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BACTERIAL SYNTHESIS OF SILVER NANOPARTICLES (AGNPS): THE POWERFUL NANOWEAPON

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ABSTRACT

The development of quick and trustworthy processes for the synthesis of nanosized materials is of countless importance in the field of nanotechnology. Biosynthesis of Silver Nanoparticles (AgNPs) using bacteria has arriving weighty interest because of their potential to synthesize nanoparticles. In the current study, synthesis of silver nanoparticles by a bacterial strain (PP-11) isolated from soil is reported. The bacterium was isolated, screened and characterized by morphological, biochemical and 16S rRNA analyses. Molecular identification of the isolate was done which showed a strain is *Bacillus thioparans PP-11*. When treating the isolated bacteria with 1mM Silver nitrate ($AgNO_3$), it was found to have the proficiency to form silver nanoparticles at 37^0C within 24 hours of incubation. This was confirmed by the visual observation and FT-IR analysis. Therefore, the current study is a march of an effective synthesis of silver nanoparticle by the present Bacillus strain. Silver nanoparticles were also tested against antibacterial potential of some human pathogens. Kinetic study of growth parameters of silver nanoparticles producing bacteriumwas carried out with respect to pH, temperature and incubation conditions. It was found that the optimum pH and temperature for the maximum growth of the present isolate were 7.0% and 37^0C respectively.

KEYWORDS: Silver nanoparticles (AgNPs), *Bacillus thioparans*, FT-IR, Antibacterial Potential.

INTRODUCTION

Now-a-days, Silver Nanoparticles are evolving as a new generation of antibacterial agent, which has been used in medical applications, hygiene and antibacterial water filter. Silver Nanoparticles have proved to be the most effective as it has good antibacterial efficiency against bacteria, other eukaryotic micro-organisms and viruses. Microorganisms affecting the mobility and reactivity of metals can be used for remediation and detoxification. Many organisms both eukaryotes and prokaryotes are known to produce many inorganic materials either extracellularly or intracellularly (Ahmad *et al*., 2003).[1] These specific characteristics of organisms particularly of microorganisms can be used in the synthesis of nanoparticles. The metallic nanoparticles such as titanium, magnesium, copper, gold, zinc and alginate have a robust bactericidal potential owing to their large surface-area-to-volume ratio (Gu *et al*., 2003[2]; Ahmad *et al*., 2005).[3] Silver Nanoparticles (AgNPs) have an significant advantage over antibiotics made conventionally in that it kills all pathogenic microorganisms and no organism has ever been described to readily develop resistance to it (Dameron 1989).[4] The silver nanoparticles synthesized by microbes can be used in target oriented as an antiseptic in waste water treatment, food industry, drug delivery, making electronic devices, in curing and detecting many diseases. Biologically synthesized nanoparticles find application in the fields of bioremediation, bio-sensors biolabelling and many more (Nair *et al*., 2002).[5] Nanoparticles produced by the chemical processes had toxic effect hence there is a rising need to develop eco- friendly, cost competitive and conveniently reproducible green methods of nanoparticle synthesis. Biological synthesis process provides a wide range of environmentally acceptable methodology, minimum time required and low cost production. Bioscience can be employed to discover medical sciences in targeted drug delivery (Langer, 2001)[6], artificial implants (Streicher, 2007)[7],fighting pathogens (Stoimenov, 2002)[8] and understanding the role and applications of microorganisms for the remediation of toxic and radionuclide contaminated sites and antibacterial effects. In the present work, a bacterium was isolated and molecularly identified as *Bacillus thioparans PP-11*by 16s rRNA sequencing. This bacterium was further used for the synthesis of AgNPs and which was found to be fairly dispersed and exhibited effective antimicrobial effect. The characterization of silver nanoparticles was done by Fourier Transform -Infra Red (FTIR) analysis.

MATERIALS AND METHODS

Chemicals and Reagents: Analytical grade silver nitrate ($AgNO_3$) used in the project was procured from laboratory.

About 100mM solution of silver nitrate (1.6987g of $AgNO_3$ in 10ml deionized water) was prepared and stored in clean amber bottles in dark. It was diluted accordingly to required molar concentrations further upon usage.

Collection of soil sample: The agricultural soil sample was collected from the root zone region of the different plants from Barshi.

Isolation of bacteria: Agricultural field soil sample was collected. Microbial analysis of soil sample was done by serial dilution method and the bacterial ecology of the soil was studied. From the field sample around different bacterial colonies were isolated and sub cultured as pure culture and spread on nutrient agar plates. The plates were then incubated for five days at room temperature. After incubation, the plates were observed for the development of colonies and the number of colony forming units was recorded. The selected strain was streaked on the Petri plate and continued for the further process.

Molecular identification

Molecular identification of the isolated strain was carried out by 16S rRNA sequence-based method. Total genomic DNA was isolated from a selected strain for PCR. Quality of the isolated DNA was checked by agarose gel electrophoresis and was further quantified using UV–Vis spectrophotometer (Hitachi U5100). The sequence data of 16S rRNA thus obtained was further aligned using BioEdit program. This sequence was then used for BLAST analysis. The 16S rDNA sequence of CS 11 was also used for phylogenetic analysis using neighbor-joining method in MEGA5 (Tamura *et al*., 2007).[9]

Kinetic Study of AgNPs Producing Bacterium

Effect of pH: Almost all microorganisms need an optimum pH for their maximum growth. Therefore, it is essential to regulate the pH of the microbiological processes. In the present work the silver nanoparticles producing bacterium was grown in nutrient medium having different pH, all the other optimal conditions were kept constant except pH and the effect of pH was studied. The physical parameter like pH was optimized for the AgNPs production. The nutrient medium having different pH 3.0, 5.0, 7.0, 9.0 and 11 was inoculated in separate tubes with a 24 hours fresh culture of the potential strain and kept for incubation for 24 hours.

Effect of Temperature: Temperature is the most vital factor. Growth of microorganism is a collective activity of a large number of metabolic reactions which was mediated by enzymes.

Therefore, the rate of microbial growth and the enzymatic reactions is always directly proportional to each other. However, in most of the cases with increase in the temperature growth also increases but it decreases rapidly at extreme upper and lower limits of the temperature. In order to determine the effect of temperature on growth of AgNPs producing promising bacterial strain, a series of experiments were executed with different temperatures *Viz.* 25^0C, 37^0C, 40^0C, 45^0C and 50^0C. All these tubes of nutrient medium were incubated at the respective temperatures for 24 hours. The growth of AgNPs producing bacterium further monitored spectrophotometrically.

Static and Shaking condition: The nutrient medium was inoculated with a promising bacterium and incubated at optimum temperature and optimum pH for 24 hours under static conditions. While all the experimental conditions were kept constant as described above. Then the second set of tubes was incubated at constant shaking conditions. These tubes were inoculated separately with specific promising isolate. After optimum incubation time, samples were withdrawn from the tubes and the growth of AgNPs producing bacterium was measured spectrophotometrically at its specific absorbance maxima. A comparison was made between shaking vs. static condition.

Biosynthesis of Ag-NPs

Bacterial strain was grown in nutrient broth. The final pH was adjusted to 7.0. The flask was then incubated at 200rpm at 37^0C. After 24 hours of incubation, the biomass was separated by centrifugation. The supernatant and pellet was then challenged with silver nitrate (prepared in deionized water) and incubated in dark condition at 37^0C. Simultaneously, a positive control of silver nitrate solution and deionized water and a negative control containing only silver nitrate solution were maintained under same conditions.

Fourier-Transform Infra-Red (FT-IR) Analysis

The biotransformed products formed in the present study was monitored by FTIR spectroscopy. The products present in the extracellular filtrate were then freeze-dried and diluted with potassium bromide in ratio of 1:100 (Saifuddin *et al*., 2009).[10] The FT-IR spectrum of sample was monitored on Fourier Transform Infrared Spectrometer and compared with control on a FT-IR instrument (Perkin Elmer Spectrum 65).The samples were mixed with spectroscopically pure KBr in the ratio of1:100 and pressed to obtain IR-transparent pellet. The pellet was placed in sample holder and the analysis was carried out in the mid IR region of 450-4000 cm -1 with 16 scan speed.

Antibacterial Activity of Silver Nanoparticles (AgNPs)

The antibacterial activity of the biologically synthesized AgNPs against pathogenic organisms such as Gram positive was measured using the Agar Well Diffusion Assay. The Muller Hinton Agar was sterilized by autoclaving at 121^0C (15 psi pressure) for 15 minutes and further used to determine the antibacterial activity of silver nanoparticles from isolated bacterium (Seema Sharma *et al*., 2011).[11] *In Vitro* profiling of antibacterial activity of the Silver Nanoparticles was studied. The sterile molten agar was cooled to (45^0C) and was poured aseptically into sterile petri dishes (15ml in each) and these plates were then allowed to solidify at room temperature. After solidification of agar the plates were seeded with appropriate bacteria by spreading evenly on to surface of the agar medium with a sterile glass spreader and wells having 8mm diameter were prepared out from the agar using a sterile cork borer which was filled with 0.1ml of the synthesized AgNPs solution in the respective wells. These plates were then incubated at 37^0C for 24 hours. The zones of inhibition were measured with a measuring scale. This experiment was carried out in triplicate for their confirmation. The results were recorded by the presence or absence of zone of inhibition.

RESULTS AND DISCUSSIONS

The biological agents in the form of microorganisms are proficient candidates for the synthesis of nanoparticles. These biogenic nanoparticles are simpler to synthesize, cost-effective and the method is greener in approach.

Isolation and Characterizations of the Bacteria

Microorganism plays a very important role in maintaining soil health, ecosystem function and production. Pure colonies of the isolated organisms were obtained. The morphological and cultural characteristics of organisms were performed according to protocol. A total of 06different bacterial strains were isolated, purified and preserved. Among the different isolated strains, the strain with good antibacterial activity against both Gram positive and Gram negative bacteria was selected for further studies and designated as PP-11.The potential strain was phylogenetically identified as *Bacillus thioparans PP-11* on the basis of its colony characterizations (**Table 1**), biochemical characterizations (**Table 2**) and 16s rRNA gene sequence (**Fig. 1**).

Table 1-Colony Characterizations of the isolates on nutrient agar medium incubated at 37^0C after 24 hours

Sr. No.	Culture Code	Size in mm	Shape	Margin	Elevation	Consistency	Opacity	Colour	Gram nature	Motility
1.	PP -1	1	Circular	Entire	Slightly convex	Moist	Transparent	Creamy	Gram positive Short rods	Highly motile
2.	PP–2	2	Irregular	Irregular	Flat	Moist	Semi Transparent	Creamy White	Gram positive Short rods	Motile
3.	PP–3	1	Circular	Regular	Slightly Convex	Moist	Opaque	White	Gram positive Short rods	Motile
4.	PP–4	1	Circular	Slightly irregular	Raised	Moist	Opaque	Yellowish White	Gram positive Short rods	Motile
5.	PP -5	2	Irregular	Entire	Flat	Moist	Opaque	Creamy White	Gram positive Short rods	Motile
6.	PP-11	2	Circular	Regular	Raised	Moist	Opaque	Creamy	Gram positive Short rods	Highly Motile

Table 2-Biochemical Characterizations of the isolates

Name of Biochemical Tests	Results					
	PP- 1	**PP-2**	**PP-3**	**PP- 4**	**PP-5**	**PP-11**
Fermentation of-						
Glucose	A, G	A	A, G	A	A, G	A, G
Fructose	A, G	A	A	A, G	A	A, G
Lactose	A	A, G	A	A	A	A, G
Maltose	A, G	A	A	A	A, G	A
Sucrose	A	A	A, G	A	A	A, G
Hydrolysis of-						
Starch	+	+	+	+	+	+
Casein	+	+	-	+	-	+
Nitrate Reduction	+	+	+	+	+	+
Enzyme Activity-						
Amylase	+	+	+	+	+	+
Catalase	+	+	+	+	+	+

Where, A, G= Acid and gas, A=Acid, + = Positive test, - = Negative Test

Molecular Identification

The bacterial DNA was isolated and 16S rRNA sequence was then amplified and sequenced. The sequence of the bacterium obtained after 16S rRNA was compared with the BLAST database to obtain the sequences that displayed maximum similarity. All the sequences obtained by BLAST revealed that the present bacterial species showed a very high percentage of sequence similarity (99%) with the sequences of *Bacillus thioparans* with a reasonably high score. The sequences having the maximum sequence similarity were then further used for the alignment using CLUSTAL X2 to construct the phylogenetic relationship.

Fig. 1- Phylogenetic Identification of the isolate PP-11

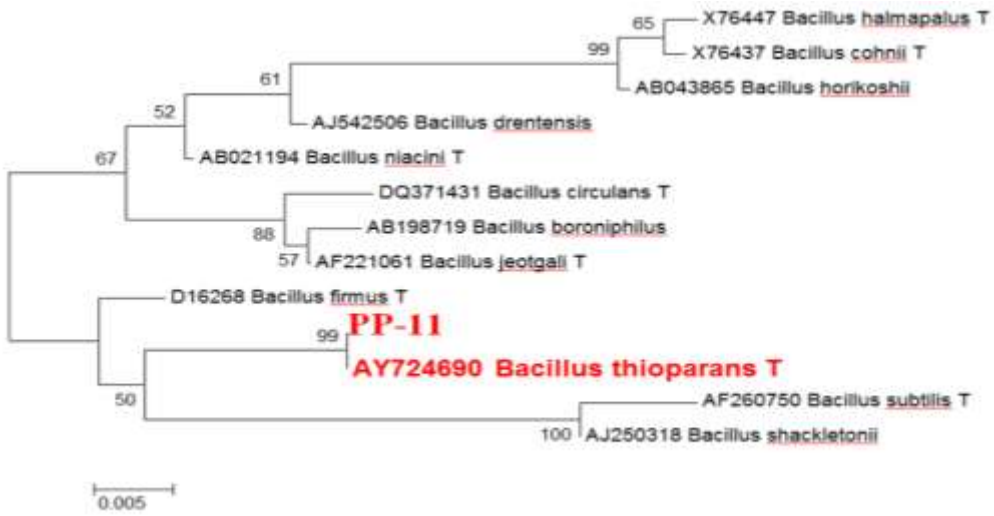


Figure 1:- Phylogenetic analysis of 16s rRNA gene sequence of *Bacillus thioparans PP-11*. The percent numbers at the nodes indicate the levels of bootstrap support based on neighbor-joining analyses of 1,000 replicates. The scale bar (0.005) indicates the genetic distance.

Kinetic Study of AgNPs producing Bacterium

Effect of pH

The optimum pH for AgNPs producing isolate was studied by inoculating the promising organisms in a medium having different pH 3.0, 5.0, 7.0, 9.0 and 11 for 24 hours of incubation. It is apparent from the **Fig. 2** those, the promising isolate showed maximum growth at pH 7.0 in 24 hours. Therefore, the optimum pH for maximum growth of AgNPs producing PP-11was found to be 7.0.

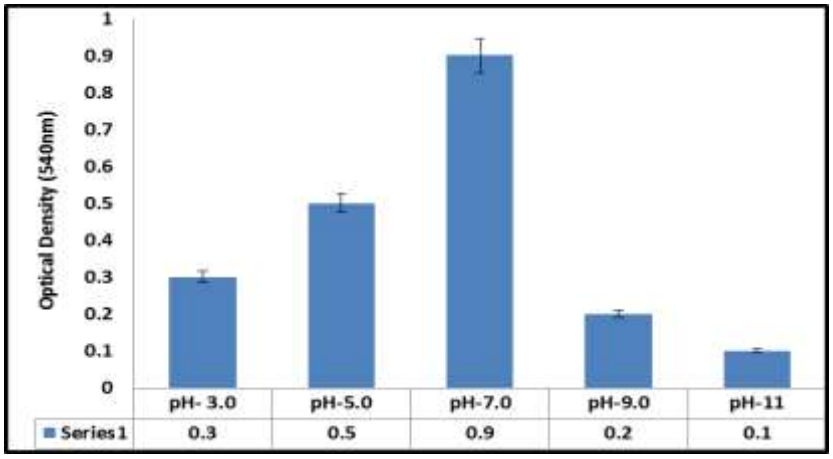


Fig. 2- Effect of pH on growth of AgNPs producing PP-11

Effect of Temperature

The optimum temperature for maximum growth of AgNPs producing bacterium was also evaluated by inoculating the promising isolate in nutrient medium and incubating the promising organism at different temperatures for 24 hours. From the **Fig. 3** it is evident that the promising isolate showed good growth at temperature 37^0C in 24 hours. From the results it is clear that the optimum temperature for maximum growth of AgNPs producing bacterium was 37^0C.

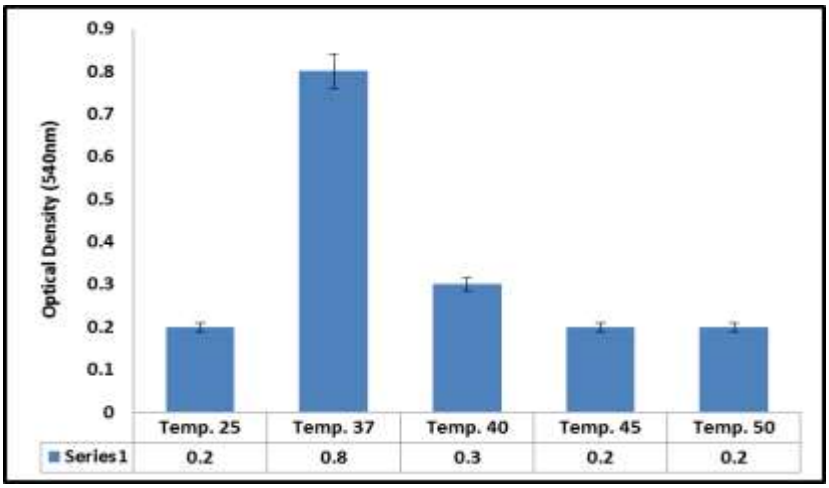


Fig. 3- Effect of temperature on growth of AgNPs producing PP-11

Static and Shaking condition

The effect of different incubation condition on growth of Silver nanoparticles producing organism was also studied by inoculating test isolate in nutrient medium and incubating at 37^0C for 24 hours. It is evident from the **Fig. 4** that maximum growth of promising isolate was found to be at shaking condition.

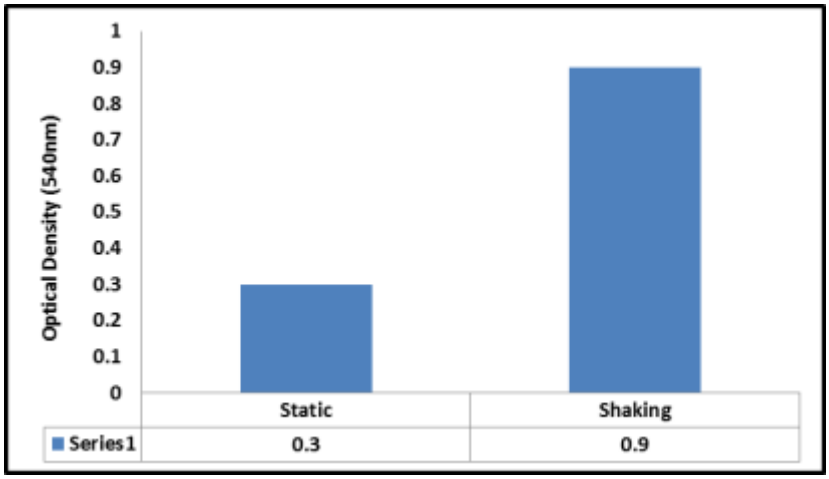
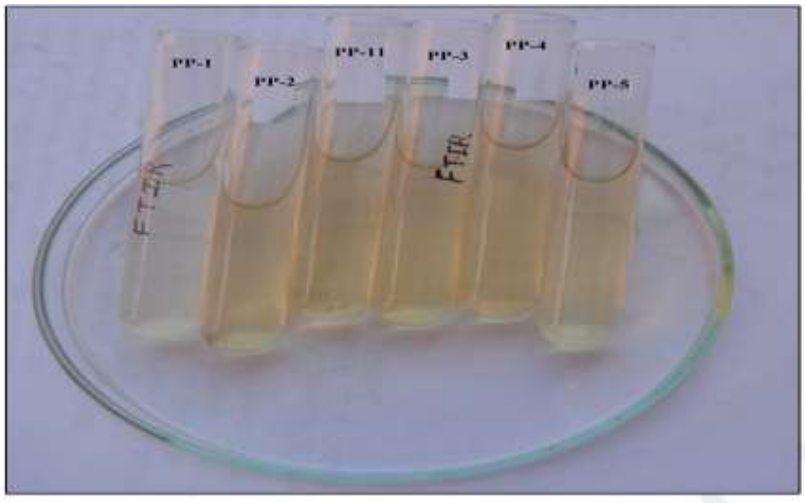


Fig. 4- Effect of incubation conditions on growth of AgNPs producing PP-11

Synthesis of Silver Nano Particles (AgNPs): Vidhya Lakshmi Das *et al*., (2014)[12] reported the synthesis of silver nanoparticles by bacterial strain isolated from heavy metal contaminated soil. In the present study AgNPs production was studied by bacterial strain isolated from soil. The isolated bacterial strain showed the ability to synthesize silver nanoparticles. This was observed by the change in color from white to sunset yellow. The observation on color change is a method generally used for screening microbial isolates for the synthesis of silver nanoparticle (Kalimuthu *et al*., 2008).[13] Similar observation was previously reported for the supernatant of *Bacillus megaterium*, where a pale yellow to brown colour was formed due to the reduction of aqueous silver ions to silver nanoparticles (Saravanan *et al.*, 2011).[14] This supports the fact that the change in color as observed in the experiment can be considered as an indication of silver nanoparticles formation. Similar visual detection method is used in the present study. It was characterized by changes in the color of reaction mixture from white to sunset yellow after 24 hours of incubation. Addition of Silver (Ag+) ions to supernatant and the pellet culture, sample showed the result as color formation to sunset yellow, (**Photoplate 1**).The intensity of color increased with increasing period of incubation due to the reduction in Ag^+ ions. The control (without silver nitrate) showed no formation of color in the culture which when incubated for the same period of time and experimental conditions. The supernatant culture showed no color changes observed on incubation period and the pellet culture containing Ag+ ions shown the change in color to sunset yellow as shown in Photoplate 1. The synthesis of AgNPs also depends on incubation period of the culture which was stated in the previous studies of Vaishali *et al*., (2012).[15] The color changed to profound orange after 24 hours of shaking with silver nitrate ($AgNO_3$) which further blackened in 72 hours of incubation (**Photoplate 2**).

Photoplate1-Visual observation of the biosynthesis of silver nanoparticles by *Bacillus thioparans PP-11*selected for the present study.

Photoplate 2- Photoplate showing change in color during different time period after addition of Silvernitrate solution (AgNO3) (from left to right): A-control, B- 24 hrs, C-48 hrsand D-72 hrs.



Photoplate 3- Sample Preparation for FTIR Analysis

Antibacterial Activity of AgNPs

The biological synthesized AgNPs inhibited different pathogenic microorganisms. *Escherichia Coli, Klebsiella spp., Staphylococcus aureus* were effectively inhibited by the Silver Nanoparticles. The mechanism behind the bactericidal effect of the Silver Nanoparticles against bacteria is not well known. It has been proposed that AgNPs act similarly to the antibacterial agents used for the treatment of bacterial infection by different mechanisms. The Silver Nanoparticles release silver ions, which contribute the bacterial effect. The mechanism of inhibition by silver ions on microorganisms is partially known. It is believed that DNA loss its replication ability and cellular proteins become inactivated upon silver ion treatment furthermore, higher concentration of Ag^+ ions have been shown to interact with cytoplasmic components and nucleic acids.

Confirmation of Synthesis of Silver Nanoparticles by FTIR Spectroscopic Analysis

The synthesis of AgNPs was further confirmed by FT-IR analysis. The FTIR is a powerful device for identifying types of chemical bonds in a molecule by making an infrared absorption spectrum that is like a molecular "fingerprint" Senapati *et al*., (2005).[16] The FTIR analytical study was carried out to explore the functional groups and mechanism of AgNPs formation chiefly to identify likely interaction between protein molecules and silver precursor salt leading to the reduction of silver ions and stabilization of AgNPs (Theivasanthi and Alagar, 2013).[17] The characteristic spectrum of silver nanoparticles is shown in **Fig. 5** It displays absorption peaks at wavelengths 458, 1335, 1338, 1407, 1456, 1506, 1558, 1635, 1653, 1670 and 3442 cm-1. The absorption peaks nearby 1600 and 1350 cm-1 embodies the presence of NO_2 which may be from silver nitrate solution and the metal precursor associated in the AgNPs synthesis process. The strong interaction of H2O with the surface of Ag could be the reason for the O-H group which is denoted by peaks around 1338 cm-1 (Nakamoto, 2006).[18] The peak at 3442 cm-1 includes stretching of OH. The carbonyl groups / carboxyl and NO2 groups (such as nitrates, nitro compounds and nitramines) normally display vibrations at 1660 to 1500 and 1390 to 1260 cm-1 region (Augustine and Rajarathinam, 2012).[19] The peak at 1558 cm-1 shows the formation of metal carbonyl group because of the stabilization of silver nanoparticles (AgNPs) by –COO group. The protein might play a vital function in the stabilization of AgNPs. The presence of bands at 1653 and 1635 cm-1 are due to stretch of carbonyl in the linkages of amide of the proteins. It is well known that proteins can attach to Silver nanoparticles by means of free amine groups of proteins (Theivasanthi *et al*., 2011).[20] The bands at 1457 cm-1 indicate C-N vibrations stretching and amino-methyl

groups / amine respectively. The band seen at 1635 cm-1 is property of –C=C- stretching and –C=O carbonyl groups. This confirms the existence of protein in samples of silver nanoparticles. It reports that protein can bind to nanoparticles either through their free amine groups or cysteine residue and stabilize them. The wave numbers related to amides, amines and amino acids in the FTIR spectrum signify the presence of protein (Ramajo e*t al*., 2009[21], Gopinath *et al*., 2013).[22]

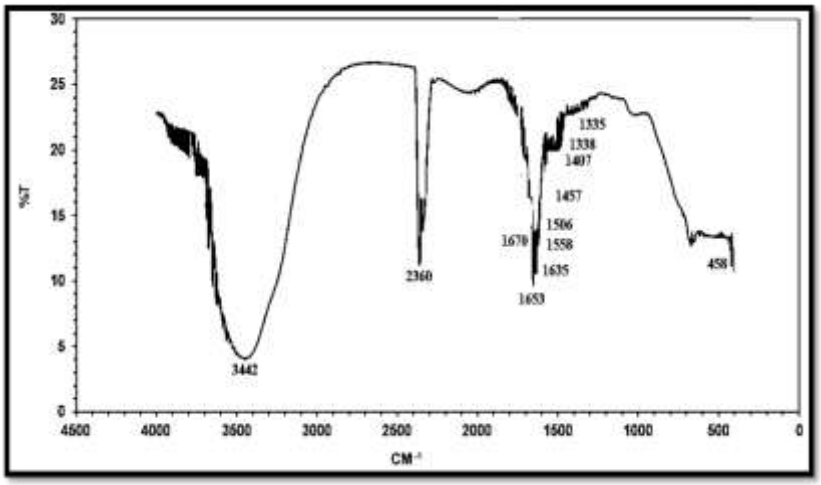


Fig. 5-FT-IR spectra recorded with synthesized silver nanoparticles

The mechanism behind the extracellular synthesis of nanoparticles using microbes is still not fully recognized. But it is considered that the enzyme like nitrate reductase secreted by microbes help in the bio reduction of metal ions to metal nanoparticles (Duran *et al*., 2005).[23] Which was further reported in *Bacillus licheniformis* where nitrate reductase secreted by the bacteria was found to be responsible for the reduction of Ag+ to nanoparticles (Kalimuthu *et al*., 2008).

***In Vitro* Evaluation of Antimicrobial Activity:** Ansar Mehmood *et al*., (2016)[24] also studied the antimicrobial activity of synthesized silver nanoparticles which was determined by Paper Disc Diffusion method against some common selected pathogens (*Klebseilla pneumoniae, Pseudomonas aeruginosa, Escherichia coli, Bacillus* and *subtilis Staphylococcus aureus*). The results of their study showed noticeable antimicrobial activities against both Gram positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*) and Gram negative bacteria (*Klebseilla pneumoniae, Escherichia coli*, *Pseudomonas aeruginosa*). Our results were in strong agreement with their results. The Silver nanoparticles are used as effective antimicrobial agents. They have bactericidal potential against MDR organisms (Rai

et al., 2012).[25] The Silver and its derivatives are extensively used in the medicine for a long time in the therapy of infections caused by bacteria. The method used in the study carried out by P. Shivakrishna *et al*., 2013)[26] is used in the same study. Accordingly, it is now essential to explore the antibacterial activity of synthesized nanoparticles by the present isolated bacterium.

Table 3-*In Vitro* Evaluation of Antimicrobial Activity of AgNPs synthesized from bacterial pellet

Strain Name	Zone of inhibition (ZOI in mm)			
	AgNPs	Culture Filtrate	Silver Nitrate	Streptomycin
Bacillus cereus	24	08	16	32
Staphylococcus aureus	26	14	22	30
Escherichia coli	20	16	18	24
Proteus mirabilis	24	14	13	28
Salmonella paratyphi	24	15	18	30
Klebsiella pneumoniae	28	22	20	30

In Vitro evaluation of antibacterial activity of Silver Nanoparticles was studied against *Bacillus cereus, Staphylococcus aureus, Escherichia coli, Proteus mirabilis, Salmonella paratyphi* and *Klebsiella pneumoniae*. The antibacterial activity of silver nanoparticles was then studied against the pathogenic bacterial strains using Agar Well Diffusion method. The highest activity was 28.00mm diameter of zone inhibition observed against *Klebsiella pneumoniae* followed by 26.00mm diameter of zone inhibition against *Staphylococcus aureus* then *Bacillus cereus, Proteus mirabilis* and *Salmonella paratyphi* having 24.00mm diameter of zone of inhibition. The zone of inhibitions produced by the synthesized silver nanoparticles was observed to compare with the standard streptomycin (P. Shivakrishna *et al*., 2013).

Photoplate 3- Crystals of Silver Nanoparticles by isolates

CONCLUSIONS

This research work demonstrates the silver nanoparticles (AgNPs) producing property of bacterium isolated from soil. The isolated PP-11 was found to have the potential to form silver nanoparticles at 37^0C within 24 hours. The synthesized silver nanoparticles were characterized and confirmed by FT-IR analysis. The nanoparticles have proved the excellent antibacterial activity against common human pathogens. Hence, the biological approach appears to be cost effective alternative to conventional chemical and physical methods of silver nanoparticles synthesis and would be suitable for developing a biological process for commercial large scale-production. The infections caused by such human pathogens need a multiple treatment, having broad spectrum antibiotics. Actually, these types of treatments are less effective, expensive and also more toxic. Nanotechnology offers a good platform to overcome the problem of antibiotics resistance with the help of the powerful Nano weapon *Viz.* silver nanoparticles (AgNPs). Ions of Silver are very reactive and are well known to bind to the important cell components inducing cell death. Conversely, production of silver nanoparticles (AgNPs) through chemical method is very tiresome while, through bacterium such as *Bacillus thioparans PP-11*, it is a faithful and an eco-friendly approach. These can be further useful in several biomedical, as well as industrial many applications.

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