM 2326) were found to be effective against multidrug resistant clinical pathogens like S. pneumoniae and S. typhi (Saravanan et al., 2011). Methicillin-resistant Staphylococcus aureus, methicillin-resistant Staphylococcus epidermidis and Streptococcus pyogenes were found to be susceptible to silver nanoparticles with size ranges from 160-180 nm (Nanda and Saravanan, 2009).

A fungal strain KSU-09 isolated from the roots of date palm (Phoenix dactylifera) identified as Amylomyces rouxii found to synthesize silver nanoparticles and possess antimicrobial activity against Shigella dysenteriae type 1, S. aureus, Citrobacter sp., E. coli, P. aeruginosa, B. subtilis, C. albicans and Fusarium oxysporum (Musarrat et al., 2010). Similarly, silver nanoparticles synthesized from Mentha piperita leaves extract possess antimicrobial activity against clinically isolated human pathogens (Mubarakali et al., 2011). Again, biologically synthesized silver nanoparticles from Acalypha indica leaf extracts were found to possess antimicrobial activity against Vibrio cholera and E. coli (water borne pathogens). MIC was 10 μg/ml for both of the pathogens (Krishnaraj et al., 2010).
Silver nanoparticles of 20-30 nm from leaves of *Acalypha indica* showed antimicrobial activity against *E. coli* and *Vibrio cholera* (Krishnaraj *et al.*, 2010), while silver nanoparticles of 3-12 nm from peels of *Citrus sinensis* have been reported to show activity against *Bacillus subtilis* (Konwarh *et al.*, 2011). AgNPs of size 33.67 nm from *Allium cepa* stem show antimicrobial activity against *E. coli* and *S. typhimurium* (Saxena *et al.*, 2010). Silver nanoparticles of size 8 nm from leaves of *Nicotiana tobaccum* inhibits *Pseudomonas putida*, *P. vulgaris*, *Escherichia coli DH5α*, *B. subtilis*, *P. aeruginosa* and *Salmonella typhi* (Suranjit *et al.*, 2011).

Few studies related to antifungal effects of silver nanoparticles have been published (Falletta *et al.*, 2008; Roe *et al.*, 2008; Zeng *et al.*, 2007). AgNPs showed powerful antimicrobial properties even in at lower concentration (Mishra and Kumar, 2009). The first study related to antifungal activity of silver nanoparticles against clinical isolates and ATCC strains of *Trichophyton mentagrophytes* and *Candida* spp. was published (Kim *et al.*, 2008). Panacek *et al.*, (2009) reported the inhibitory effect of silver NPs against the tested yeasts (*Candida albicans*, *C. tropicalis* and *C. parapsilosis*) even at very low concentration. The inhibitory effect of silver NPs was enhanced through their stabilization. Nasrollahi *et al.*, (2011) reported that the MIC50 of silver nanoparticles against *S. cerevisiae* (ATCC 5027) was significantly lower than that of amphotericin B (16 mg/ml) and fluconazole (64 mg/ml). AgNPs were found to be effective against *Candida* sp., dermatophytes and a few phytopathogenic fungi (Krishnaraj *et al.*, 2012). Nanosilver is an effective and a fast-acting fungicide against a broad spectrum of common fungi including genera such as *Aspergillus*, *Candida* and *Saccharomyces* (Wright *et al.*, 1999). Ag-NPs have considerable antifungal activity in comparison with other antifungal drugs. So, further investigation for clinical applications is required (Nasrollahi *et al.*, 2011). Kim *et al.*, (2012) reported that AgNPs possess antifungal properties against plant pathogens at various levels. On the basis of available literature related to antimicrobial activity of silver nanoparticles, AgNPs could be considered as an excellent broad-spectrum antibacterial agent. More importantly, the AgNPs produced by *A. terreus* exhibited potent antifungal activity against *Candida* species, which were the most important pathogenic fungi. Additionally, the AgNPs showed good inhibition activity towards two kinds of filamentous fungus, which were naturally resistant to the common antifungal agent fluconazole (Espinel-Ingroff *et al.*, 2000).
AgNPs are also known for their antimicrobial potential against several viruses, including hepatitis B (Lu et al., 2008), respiratory syncytial virus (Sun et al., 2008), herpes simplex virus type 1 (Baram-Pinto et al., 2009) and monkey pox virus (Rogers et al., 2008). AgNPs and ions have been shown to possess intrinsic cytotoxic activity (Kim et al., 2007; Baker et al., 2005) and exhibit an enhanced antimicrobial effect, when applied on silicon structures (Furno et al., 2004). Anti-viral properties of biologically formed AgNPs are found to be more effective than chemically synthesized AgNPs (De Gusseme et al., 2010). Vero cells co-incubated with AgNPs were reported to prevent plaque formation after being infected with the Monkey pox virus (Rogers et al., 2008). Silver nanoparticles (diameter 5-20 nm, average diameter 10 nm) inhibit HIV -1 (Sun et al., 2005). The antiviral effects of AgNPs on the hepatitis B virus (HBV) have been reported using a HepAD38 human hepatoma cell line (Lu et al., 2008). The capacity of AgNPs to inhibit an influenza virus was determined in a MDCK cell culture and was demonstrated that with AgNPs at 0.5 μg/ml concentration viral infectivity was reduced. Metallic nanoparticles have also been described as a possible HIV preventative therapeutic (Bowman et al., 2008). Human cervical tissue when challenged with AgNPs as an anti-HIV-1 agent, AgNPs provided protection against the transmission of cell-free and cell-associated HIV-1 (Lara et al., 2010c).

**Mechanism of antimicrobial activity**

The mechanism of inhibitory effects of Ag⁺ ions on microorganisms is not completely clear. Silver nanoparticles can either leads to inhibition of growth/loss of infectivity or to cell death. The bactericidal properties of silver nanoparticles are due to the release of silver ions from the particles, which confers the antimicrobial activity (Amarendra et al., 2010). With respect to the microbes, the silver nanoparticles get attached to the cell wall, thereby disturbing the permeability of cell wall and cellular respiration. The nanoparticles may also penetrate deep inside the cell wall, thus causing cellular damage by interacting with phosphorus and sulphur containing compounds, such as DNA and protein. The mechanisms behind the activity of nano-scaled silver on bacteria are not yet fully elucidated. The most common mechanisms of toxicity proposed to date are: (1) uptake of free silver ions followed by disruption of ATP production, (2) silver nanoparticle and silver ion generation of ROS, (3) silver nanoparticle direct damage to cell membranes/wall and 4) inhibition of enzyme activity and DNA synthesis (Weir et al., 2008; Li et al., 2008; Kim et al., 2007; Rabea et al., 2003). The brief
mechanisms of antimicrobial activity of metal nanoparticles are shown in figure 2.2 (Damm and Munstedt, 2008; Neal, 2008).

**Fig. 2.2 Diagram summarizing nano-scaled silver interaction with bacterial cells**

Besides, the potency of antibacterial effects corresponds to the size of nanoparticles. Smaller particles have higher antibacterial activities. Antimicrobial activity of silver nanoparticles depends on shape (Pal et al., 2007), size (Yen et al., 2009), concentration of Ag NPs (Asharani et al., 2009) and the sensitivity of microbial species to silver (Ruparelia et al., 2008). The respiratory chain and cell division are affected by AgNPs which finally lead to cell death (Klasen and Burns, 2000). Morones et al., (2005) also reported that antimicrobial property of silver nanoparticles is related to their size and shape. The antimicrobial efficacy of the nanoparticles depend on the shapes of nanoparticles also, this can be confirmed by studying the inhibition of bacterial growth by differentially shaped nanoparticles (Morones et al., 2005).

Several studies have reported that the positive charge on Ag$^+$ ion is crucial for its antimicrobial activity. The electrostatic attraction between the negatively charged cell membrane of the microorganism and the positively charged nanoparticles is responsible for antimicrobial effect of silver nanoparticles (Kim et al., 2007). In contrast, Sondi and Salopek-Sondi, (2004) reported that the antimicrobial activity of AgNPs on gram-negative bacteria depends on the concentration of AgNPs and is closely associated with the formation of pits in the cell wall of bacteria. Silver nanoparticles accumulated in the bacterial membrane disturbing the membrane permeability, resulting in cell death. However, because those studies included both positively charged Ag$^+$ ions and negatively charged AgNPs, this data is insufficient to explain the antimicrobial mechanism of positively charged silver
nanoparticles. The rapid breakdown of silver nanoparticles releases ionic silver that inactivates vital bacterial enzymes by interacting with essential thiol groups. In a very interesting study, antibacterial effects of silver nanoparticles synthesized by the sodium boro-hydride method was evaluated on recombinant *E. coli*, bacteria expressing green florescent protein (GFP) used as the model system. It was observed that silver nanoparticles above a certain concentration were not only bactericidal but also found to reduce sizes of treated bacteria compared to untreated ones. However, no direct effect on DNA/protein profile was observed in electrophoretic studies (Gogoi *et al.*, 2006).

Several reports are available that confirms mechanism of antimicrobial action. Silver ions can inhibit bacterial DNA replication, damage bacterial cytoplasm membranes, depleting levels of intracellular adenosine triphosphate (ATP) and finally cause cell death (Feng *et al.*, 2000). Silver nanoparticles have the ability to anchor to the bacterial cell wall and subsequently penetrate it, thereby causing structural changes in the cell membrane like the permeability of cell membrane and death of the cell. There is formation of ‘pits’ on the cell surface and accumulation of the nanoparticles on the cell surface (Sondi and Salopek-Sondi, 2004). Amro *et al.*, (2000) suggested that metal depletion may cause the formation of irregularly shaped pits in outer membrane and change membrane permeability, which is caused by the progressive release of lipopolysaccharide molecules and membrane proteins. The formation of free radicals by the silver nanoparticles may be considered to be another mechanism by which the cells die. Sondi and Salopek- Sondi, (2004) speculated that a similar mechanism may cause the degradation of membrane structure of *E. coli* during treatment with Ag NPs. Although it is assumed that AgNPs are involved in some sort of binding mechanism, the mechanism of interaction between AgNPs and components of outer membrane is still unclear.

Some studies have reported that, the positive charge on Ag\(^+\) ion is crucial for its antimicrobial activity through the electrostatic attractions between the negatively charged cell membrane of microorganisms and the positively charged nanoparticles (Sondi and Salopek-Sondi, 2004; Tiwari *et al.*, 2008; Vijayakumar *et al.*, 2013). In addition, Ag NPs not only interact with the surface of membrane, but can also penetrate inside the bacteria (Sharma *et al.*, 2009). AgNPs have also intensive tendency to react with sulfur and phosphorus groups, thus the cell membrane proteins containing sulfur and compounds containing phosphorus
such as DNA are likely to be the preferential sites for AgNPs (Dehkordi et al., 2011). AgNPs have not been shown to cause bacterial resistance currently complicating antibiotic therapy of bacterial infections.

Nasrollahi et al., (2011) confirmed extensive damage to cell membranes with the formation of ‘pits’ and conclude this to be the primary cause of cell death. Lokini and Narayanan, (2013) showed that AgNPs could destabilize the outer membrane and rupture the plasma membrane, thereby depleting intracellular ATP. Silver has a greater affinity to react with sulfur or phosphorus-containing biomolecules in the cell; therefore, sulfur-containing proteins in the membrane or inside cells and phosphorus containing elements like DNA are likely to be preferential sites for binding AgNPs. Danilczuk et al., (2006) reported that Ag-generated free radicals derived from the surface of Ag NPs were responsible for antimicrobial activity. The Ag-NPs can easily reach to nuclear content of bacteria and they present the large and impressive surface area (Chudasama et al., 2009; Chen et al., 2010). This could be the reason behind their excellent antibacterial effect.

Petica et al., (2008) reported that the mechanism of antibacterial effects of silver ions (Ag+) involves their absorption and accumulation by bacterial cells and shrinkage of the cytoplasmic membrane and its detachment from the cell wall. Due to the infiltration of cell by Ag+ ions, DNA molecules become condensed and incapable of replication. It has been reported that the mode of action of silver nanoparticles is similar to that of silver ions. However, the effective bactericidal concentration of silver nanoparticles is at a nanomolar level as compared to a micromolar level for silver ions (Kong and Jang, 2007). The phosphotyrosine profile of bacterial peptides is altered by nanoparticles. It was found that the nanoparticles dephosphorylate the peptide substrates on tyrosine residues, which leads to the inhibition of signal transduction and eventually leads to stoppage of growth.

However, Lara et al., (2010 a) in another report, proposed another mechanism of bactericidal action based on the inhibition of cell wall synthesis, protein synthesis mediated by the 30S ribosomal subunit and nucleic acid synthesis. The proteomic data revealed that a short exposure of E. coli cells to antibacterial concentrations of AgNPs resulted in an accumulation of envelope protein precursors, indicative of the dissipation of proton motive force (Lok et al., 2006). Consistent with these proteomic findings, AgNPs were shown to destabilize the outer membrane, collapse the plasma membrane potential and deplete the
levels of intracellular ATP (Dibrov et al., 2002). The mode of action of AgNPs was also found to be similar to that of Ag$^+$ ions; however, the effective concentrations of silver nanoparticles and Ag$^+$ ions were at nanomolar and micromolar levels. Therefore results in *E. coli* suggested that silver nanoparticles may damage the structure of bacterial cell membrane and depress the activity of some membranous enzymes, which cause *E. coli* bacteria to die eventually (Li et al., 2010).

As this is evident from the above discussion, it is unlikely that AgNPs mediated inhibition of microorganisms involves a simple, one-dimensional route. Analysis of intercellular metabolites and gene expression levels could provide information about the effect of nanoparticles on the organism as well as the possible causes. Niazi *et al.*, (2011) did genome wide analysis of *S. cerevisiae* under silver nanoparticle stress and identified 90 genes affected by both Ag NPs and Ag-ions, among these; metalloprotein mediating high resistance to metal were strongly induced by AgNPs (45-folds) and Ag-ions (22-folds) respectively. A total of 17 genes, responsive to chemical stimuli, stress and transport processes were differentially induced by AgNPs. The differential expression was also seen with Ag-ions that affected 73 up and 161 down regulating genes and most of these were involved in ion transport and homeostasis.

Although the interaction of AgNPs with viruses is still an unexplored field, the mechanism of action of AgNPs as an antiviral and virucidal has been studied against several enveloped viruses. Nanosilver may interfere with the fusion of the viral membrane, inhibiting viral penetration into the host cell (Mehrbod *et al.*, 2009). Elechiguerra *et al.*, (2005) has suggested that nanoparticles bind with a viral envelope glycoprotein and inhibit the virus by binding to the disulfide bond regions of the CD4 binding domain within the HIV-1 viral envelope glycoprotein gp120. This fusion inhibition was later elegantly demonstrated by Lara *et al.*, (2010 b). It has been suggested that silver nanoparticles interact with virus via preferential binding to the gp120 glycoprotein knobs. Due to this interaction, silver nanoparticles inhibit the virus from binding to host cells as demonstrated *in vitro* (Jun *et al.*, 2007). It has been reported that the HIV-1 virus binds exclusively to silver nanoparticles whose size is in the range of 1-10 nm (Elechiguerra *et al.*, 2005).

### 2.5 Applications of silver nanoparticles
The use of silver nanoparticles is expanding rapidly primarily due to the benefits promised by their incorporation into products. The electrochemical properties of AgNPs incorporated them in nanoscale sensors that can offer faster response times and lower detection limits. For instance, Manno et al., (2008) electrodeposited AgNPs onto alumina plates gold micro-patterned electrode that showed a high sensitivity to hydrogen peroxide (Hahm and Lieber, 2004). Catalytic activities of nanoparticles differ from the chemical properties of bulk materials. Furthermore, AgNPs was found to catalyze the chemiluminescence from luminal-hydrogen peroxide system with catalytic activity better than Au and Pt colloid (Guo et al., 2008). Silver nanoparticles supported halloysite nanotubes (Ag/HNTs), with Ag content of about 11% to catalyze the reduction of 4-nitrophenol with NaBH₄ in alkaline aqueous solutions (Liu and Zhao, 2009). The optical properties of a metallic nanoparticle depend mainly on its surface plasmon resonance, where the plasmon refers to the collective oscillation of free electrons within the metallic nanoparticle. It is well known that the plasmon resonant peaks and line widths are sensitive to the size and shape of nanoparticles, metallic species and surrounding medium. For instance, nanoclusters composed of 2-8 silver atoms could be the basis for a new type of optical data storage. Moreover, fluorescent emissions from the clusters could potentially be used in biological labels and electroluminescent displays (Berciaud et al., 2005; El-Sayed, 2001; Kelly et al., 2003; Jin et al., 2001; Mulvaney, 1996; Kossyrev et al., 2005).

The nanoparticles synthesized through biological synthesis (diatoms, fungi, bacteria and yeast) were found to be more biocompatible (Guidelli et al., 2011). The application of Ag NPs in medical can be divided into two types i.e. diagnostic and therapeutic uses. Recent studies of AgNPs lead to the utilization in some important applications such as diagnostic imaging, therapy, bio-sensing and cancer diagnosis (Majdalawieh et al., 2014). Lim et al., (2011) reported that Surface Enhanced Raman Spectroscopy (SERS) based on AgNPs can be used in cancer detection in non-invasive way. The applications of silver nanoparticles has been reported in wound dressing, scaffolds, eye treatment, dental hygiene (Cao et al., 2010) and bone substitute biomaterials. The exact mechanisms of wound healing have not yet known, however silver nanoparticles also increased mechanical strength by improving collagen alignment (Kwan et al., 2011). Acticoat, made of two layer of polyamide ester membranes coated with silver nanoparticles is most popular wound dressing (Trop et al.,
By Incorporating silver nanoparticles at surface antibacterial properties can be increased without affecting biocompatibility (Cao et al., 2010) and also provides superior cosmetic after wound healing and better efficacy. Silver contained materials are used for surgical meshes. Central Venous Catheters (CVC) coated with silver nanoparticles are less infectious in blood stream (Wong and Liu, 2010). Albumin stabilized silver nanoparticle has improved antiviral properties of human serum (Sun et al., 2005). AgNPs are considered to be used as drug delivery vehicles and cancer therapeutic agents. Interferon gamma and tumor necrosis can also be inhibited by Ag nanoparticles (Shin et al., 2007). Nano silver can be used for destroying unwanted cells due to its plasmonic nature. The cells can be conjugated to the target cells and then be used to absorb light and convert it to thermal energy. The thermal energy can lead to thermal ablation of the target cells (Loo et al., 2005). Recently, the outbreak of infectious diseases such as cholera (*V. cholera*), influenza (A/H5N1), diarrhea (*E. coli*) etc. around the world has become a burden on public health and economics. Transmission of pathogens like bacteria, fungi, virus etc. causes outbreaks of such infectious diseases. Disinfectant products based on silver nanoparticles have been recommended for the cures of environment containing such pathogens (Majdalawieh et al., 2014).

AgNPs have been used extensively as anti-bacterial agents in the health industry, food storage, textile coatings, disinfecting medical devices/home appliances, water treatment and a number of environmental applications (Bosetti et al., 2002; Cho et al., 2005; Gupta and Silver, 1998; Jain and Pradeep, 2005; Li et al., 2008). Apart from the diverse applications in research and medicine (Salata, 2004), silver nanoparticles are found in an increasing number of consumer products such as food packaging, odour resistant textiles, household appliances and cosmetics. Silver nanoparticles are used in products ranging from vacuum cleaners and washing machines to wound dressing and medical devices. It is also popular as a coating in antibacterial kitchenware, socks, textiles, cleaning products, air filters, teeth cleaners, toothpaste, baby products and nutritional supplements. According to the Nanoparticles News Review (2009), the global market for nanotechnology increased from $11.6 billion in 2007 to $12.7 billion in 2008. It should reach 91.1 Million USD by 2020, at a compound annual growth rate of 5.4% from 2015 to 2020. Electronics, biomedical and consumer applications will have high projected growth rates of 30.3 %, 56.2 % and 45.9 % over the next 5 years.
Antimicrobial activity of silver nanoparticles encouraged the textile industry to use AgNPs in different textile fabrics. In this direction, silver nanocomposite fibers were prepared containing silver nanoparticles incorporated inside the fabric (Yeo et al., 2003). The cotton fibers containing AgNPs exhibited high anti-bacterial activity against *Escherichia coli* (Yeo et al., 2003; Duran et al., 2007; Chen and Chiang, 2008). The catalytic and antimicrobial properties of nanoparticles are now used for the waste water treatment or water disinfectant. A number of contaminants such as lead and pesticides are affecting water supplies globally due to their widespread use; pollutants of geochemical origin such as arsenic and fluoride are currently found in selected countries. It is quite manifested that pesticides contain highly toxic recalcitrant groups and hence are extremely difficult to break through normal synthetic routes of degradation. A useful example of size-dependent catalysis has been demonstrated through AgNPs-catalyzed reduction of nitrophenol to aminophenol (Pitsillides et al., 2003).

Although, it has been demonstrated that AgNPs function as broad-spectrum virucidal, bactericidal agents and in addition, increase wound healing, but safety has not been demonstrated extensively in animal models and therefore, additional testing of AgNPs is needed before they can be used in clinical applications. Moreover, in order to propose any biological applications of AgNPs, it is mandatory to investigate the toxicity of this nanomaterial. It soon became more important for antimicrobial finished fabrics to protect the wearer from bacteria than it was to simply protect the garment from fiber degradation (Yadav et al., 2006). The need for antimicrobial fabrics increased with the rise in resistant strains of microorganisms. The functional textiles include everything from antimicrobial finished textiles, to durable, permanent press finished garments, textiles with self-cleaning properties and textiles with nanotechnology (Rajendran et al., 2010). The coated antimicrobial sutures have also been developed to aid fast wound heal without microbial infection (Dubas et al., 2011). The antimicrobial effect of biologically synthesized silver nanoparticles from *Fusarium oxysporum* was observed, when incorporated in cotton fabrics against *S. aureus* (Duran et al., 2007). Nanoparticles also aid in dye degradation and it has been found that AgNPs are good, highly efficient and stable photocatalysts under ambient temperature for degradation of organic compounds and dyes (Wang et al., 2008). The bleaching of organic
dyes by application of potassium peroxodisulphate in aqueous solution at room temperature is enhanced strongly by the application of silver nanoparticles (Kohler et al., 2008).

Soloviev, (2007) have reported the most promising applications of biologically inspired nanoparticles in nanobiotechnology and in tissue and cell specific drug delivery. Unlike liposomes, dendrimers, metal and semiconductor nanoparticles, the nanoparticles made of biopolymers, such as bacterial spores, viruses and alike are naturally uniform in size and offer precise control for the surface displayed targeting groups and their components. Nanoparticles have also been modified for early detection of Alzheimer's disease biomarkers in biological fluids as well as delivery of bioactive molecules directly to brain. Although nanotechnology is expected to have a huge impact on the development of “smart” drug delivery and devices against Alzheimer's disease, a crucial gap still to be filled concerns the elucidation of its etiology (Brambilla et al., 2011). The cytotoxicity of silver nanoparticles synthesized by using Iresine herbstii leaf aqueous extracts toward the HeLa cervical cancer cell line was studied (Dipankar and Murugan, 2012).

Silver oxide nanoparticles exhibited antitumor properties in transplanted Pliss lymphosarcoma tumor models when administered by intravenous injection in the form of aqueous dispersions (Rutberg et al., 2008). Silver oxide nanoparticles exhibited antitumor properties in transplanted Pliss lymphosarcoma tumor models, when administered by intravenous injection in the form of aqueous dispersions (Rutberg et al., 2008). It is necessary to use designed and engineered NPs especially in therapy that can be targeted to tissues of interest, also can produce specific, desired effects. The function of AgNPs as antibacterial agents has been well established.

2.6 C2C12 cell line

C2C12 cells are a mouse myoblast cell line derived from satellite cells, whose behavior corresponds to that of progenitor lineage. These cells are a subclone of C2 myoblasts (Yaffe and Saxel, 1977), which spontaneously differentiate in culture after serum removal (Blau et al., 1983). Under appropriate conditions, these cells differentiate into contractile myotubes and produce characteristic muscle proteins. In the undifferentiated condition, C2C12 myoblasts are flat, fusiform or star-shaped mono-nucleated cells.

2.7 Cytotoxicity of silver nanoparticles
The rapid development of nanotechnology has lead to an increasing concern related to possible toxic effects of nanomaterials, including human health and environmental impact direct and indirect exposures to nanoparticles (Panda et al., 2011). As production and the use of nanoparticles is increasing exponentially, the chances of unplanned events leading to their dissemination and accumulation in the environment is also increasing and could lead to unpredicted changes to biological systems (Williams et al., 2006). The small scale of these materials coupled with their unique physicochemical properties may cause some engineered nanomaterials to pose potential risks to human health and the environment. In fact, nanoparticles may have higher toxicity than bulk materials (Donaldson et al., 1999). There is a huge need to understand the potential risks, which nanoparticles may pose to human health as a result of consumer, medical, occupational and environmental exposure.

Information on the toxicological implication of the use of silver nanoparticles is limited in contrast to potential applications of silver nanoparticles (Wijnhoven et al., 2009). Nanoparticles in the wider environment could pose a hazard to a large number of species including plants, microorganisms, invertebrates, fish and mammals, potentially acting at the individual or population level and impacting on the structure and function of ecosystem as a whole. Silver nanoparticles intentionally or unintentionally may be released in the environment. So, it is quite necessary to study the effect of AgNPs to ecological components.

Nanoparticle toxicity, including human health and environmental implications is still considered not completely elucidated and relatively unexplored (Nel et al., 2006; Lewinski et al., 2008). Silver has been reported as toxic to humans or animal cells, when in nanoparticle form, with reported observations of a cytotoxic response nearly identical to that for chrysotile asbestos (Soto et al., 2005). Nanosilver aggregates are said to be more cytotoxic than asbestos (Soto et al., 2008). Human health, studies have demonstrated that nanoparticles have toxic effects at the cellular, subcellular and biomolecular levels, such as genes and proteins (Gurr et al., 2005; Chi et al., 2009). Only few studies have been conducted to assess the toxicity of silver nanoparticles. Inflammation, oxidative stress, genotoxicity and cytotoxicity are the main consequences of silver nanoparticles exposure. Irreversible pigmentation in the skin (argyria) and eyes (argyrosis) with other toxic effects like irritations, organ damage and blood count change are the possible adverse effects due to prolonged exposure of Ag+ ions (Panyala et al., 2008). Even commercially available silver-based dressings have been proved
to have cytotoxic effects on various experimental models (Burd et al., 2007). The smaller sized nanoparticles were found to be more toxic, compared with larger nanoparticle sizes (Gaiser et al., 2012). Nanomaterials including nanosilver, because of their extremely small size, which is comparable to the size of viruses, may have the ability to enter, translocate within and damage living organisms. Some nanoparticles are able to penetrate the lung or skin and may also enter the circulatory and lymphatic systems of humans and animals, finally may also enter body tissues and organs and can disrupt cellular processes that can leads to fatal consequences. The silver nanoparticles primarily accumulate in the liver (Johnston et al., 2010) but have also been reported to be toxic in other organs including the brain (Ahmed et al., 2010). Some studies have reported that NPs are able to cross the blood-testis and blood-brain barrier (De Jong et al., 2008; Lankveld et al., 2010).

There are various routes from where the nanoparticles can enter inside the human body like the respiratory tract, gastrointestinal tract (GIT) and skin. Another possible route of exposure is systemic administration as nanoparticles have been explored for therapeutic applications and diagnostic imaging. After absorption across the lung epithelium, nanomaterials can enter the blood and lymph to reach cells in the bone marrow, lymph nodes, spleen and heart (Hagens et al., 2007; Oberdorster et al., 2005 a). Silver nanoparticles have been found in the blood of patients with blood diseases and in the colon of patients with colon cancer (Gatti, 2004). After Inhalation, silver nanoparticles may migrate to the olfactory bulb, where they may locate in mitochondria and can translocate to the circulatory system, liver, kidneys and heart (Oberdoster et al., 2005 a, 2005 b; Takenaka et al., 2001). At these sites, nanoparticles can undergo a series of processes like binding and reacting with proteins, phagocytosis, deposition, endosome formation and translocation. The nanoparticles on interaction with tissues can elicit the cellular responses such as activation of cell, reactive oxygen species (ROS) generation, inflammation or apoptosis and cell death (Chen et al., 2006; Xia et al., 2006; Ahn et al., 2005).

Numerous studies have documented the toxicity of silver nanoparticles to cultured mammalian cell lines. Silver nanoparticles have been reported to impair mitochondrial function and to increase membrane leakage of mouse spermatogonial stem cell and rat liver cells (Braydich-Stolle et al., 2005; Hussain et al., 2005). In vitro toxicity assay of silver nanoparticles in rat liver cells has shown that even low-level exposure to silver nanoparticles
resulted in oxidative stress and impaired mitochondrial function (Hussain et al., 2005). Silver nanoparticles also proved to be toxic to In vitro mouse germ line stem cells as they impaired mitochondrial function and caused leakage through the cell membranes. Aggregated AgNPs have been reported to be cytotoxic to alveolar macrophage cells as well as epithelial lung cells (Soto et al., 2007).

Silver is lethal for bacteria, keratinocytes and fibroblasts (Poon and Burd, 2004). In vitro experiments demonstrated that AgNPs exhibited significant cytotoxicity to human cells (AshaRani et al., 2009; Kawata et al., 2009). Previous studies reported that AgNPs induced genotoxicity and cytotoxicity in both cancer and normal cell lines (Yoon et al., 2007). Greulich et al., (2009) have reported the concentration-dependent activation of human mesenchymal stem cells (hMSCs) and cytotoxicity was observed at Ag-NPs concentrations above 5 μg ml⁻¹. Zanette et al., (2011) reported a concentration and time-dependent decrease of viability in human-derived keratinocyte HaCaT cell line model. In another publication, Kawata et al., (2009) have reported the In vitro toxicity of Ag-NPs at non-cytotoxic doses to HepG2 human hepatoma cells. Ag nanoparticles induce oxidative stress and DNA damage in hepatoma cell line, HepG2 and lead to cell death (Kim et al., 2009). It has also been reported that Ag nanoparticles are highly toxic to the osteoblast cell lines (Moaddab et al., 2011). Silver nanoparticles also showed cytotoxic effect against human fibrosarcoma and human skin/carcinoma cells (Arora et al., 2008). Vivek et al., (2012) reported the In vitro cytotoxicity of biogenic AgNPs against normal epithelial cells (HBL-100) and human breast cancer cell (MCF-7). AgNPs exhibited a toxic effect on cancer cells with 88 % death of HeLa cells upon treatment with 300 μg/ml AgNPs (Dipankar and Murugan, 2012).

Significant evidence has been reported in relation to the toxicity of silver nanoparticles to higher organisms. It has been shown toxic to fish such as zebrafish (Asharani et al., 2009; Yeo and Kang, 2008; Yeo and Yoon, 2009), Diptera species such as Drosophila melanogaster (Ahmed et al., 2010) and different mammalian cell lines of mice (Braydich-Stolle et al., 2005; Hussain et al., 2005), rats (Kim et al., 2008) and also humans (Asharani et al., 2009; Braydich-Stolle et al., 2005; Hsin et al., 2008; Hussain et al., 2005). Highly purified silver nanoparticles affected early development of zebra fish embryos (Lee et al., 2007). In vivo studies on the oral toxicity of nanosilver on rats have revealed that the target organ in mouse for AgNPs was liver. Histopathological studies showed that there was a
higher incidence of bile duct hyperplasia, with or without necrosis, fibrosis and pigmentation in the study animals (Kim et al., 2010). Nanosilver also has toxic effects on aquatic animals because silver ions can interact with the gills of fish and inhibit basolateral Na\(^{+}\)-K\(^{+}\)-ATPase activity, which can in turn inhibit osmoregulation in the fish (Wood et al., 1993). Silver nanoparticles have to be classified under ‘category acute 1’ as per the Globally Harmonized System of Classification and Labelling of Chemicals, suggesting that the release of nanosilver into the environment has to be carefully considered (Asghari et al., 2012).

Liu et al., (2010) reported that the cytotoxicity of silver nanoparticles depends on the size of nanoparticles; as smaller nanoparticles were found to be more toxic than larger, as earlier can easily enter cells. Kittler et al., (2010) have demonstrated that the toxicity of Ag-NPs increases during storage because of slow dissolution under release of silver ions. The modification of nanoparticles can also affect cytotoxicity of silver nanoparticles, lactose modified AgNPs caused more DNA damage in A549 cells compared to oligonucleotide-modified AgNPs (Sur et al., 2012).

The nanoparticles in plants can enter cellular system via roots and stomata, effect transpiration, plant respiration, photosynthesis and interfere with translocation of food material (Rana and Kalaichelvan, 2013). AgNPs increased the number of chromosomal aberrations, micronuclei and decreased the mitotic index in Vicia faba (Patlolla et al., 2012). Nanosilver with its antimicrobial activity can hinder the growth of many ‘friendly’ bacteria in the soil. By showing toxic effects on denitrifying bacteria, silver can disrupt the denitrification process, which involves the conversion of nitrates into nitrogen gas, which is essential for the plants. Loss of environmental denitrification through reduction of plant productivity can lead to eutrophication of rivers, lakes, marine ecosystems and destroy the ecosystem (Senjen, 2007).

Recently, In vitro tests were performed to evaluate the cytotoxicity and mechanisms involved in the toxicity of different metallic nanoparticles. NPs are capable of binding to cells as well as macromolecules like proteins and DNA (AshaRani et al., 2009). When NPs come in contact with cells, they are taken up by a variety of mechanisms that can lead to activation of cellular signalling processes producing reactive oxygen species (ROS), inflammation and finally cell cycle arrest or cell death (Ahmed et al., 2008, 2010; Asha Rani et al., 2009). Notably, AgNPs are capable of entering the nucleus and directly or indirectly interacting with
nuclear material (Kruszewski et al., 2011), leading to alterations in DNA integrity or affecting its synthesis. ROS formation was suggested to be a key event in DNA damage induction in A549 cells, a human lung cancer cell line, exposed to AgNPs (Foldbjerg et al., 2011). Silver nanoparticles decreased levels of glutathione, indicating oxidative stress that results in cellular damage and lipid peroxidation in on human fibrosarcoma and human skin/carcinoma cells (Arora et al., 2008). There is evidence that shows that silver ions cause changes in the permeability of cell membrane to potassium and sodium ions or mitochondrial activity (Kone et al., 1988). Silver nanoparticles have demonstrated inhibition of the formation of new blood vessels (angiogenesis) in mice with tumors, possibly due to inactivation of PI3K signalling pathways. (Gurunathan et al., 2009). Silver nanoparticles can induce toxic effects on the proliferation and cytokine expression by peripheral blood mononuclear cells (Shin et al., 2007).

2.7.1 In-vitro cytotoxicity assays
Measurement of cell viability and proliferation forms the basis for numerous In vitro assays of a cell population’s response to external factors. Different in-vitro toxicity assays e.g. tetrazolium salts (MTT, MTS, WST, XTT), Lactate dehydrogenase (LDH) and typan blue can be used for the toxicity studies on mammalian cell. The reduction of tetrazolium salts is widely accepted as a reliable way to examine cell proliferation. The yellow 3-(4, 5-dimethylthiazolyl-2)-2, 5- diphenyltetrazolium bromide (tetrazolium MTT) is reduced by metabolically active cells by dehydrogenase enzymes to generate reducing equivalents such as NADH and NADPH. The reduction process forms intracellular purple formazan which can be solubilised and quantified by spectrophotometric analysis (Moshmann, 1983). The MTT assay measures the cell proliferation rate, when metabolic events lead to necrosis or apoptosis. In absence of cells the MTT reagent doses not yield high background absorbance values.

The proliferation assays like MTT has two major limitations: they can give false positive results where specific aspects of cellular metabolism are affected (Berridge et al., 2005) and they are unable to differentiate cell cycle inhibition and cellular death (Galluzzi et al., 2009; Kroemer et al., 2009). To overcome these, multiplexing the assay with a LDH (lactate dehydrogenase) based cytotoxicity assay to allow for further quantification LDH catalyzes the conversion of lactate to pyruvic acid and back, as it converts NAD⁺
to NADH and back. The lactate dehydrogenase (LDH) assay accurately quantifies cytotoxicity of chemicals via the measurement of LDH released from damaged cells. In the assay, LDH catalyzes formation of a reporter chromophore that can be quantified spectrophotometrically at 490 nm peak, a standard assay and related to the released LDH concentration.