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International Journal of Current Research
Vol. 9, Issue, 11, pp.61849-61856, November, 2017

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

ISSN: 0975-833X

RESEARCH ARTICLE

EXTRACELLULAR BIOSYNTHESIS AND CHARACTERIZATION OF SILVER NANOPARTICLES BY USING *BACILLUS CEREUS* GAD 20 AND THEIR ANTIBACTERIAL POTENTIAL AGAINST *E.COLI* AND *S.AUREUS*

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ARTICLE INFO

Article History:
Received 23rd August, 2017
Received in revised form
19th September, 2017
Accepted 05th October, 2017
Published online 30th November, 2017

Key words:

Bacillus cereus, SEM,
FTIR, Antibacterial activity.

ABSTRACT

The use of bacterial strains in the synthesis of silver nanoparticles emerges as an eco-friendly and exciting approach towards the field of research in life sciences. In this present work, microbial production of silver nanoparticles was investigated using the bacterial strain *Bacillus cereus* GAD 20. The test bacterium was isolated from soil samples from Gadchiroli district of Maharashtra state grown on Hichrome Bacillus Agar and Bacillus Differentiation Agar and further identified on the basis of 16S rRNA. Synthesized silver nanoparticles were characterized by UV-Vis spectroscopy and the maximum absorbance was found to be around λ-427nm. The particle size of silver nanoparticles was studied by Scanning Electron Microscopy (SEM). FTIR analysis confirms the presence of proteins as stabilizing agents. The antibacterial activity of silver nanoparticles was studied against multi drug resistant bacterial strains of *Escherichia.coli* and *Staphylococcus.aureus.* Zone of inhibition of microbes in presence of silver nanoparticles showed inhibition of growth suggesting antibacterial property of the silver nanoparticles.

Citation: Nikhil A. Kolte, P. M. Tumane and Wasnik, D. D. 2017. "Extracellular biosynthesis and characterization of silver nanoparticles by using *bacillus cereus* gad 20 and their antibacterial potential against *e.coli* and *s.aureus*", *International Journal of Current Research*, 9, (11), 61849-61856.

INTRODUCTION

There is a growing concern on the emergence and re-emergence of drug-resistant pathogens like multi-resistant bacterial strains. The development of new resistant strains of bacteria to current antibiotics (Kyriacou *et al.,* 2004) has become a critical serious problem considering with respect to public health; therefore, there is a need of innovative approach to develop new bactericides (Panacek *et al.,* 2006). Improper use of antibiotics (Dos Santos *et al*., 2014) leads to the emergence of resistance genes (D'Costa *et al*., 2011). Today, there is a need of growing concern for alternative treatments(Chen *et al*., 2008).In such situation, non-traditional antibacterial agents are of great interest to overcome the problems associated with resistance that develops from several pathogenic microorganisms against most of the commonly used antibiotics (Dos Santos *et al*., 2014). In recent days nanotechnology has induced great scientific advancement and achievements in the field of research and technology. Nanotechnology can be termed as the synthesis, characterization, exploration and application of nano sized (1-100nm) materials for the development of science. Nanotechnology involving synthesis and applications of nanoscale materials is an emerging field of nanoscience with significant applications in biology, medicine and electronics owing to their unique particle size and shape dependent physical, chemical and biological properties (Albrecht MA *et.al*, 2006). Nanotechnology is also being utilized for human welfare with respect to medicine for diagnosis, therapeutic drug delivery and the development of treatments for many diseases and disorders. Nanotechnology is an enormously powerful technology, which holds a huge promise to design and development of many types of novel products with its potential having medical applications on early disease detection, treatment, and prevention. Nanoparticles are defined as the substances that are intentionally produced, manufactured or engineered to have specific properties and one or more dimensions typically between 1 to 100 nanometres. Synthesis of nanomaterials by biological approach is innovative, cheaper and environmental friendly. Silver nanoparticles are applied in nanomedicine from time immemorial and are still used as powerful antibiotic and anti-inflammatory agents. The non-polluting nanotechnologies have revolutionized the production of nanomaterials as environmentally safe products. Several chemicals used in the synthesis of nanoparticles are toxic which leads to environmental pollution (Esumi, *et al*., 2001).

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Therefore, an alternative methodology is mandatory to trounce to overcome the toxic and polluting chemicals, along with various reducing and stabilizing agents. In this respect, biological methods for the synthesis of silver nanoparticles involving microorganisms or plant extracts are more effective. The naturally available biological resources can be an alternative source for the biosynthesis of nanoparticles (Prathna *et al.,* 2010, Singaravelu *et al.,* 2007 and Mubarak Ali *et al.,* 2011). Recently, biosynthesis methods by employing microorganism such as bacteria (Joerger *et al.,* 2000) and fungus (Shankar *et al* 2003) or plants extract (Shankar *et al* 2003, Chandran *et al* 2006 and Gardea-Torresdey *et al.,* 2002), have emerged as a simple, viable and valuable alternative to more complex chemical synthetic procedures to obtain nanomaterials.

MATERIALS AND METHODS

Isolation and identification of *Bacillus cereus* on the basis of Morphological, Cultural and 16S rRNA: The identification and characterization of the culture was performed on morphological and biochemical pattern. Serial dilution method was performed to get isolated colonies of *Bacillus cereus.* Hichrome Bacillus agar plates were made and the pure culture was isolated after the requisite period of incubation. These colonies are inoculated on Bacillus Differential Agar medium. After that, the plates were examined and the colonies were stained by Gram staining method. Subsequent identification tests including, citrate utilization, motility, Sugar fermentation, Voges-Proskauer, Indole production, Catalase, Oxidase and production of H_2S were performed. Further molecular identification of *Bacillus cereus* by 16S rRNA was done.

Production of cell free supernatant from *Bacillus cereus* GAD 20: For the extracellular synthesis of silver nanoparticles using *Bacillus cereus* GAD 20 involve extraction of cell free supernatant. *Bacillus cereus* was grown in LB medium. The culture flasks were incubated on a shaker and agitated at 120 rpm. The cell supernatants were collected after 24 hours by centrifugation at 5,000 rpm for 10 minutes at 6˚C.

Synthesis of silver nanoparticles: Extracellular synthesis of silver nanoparticles using *Bacillus cereus* supernatant was carried out as described by Shahverdi *et al*., 2007 with slight modifications as described below. 1mM silver nitrate (Final concentration) solution was prepared in double distilled water. 200 ml of aqueous solution of 1mM silver nitrate was treated with 100 ml of *Bacillus cereus* supernatant in a 500 ml Erlenmeyer flask. The whole sample kept in the shaker at 120 rpm and maintained in dark condition. The control was maintained without addition of silver nitrate with the experimental flask containing cell filtrate. The reduction of silver nitrate was coined by visible colour change of the solution.

Characterization of Silver nanoparticles: For the characterization of silver nanoparticles several techniques are used. In the present investigation; UV-VIS spectroscopy, Fourier transforms infrared (FTIR) spectroscopy and scanning electron microscopy (SEM) were used for the characterization of silver nanoparticles.

Antibacterial study of the synthesized silver nanoparticles by Well plate method: The synthesized AgNPs were tested for their antibacterial activity by the agar well diffusion method (Bauer *et al*., 1966) against different kinds of pathogenic multidrug resistant bacteria isolated from clinical samples. The tested strains included; *Staphylococcus aureus* as Gram positive bacteria and *Escherichia coli* as Gram negative bacteria. After adjusting the turbidity of the inoculums suspension (0.5 McFarland Turbidity Standards), a sterilized swab was aseptically dipped into the suspension, rotated several times and pressed firmly on the inside wall of the test tube to removed the excess of the inoculums from the swab. The dried surface of the Muller-Hinton agar plates were inoculated by swabbing over the entire sterile agar surface with the bacteria. This procedure was repeated for two more times by rotating the plate approximately 60^o each time to ensure an even distribution of inoculums. Wells of 6 mm diameter were bored into agar medium using a sterilized cock borer. Using a micropipette, 50 µl (0.2 mg/ml) silver nanoparticles solution was added into each well. After incubation at 37˚C for 24hr, zone of inhibition were measured in mm with zone measuring scale.

RESULTS AND DISCUSSION

A study on extracellular biosynthesis of Silver nanoparticles by the culture supernatant of *Bacillus cereus* GAD 20 was carried out in this work.

Identification of *Bacillus cereus* GAD 20

Morphological and biochemical characteristics of *Bacillus cereus* GAD 20 were outlined in Table 1. On the basis of morphological, biochemical characteristics as well as 16S rRNA study, the isolate was identified as *Bacillus cereus.*

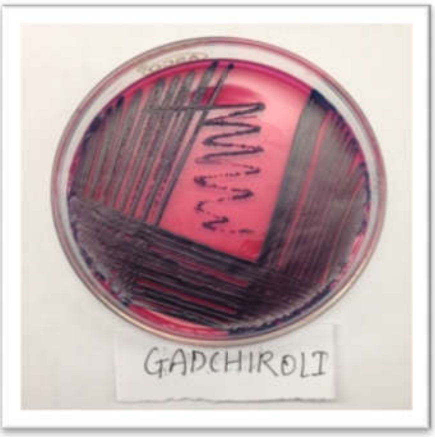
Table 1. Morphological and biochemical characteristics of *Bacillus cereus* GAD 20

Character / Test	*Bacillus cereus* GAD 20
Morphological characteristics:	
Cell shape	Rod
Gram reaction	+
Motility	Motile
Cultural Characteristics:	
Hichrome Bacillus Agar	Pink colored colonies
Bacillus Differentiation Agar	Transparent colonies
MacConkey Agar	White-yellowish colonies
Catalase	-
Oxidase	+
IMViC Test	
Indole production	-
Methyl red	-
Voges- Proskauer	-
Citrate	+
Urease	+
H2S production	-
Utilization of carbon Source	
Glucose	A: + G: -
Sucrose	A: + G: -
Mannitol	-
Lactose	-

+ : Positive Test - : Negative Test
A : Acid production G : Gas production

Visual observation

The detection of synthesized silver nanoparticles was primarily done on the basis of visual observations. Upon addition of Silver nitrate (1 mM Final concentration) into the supernatant (free of any kind of precipitates) of *Bacillus cereus* GAD20 were used as a catalyst for the synthesis of silver nanoparticles



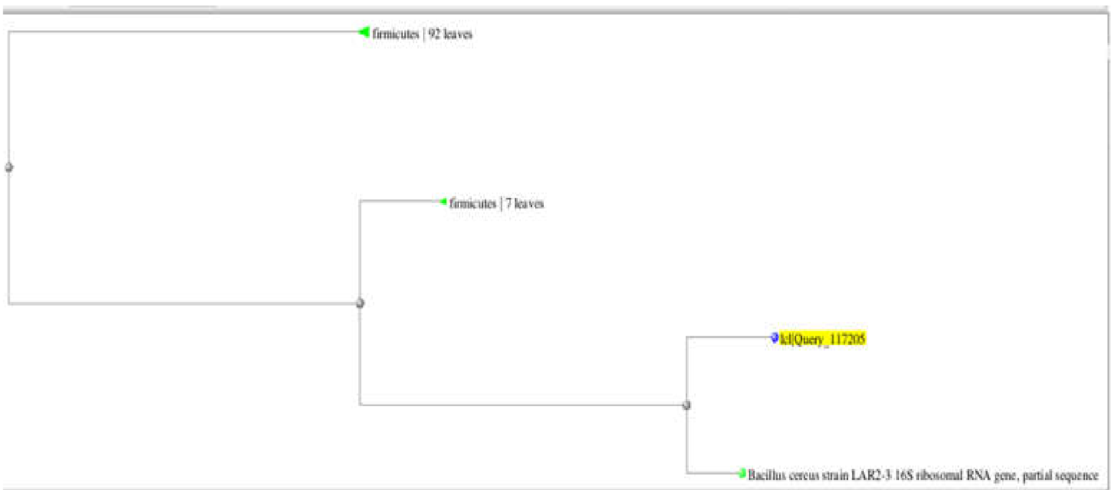
A

B

Figure 1. A and B: *Bacillus cereus* GAD20 Colonies on Hichrome Bacillus Agar

Sequence results of 16S rRNA :

T G C G G A T G GCC T A CA A T G C A G T C G AAC G G CAG CACA G G AG AGCT T GC T C T C T G G GT G GC G
AG T G GC G G AC G G GT G AG G AATACAT C G G AAT C TAC T T T T T C C G T G G G G G ATAAC GT A G G G
AAAC T TAC G C TA AT AC C G CAA A C G AC C TAC G G G T G AAA GCA G GG G AC C T T C G G G C C T TG C G
C G AT T G AAT G A G C C G ATG T C G G AT TA A G C TA G T T G GC G G G G TA AA G GC C CAC CA A G G C G
AC G AT C C G TAG C T G G T C T G AG A G G AT G AT CA G C CA CA C TG G A AC T G A G AC AC G G TC CA G
AC TC C TA C G G G AG G GCA G C A G T G G G G AAT AT T G G ACA A T G G G C G CA AG C C TG AT C CA G C C
A TA C C G C G T G G G T G AA G A AG GC C T T C G G G T T G TAA A G C C C T T T T GT T G G GA A A GA A AT C
C C A G C T G G C T A AT AC C C G G T T G G G AT G AC G G T AC C CA A A G A ATA A G CA C C G G C TA A C T
T C G T GC CA G C AG C C G C G G TA AT AC G A AG G GT G CA A G C G T T AC T C G G A A AT TAC T G G G C G
TA A A GC GT G C G TAG GT G G TC G T T T AAG TC C G T T GT G AA A GC C C T G G G C T CAAC C T G G G A
AC T G C A G TG G ATA C T G G G C G AC TAG A G T GT G GT A GGAG G GT A GCG G A AT TC CT G GTG TA GC
A G T GA AAT GC GT A GA G AT CA G G AG GA ACAT C C AT G G C G A AG GC AG C TA CC T G G ACCA A CAC T
G ACAC T G AG G CAC GA AA GC GGT G G G G AG CAAA CAG G ATTA GAT AC C CT G GTAG T CCA C GC CC T
AA AC GATG C GA AC TG G AT GT T G G GT GCAAT T T G GC AC GCAG T ATC GAAG CT AAC GC GT T AA G T
TC GC CC GC C TG G G GA GT ACG GTCG C A G ACTGG A A C T CAG AG G AA TT G AC GGGGGCC CGCA CAA
CCG GT GGAG TAT GT GG T T T A T T CT A TG C A C GC G A GAA CCTT ACC T GGCC TTGACAT GTC GA G A CTT
T CCA A G AT G GA T G GTGGC T TC T G AACT C G AC A CA GGTA C T GC ATG G CT GT C GT CA GC T CAT GTC
T GA GA TG T G AGTG A G T G CCTC A AC GA C G C AG CCC T CG TCCCTCC T T GGC T GCGC G GT AGT G GGG
GGGA CTGCT GT GC GA ATCAT C A G G AAC AA C G A G AA GA GA G G GA T AAA GT TG G TG TT T CT G TGCC
GTTGT A G C G A G GC CTA CA TAC G A G C T ACA ACA C A TT A TGAG AC G T G G G TGC G G TGC G G C GCT
C CGT A AC CG G ATG C A G A GAC GC CG AA C G C CG A G CT C A AT TA TG TA AT G C AG AGT GA G A C G C
A T CG A TC T CGCT C T GA T G CAG T A G T G T T TG GAGT



The dendrogram of phylogenetic tree

Sr.No	Sample ID	Primer Sequence	Identification BLAST	Percentage Similarity (%)
01.	GAD 20	Forward Primer 5'- AGA GTT TGA TCM TGG CTC AG -3' Reverse Primer 5'- TAC GYT ACC TTG TTA CGA CTT -3'	*Bacillus cereus*	97

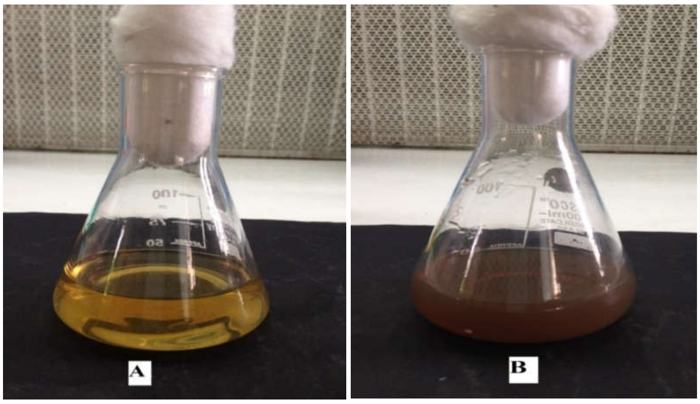


Figure 2. Colour observation during the synthesis of silver nanoparticles using *Bacillus cereus* GAD 20 (A - Control; B – Experimental)

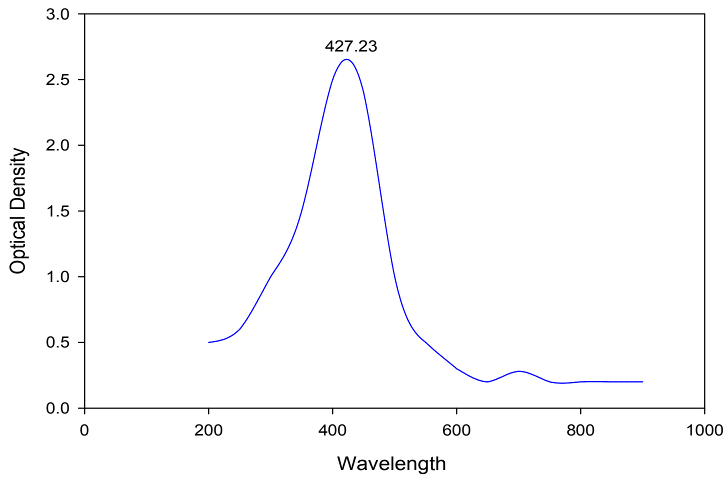


Figure 3. UV-visible spectra of synthesized silver nanoparticles

in the dark condition, respective sample changed in colour from almost yellowish to dark brown. Control (without silver nitrate) showed no change in colour of the supernatant culture when incubated in the same conditions (Figure 2). The extracellular synthesis of silver nanoparticle reaction was started when silver nitrate was added to supernatant of *Bacillus cereus* and incubated at 37ºC. When supernatant culture of *Bacillus cereus* treated with the 1mM $AgNO_3$ colour of the medium changes to dark brown due to Surface Plasmon Resonance (SPR) phenomenon as shown in figure 1. Thus, change in the colour intensity might be due to the excitation of the Surface Plasmon Resonance of the silver nanoparticles or reduction of the silver nitrate (Mulvaney, 1996; Gopinath *et al*., 2012). Similar observation of colour change was found in the reports of other researchers like Anuradha Prakash *et al*., (2011); Silambarasan and Jayanthi,(2012); Priyadarshini *et al*., (2013); Ranjitham *et al*., (2013). This supports the fact that change in colour in the experimental set as observed can be considered as an indication of AgNPs formation.

Characterization by UV-Vis Spectroscopy: *Bacillus cereus* GAD 20 mediated synthesis of silver nanoparticles shows the colour change of the nutrient broth with 1Mm Silver Nitrate medium from pale yellow to brown and further confirmation is done with the help of UV-Vis spectrophotometer operated at resolution of 1nm by scanning absorbance from 190 to 900 nm. The ability of *Bacillus cereus* for the synthesis of silver nanoparticle was characterized. The obtained absorbance peak is at λ 427 nm having the peak shift towards red region and the peak is broad and asymmetric (Figure 3). Previous reports shows that silver nanoparticles have free electrons responsible to create surface plasmon resonance at 406, 416, 430, and 448 nm (Banu *et al*. 2014; Du *et al*. 2016; Gopinath and Velusamy 2013; Sundaravadivelan and Padmanabhan 2014; Wang *et al*. 2015). The similar trends of observation were noticed by Ali Deljou and Samad Goudarzi, 2016 using thermophilic *Bacillus Sp*. Similar observations of Pal *et al*., 2007; Mohan *et al*., 2014 reported that the silver nanoparticle absorbs maximum light at the wavelength 420nm. Silambarasan and Abraham, 2012 works on the synthesis of silver nanoparticles by using *Bacillus cereus* showed the absorption maxima peak of silver nanoparticles at 440 nm which is little different from present study observations. The observation in the study of Anuradha Prakash *et al*., (2011) corroborates with the present study shows the absorption maxima peak at 435 nm.

Characterization by Fourier Transformed Infrared Spectroscopy: The FTIR spectra were carried out to find out the probable interactions between silver and bioactive factors produced by the given bacterial strain (*Bacillus cereus* GAD 20) which are responsible for the synthesis of silver nanoparticles by capping agent. FTIR spectra were recorded for silver nanoparticles synthesized from *Bacillus cereus* GAD 20 are shown in figure

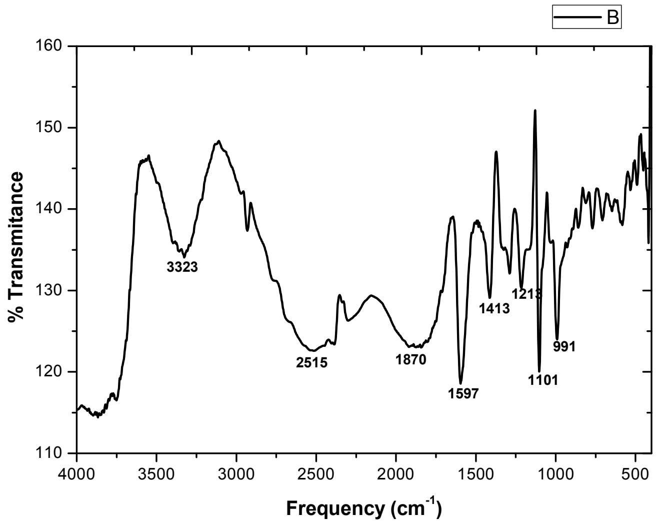


Figure 4. FTIR Spectrum of silver nanoparticles synthesized using *Bacillus cereus* GAD 20

The FTIR illustrates the absorbance band with peaks at 3323 cm^{-1}, 2515 cm^{-1}, 1870 cm^{-1}, 1597 cm^{-1}, 1413 cm^{-1}, 1219 cm^{-1}, 1101 cm^{-1},991 cm^{-1} .FT-IR spectrum of AgNPs that the bands in the range of 3000 cm^{-1} - 3500 cm^{-1}, spectrum shows a broad and strong intensity peak at nearly 3323 cm^{-1} which can be attributed to alcoholic or phenolic hydrogen bonded O-H bonds. This peak can also be due to the presence of amines (N-H) groups which signifies the presence of proteins secreted by bacteria extracellularly. The peak at 1597 cm^{-1} could also be attributed to C=C stretching vibrations about the amide C=O and conjugated C=O of ketones, aldehydes and esters which proofs as a factors for the presence of enzymes / proteins that are responsible for the reduction and stabilization of silver nanoparticles. The peak at 1413 cm^{-1} are due to the C=C stretch present in aromatic rings. It could also due to the C-H bonds of alkanes or may be related to COO- symmetrical stretch from carboxyl groups of the amino acid residues. Present study showed similar result of Priyadarshinia *et al*.,(2013) which reveals the distinct peak in the range of 3434, 1610 and1114 cm^{-1} which corresponds to strong stretching vibrations of O-H functional group, C- C stretching vibrations and functional group of amide-II respectively. These results are also supported by Singh *et al*., (2013) presence and binding of proteins with silver nanoparticles which plays an important role in stabilization and also act as reducing agent. Silambarasan *et al*., (2012) works on the synthesis of silver nanoparticles using *Bacillus cereus* and observed that two bands were present at 3441.71 cm^{-1} and 1650.10 cm^{-1}. The 1650.10 cm^{-1} band was identified as amide and this observation confirms the presence of protein in the sample of silver nanoparticles which agree with this results. Thus the overall FTIR pattern confirms the presence of proteins in synthesized silver nanoparticles. The free amine and carbonyl groups present in the bacterial protein could possibly perform the function for the formation and stabilization of silver nanoparticles (Babu and Gunasekaran, 2009; Balaji *et al*., 2009).

Characterization by Scanning Electron Microscopy (SEM): Scanning electron microscopy (SEM) was used to determine the size and shape of the synthesized nanoparticles. SEM images revealed the average size of silver nanoparticles 80 to 90 nm having spherical shape. Deepak *et al*., (2011) synthesized the nanoparticles using *Bacillus cereus* NK1 strain having the average size about 50–80 nm wth spherical shape. Silambarasan and Jayanthi, (2012) synthesized extracellular AgNPs having irregular shape with 62.8 nm in size.

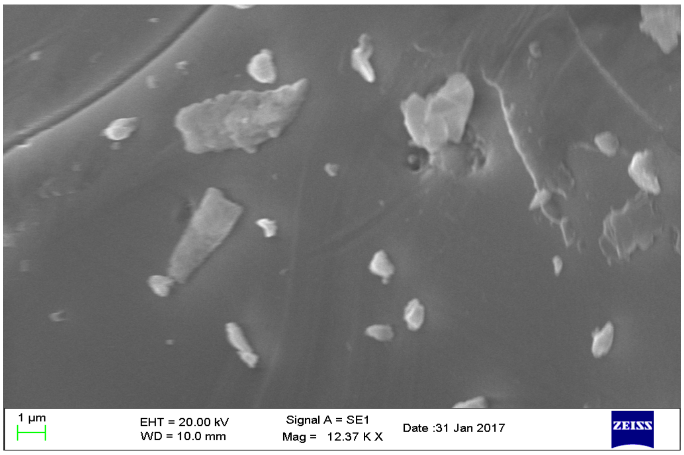


Figure 5. SEM image of synthesized silver nanoparticles

Identification of test bacteria on the basis of their Morphological, Cultural and Biochemical Characteristics: The test bacteria which are isolated from the clinical samples are identified on the basis of their morphological characteristics such as Gram staining and motility, cultural characteristics based on the cultivation of bacteria on the different biological media and biochemical characteristics by testing IMViC, Sugar fermentation, Enzymatic reaction etc. Identified bacterial strains on the basis of their characteristics were shown in Table No.2.

Table 2. Identification of Bacterial strains on the basis of morphological, biochemical and cultural characteristics

Sr. No.	Bacterial Isolates	Identified Bacteria
01.	US02	*Escherichia coli*
02.	PS01	*Staphylococcus aureus*

Antibacterial activity of silver nanoparticles

Analysis of zone of inhibition by well plate method: In the present study, the antibacterial activity of the synthesized AgNPs synthesized using *Bacillus cereus* GAD 20 against two species of pathogenic multidrug resistant bacteria were investigated. The bacterial strains including *Staphylococcus aureus* and *Escherichia coli* which are isolated from clinical samples. The synthesized AgNPs were proved to have antibacterial activity against respective tested bacterial strains.

Table 3. Zone of inhibition of synthesized silver nanoparticles against test organism

Sr. No.	Test bacterial strain	Zone of inhibition of AgNPs (in mm)
01.	*Staphylococcus aureus*	13
02.	*Escherichia coli*	20

US – Urine Sample PS – Pus sample SS – Sputum sample

Figure 6. Cultural characteristics of *E.coli* on EMB agar

Figure 7. Cultural characteristics of *S.aureus* on Mannitol salt agar

Figure 8. Zone of inhibition against *E.coli*

Figure 9. Zone of inhibition against *S.aureus*

AgNPs synthesized using *Bacillus cereus* GAD 20 shown highest zone of inhibition against *Escherichia coli* (20mm) followed by *Staphylococcus aureus* (13mm). The inhibitory impact of the AgNPs on each microorganism is specific and differs from one to another. In general, Ag ions from nanoparticles are believed to become attached to the negatively charged bacterial cell wall and lyse it, leading to protein denaturation and finally cell death (Lin *et al.*, 1998). The mechanism of action to inhibit the population of microorganisms by silver nanoparticles suggests that upon treatment, Yamanaka *et al.*, (2005) suggested that DNA loses its replication ability and expression of ribosomal subunit protein, as well as other cellular proteins and enzymes essential to ATP production hence microorganisms become inactivated. Some authors have been suggested that AgNPs are not responsible for DNA damage (Hashimoto *et al.*, 2012), while according to others (Lu *et al.*, 2010) they intercalate into the DNA. Other studies proposed that, AgNPs may attach to the surface of cell membrane disturbing permeability and respiration functions of the cell or by interfering with components of the microbial electron transport system (Percival *et al.*, 2005 and Sharma *et al.*, 2009). It has been reported that AgNPs can damage cell membranes resulting in structural changes, which makes bacteria more permeable to the nanoparticles (Lazar *et al.*, 2011; Periasamy *et al.*, 2012). AgNPs have been shown to be definitely an effective antibiotic against *E. coli*, *S. typhi*, *Staphylococcus epidermidis* and *S. aureus* (Jain *et al.*, 2009). In this perspective, Kim *et al.,*(2007) studied AgNPs antimicrobial activity against *E. coli* and *S. aureus* showing that *E. coli* was inhibited at low concentrations, while the inhibitory effects on the growth of *S. aureus* were less marked (Wu et al., 2014) which is correlates with the present study. Dipak Paul and Sankar Narayan Sinha, (2014) synthesized silver nanoparticles using *Pseudomonas aeruginosa* KUPSB12 reported highest inhibition zone of 19.0 mm diameter was formed against *Escherichia coli* and the lowest of 13.6 mm was produced against *Staphylococcus aureus* showed similar results with present study.

Conclusion

In conclusion, present study reported the simple biological way for synthesizing the silver nanoparticles using the culture supernatant of *Bacillus cereus* GAD 20. The present investigation suggests the extracellular synthesis silver nanoparticles. Synthesis of silver nanoparticles was primarily characterized by visual observation. Further, the results of FTIR suggested that the protein might have played a vital role in the stabilization and synthesis of silver nanoparticles. Synthesized stable silver nanoparticles showed a potent antibacterial activity against two pathogenic bacterial strains isolated from clinical samples. *Bacillus cereus* is a cheap and environment-friendly bio-resource for the synthesis of silver nanoparticles with antibacterial activity.

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